1

Minimally Invasive Surgery for Neurogenic Tumors of The Posterior Mediastinum in Children

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Actuality: Neurogenic tumors of the mediastinum in children occur in 15% of all tumors of the nervous system. The main method of treatment of this pathology is surgery, including minimally invasive surgery.

Objectives: To determine the possibility of minimally invasive surgery in the treatment of neurogenic tumors of the posterior mediastinum in children.

Materials and methods: From 2007 to 2016, 47 patients underwent thoracoscopic operations of the neurogenic tumor of the mediastinum. The age of the patients varied from 5 months to 16 years. The largest number of children entered the group from 1 year to 3 years - 18 people (38.3%). The sex ratio was approximately equal to: boys were 24 (51%), girls - 23 (49%).The duration of the operations was, on average, 49 minutes. In 85% of cases, the operation time was less than 60 minutes, and only 15% more than 1 hour. The blood loss during thoracoscopic operations averaged 14.3 ml. In 36 cases, blood loss was not observed. No intraoperative complications were noted. All patients within the first 24 hours after the operation were transferred to the profile department from the intensive care unit. Postoperative complications arose in 4 patients - Horner's syndrome, in connection with the localization of the tumor process in the apical part of the chest. 43 patients after surgery were in the surgical clinic for no more than 3 days, and 4 patients were detained in the hospital for up to 7 days to assess the neurological status due to manifestations of Horner's syndrome. All neoplasms were represented by neurogenic tumors: neuroblastoma-23 (49%), ganglioneuroblastoma-14 (30%) and geglioneuroma-10 (21%).

Conclusion: The advantages of using minimally invasive surgery for the treatment of neurogenic mediastinal tumors are: low traumatism, minimal blood loss, low number of complications, early activation of the patient and shortening of time in hospital, good cosmetic effect. Performing endosurgical operations in children with mediastinal tumors can be at the age of several months, while the oncological principles of performing surgical intervention are not violated, and the age of the child is not a limiting factor for performing endosurgical operations.

2

Histone Demethylases Are Novel Therapeutic Targets of Neuroblastoma

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Neuroblastoma is the most common type of cancer in infants and causes as much as 15% of cancer-related deaths in children. Although the outcomes of children with low- or intermediate-risk disease are excellent, those of children with high-risk disease remain dismal. Thus, the need to identify novel therapeutic targets and develop more effective treatments for high-risk neuroblastoma is urgent. The genetic abnormalities that drive tumorigenesis are usually coupled with epigenetic alterations, such as aberrant histone modification, which may help oncogenic drivers accelerate cancer progression, metastasis, and therapy resistance. Oncogenic MYCN amplification is the most important biological feature of high-risk neuroblastoma.

Histone lysine demethylases appear to be involved in facilitating the activity of oncogenic transcription factors such as Myc. Therefore, targeting histone demethylases may block Myc signaling central to tumorigenesis. Here we present that genetic or pharmacologic inhibition with novel inhibitors of histone demethylases is able to inhibit proliferation of neuroblastoma. Our findings provide new insight into the epigenetic regulation of Myc function via histone demethylation and indicate that pharmacologic inhibition of histone demethylases may be an effective approach for cancer therapy through Myc pathway inhibition.

3

Prognostic Value of Minimal Residual Disease in High-Risk Neuroblastoma Group: Protocol NB 2004m, End of Induction

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Background: To establish the prognostic value of the presence of a minimal residual disease (MRD) in bone marrow (BM) in the high-risk neuroblastoma group at the end of induction.

Methods: The study of MRD in BM on protocol NB2004m, end of induction, performed by flow cytometry (Syto16+CD45-CD56+CD81+ phenotype), immunocytochemistry (GD2-positive cells), molecular biology (semiquantitative evaluation of TH and PHOX2B gene expression using RT-PCR).

Results: For flow cytometry and immunocytochemistry, we used cut-off positivity and negativity. Overall survival (OS) flow cytometry (n = 5) 100% vs (n=31) 52%±11% (p=0,16); event-free survival (EFS) 50%±25% vs 37%±10%, (p=0,4). OS immunocytochemistry (n = 1) 100% vs (n = 20) 86% ± 9% (p = 0,8); EFS 0% vs 40% ± 19%, (p=0,34). The presence of MRD in BM, determined by both methods, didn't influence OS and EFS on patients with neuroblastoma.

Cut-off, determined for the level of expression of TH and PHOX2B genes, allows to classify a part of patients as ultra-high risk: for expression of TH gene - log10> 0.0018, for PHOX2B gene - positive expression in BM. In case of expression of TH gene higher than cut–off OS (n=11) 30%±15% vs (n=34) 67%±10% (p = 0,0032); EFS 11% ± 9% vs 53% ± 10%, (p = 0,0004). Positive expression of PHOX2B gene OS (n = 9) 35% ± 18% vs (n = 31) 59% ± 11% (p = 0,0032), EFS 0% vs 55% ± 15%, (p = 0,0002).

In Cox regression analysis, we used the following risk factors: age, stage, MYC-N amplification, the expression level of TH and PHOX2B genes in BM, end of induction. Two regression models for OS and EFS were constructed. In the model for EFS, the expression of PHOX2B gene had significance (p = 0,0087) with RR 3.02.

Conclusion: the presence of MRD in BM, revealed by the evaluation of the expression of TH and PHOX2B genes, in our patients at the end of induction strongly negatively influences the prognosis of the disease. For this cohort of patients, the approaches to therapy should be changed, since the standard therapy is not effective for them.

4

Outcomes and Complications of Surgery in Patients with Intermediate-Risk Neuroblastoma: Experience from an Indian Tertiary Cancer Centre

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Background: The treatment of intermediate risk (IR) neuroblastoma has evolved with the focus now on reducing the drugs, dosage, and duration of chemotherapy. The aim of this study is to present the outcomes of treatment and the complications of surgery in patients with IR neuroblastoma treated at a tertiary cancer center in India.

Procedure: All eligible patients with IR neuroblastoma treated between April 2005 and August 2016 were identified. The presence and number of image-defined risk factors (IDRF) before and after neoadjuvant chemotherapy were retrospectively analyzed as were the extent of surgery, complications, and outcomes.

Results: Of 282 neuroblastoma patients treated during the study period, 54 had IR neuroblastoma. A median of 3 IDRF's were identified at presentation which reduced to 2 after preoperative chemotherapy; however, complete disappearance of IDRF was not seen in any patient. Complete excision was achieved in 25 patients. There were 28 surgical complications in 22 patients with a similar incidence in patients with complete (n=14) or incomplete (n=13) resection (p=0.6). The most common complication was postoperative chylous leakage. There was no perioperative mortality. After a median follow-up of 47 months, the 4-year overall and event-free survival was 91.5% and 75% respectively. There was no difference in survival between patients who underwent complete resection versus those with incomplete resection (p=0.78).

Conclusion: Outcomes of IR neuroblastoma are favorable. IDRFs do not predict the extent of resection or survival. The extent of resection does not affect the survival and complications can occur even when the resection is incomplete.

5

A Kinome-Wide RNAi Screen Identifies ALK As a Target to Sensitize Neuroblastoma Cells for HDAC8-Inhibitor Treatment

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The prognosis of advanced stage neuroblastoma patients remains poor and, despite intensive therapy, the five-year survival rate remains less than 50%. We previously identified histone deacetylase (HDAC) 8 as an indicator of poor clinical outcome and a selective drug target for differentiation therapy in vitro and in vivo. Here we performed kinome-wide RNAi screening to identify genes that are synthetic lethal with HDAC8 inhibitors. These experiments identified the neuroblastoma predisposition gene ALK as a synthetic lethal candidate gene. Accordingly, the combination of the ALK/MET inhibitor crizotinib and selective HDAC8 inhibitors (6 µM PCI-34051) efficiently killed neuroblastoma cell lines with wildtype ALK (SK-N-BE(2)-C, IMR5/75), amplified ALK (NB-1), and those carrying the activating ALK F1174L mutation (Kelly). The effective dose of crizotinib ranged from 0.05 µM for ALK-amplified cell lines to 0.8 µM for cell lines with wildtype ALK. Bioinformatic analyses revealed that the mRNA expression level of HDAC8 was significantly correlated with that of ALK in two independent patient cohorts, from the Academic Medical Center (AMC; n=88) and the German Neuroblastoma Trial (n=649), and co-expression of both target genes identified patients with very poor outcome. Mechanistically, HDAC8 and ALK converge at the level of receptor tyrosine kinase (RTK) signaling and their downstream survival pathways, such as ERK signaling. Combination treatment of HDAC8 inhibitor with crizotinib efficiently blocked the activation of growth receptor survival signaling and shifted the cell cycle arrest and differentiation phenotype toward effective cell death of neuroblastoma cell lines, including sensitization of resistant models, but not of normal cells. These findings reveal combined targeting of ALK and HDAC8 as a novel strategy for the treatment of neuroblastoma.

6

Anatomic Patterns of Relapse and Progression Following Treatment with 131I-MIBG in Metastatic Neuroblastoma

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Purpose/Objectives: Neuroblastoma is the most common pediatric extracranial solid tumor. Patients with MIBG-avid relapsed or refractory neuroblastoma following therapy may exhibit significant but often transient responses to salvage treatment with 131I-MIBG. It is not known whether disease progression following 131I-MIBG treatment occurs in previously involved vs. new anatomic sites of disease. Understanding this pattern of relapse may inform the use of consolidative external beam radiation therapy following 131I-MIBG administration.

Materials/Methods: Patients with relapsed or refractory metastatic MIBG-avid neuroblastoma who received 131I-MIBG on phase II/compassionate-access protocols at UCSF were included if they had 1) stable or responding disease 6-8 weeks following 131I-MIBG infusion, but subsequently experienced disease progression and 2) serial diagnostic MIBG scans from protocol enrollment through first progression. Scans were reviewed to establish anatomic locations and temporal evolution of MIBG-avid disease. Progression was defined as development of MIBG-avid disease in a previously uninvolved site, recurrence of MIBG avidity in a previously involved site that fully cleared following treatment, or as progression in a previously involved site.

Results: 84 MIBG-avid metastatic sites were identified prior to MIBG therapy in 12 patients. Median age at first 131I-MIBG treatment was 9.1 years (4.3-51.2). Following first 131I-MIBG infusion and prior to disease progression, seven patients received additional 131I-MIBG treatments, but none received external beam radiation therapy. Median time to progression after first 131I-MIBG treatment was 0.6 years (0.3-2.5). At first progression, 101 MIBG-avid sites were identified, of which 69 (68%) overlapped with pre-treatment disease sites, while 32 (32%) represented anatomically new disease areas. Eight of 12 patients had one or more new MIBG-avid site at first progression. Of the 69 involved sites at progression that overlapped with pre-treatment disease, 11 represented relapsed sites that had cleared following MIBG therapy, 2 were persistent but increasingly MIBG-avid, and 56 were stably persistent.

Conclusions: Previously involved disease sites predominate at disease progression following 131I-MIBG treatment. Nevertheless, the majority of patients progressed in at least one new anatomic site. This suggests that consolidative focal therapies targeting residual disease sites may be of limited benefit in preventing systemic disease progression following 131I-MIBG treatment of relapsed or refractory neuroblastoma.

7

Whole Genome Sequencing for Precision Medicine in High-Risk Neuroblastoma Patients - Translation from Research to Clinical Practice in Sweden

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The overall survival of high-risk neuroblastomas is about 40 %, implying an urgent need for improvement in treatment strategies for this particular patient group. Clinical use of next-generation sequencing provides a comprehensive approach for rapid determination of genomic biomarkers for diagnosis/prognosis, and of foremost importance, to achieve precision treatment based on the patients' specific tumor targets.

Whole genome sequencing (WGS) was performed on tumor/normal-pair for 30 neuroblastoma patients, both retrospective samples and a subset of patients with progressive disease despite ongoing treatment. Sequencing was performed for an average coverage of at least 60X and 30X for tumor and normal tissue respectively. Read trimming, mapping to hg19 and variant calling were performed using the CLC Genomics Workbench software while copy number profiles were prepared through the CANVAS software. Systematic filtering was performed using the Ingenuity variant analysis tool. Variants with allele frequency above 3% in common population based cohorts were discarded as well as excluding all synonymous variants or variants in non-coding regions except those affecting canonical splice sites.

In our present setting the approximate time for handling is 2,5-3 weeks, counting from arrival of material to analyzed data. The analysis includes copy number variations, structural aberrations and mutational screening. These alterations have then been thoroughly evaluated with regards to biological relevance and druggability, looking for drugs either used in existing cancer treatment or in clinical trials. Among the 30 patients, potentially actionable targets were detected in 14 (47%) of the cases; including aberrations in ALK, CDK4/MDM2, CDKN2A, CCND1 and ROCK2. Overall interpretation was negative or inconclusive for 16 patients showing no or only weak support for targeted treatment.

Our results demonstrate the feasibility to incorporate WGS in clinical practice. A logistic and analytic pipeline has been established in order to present the results in a clinically meaningful time frame. Actionable targets that could be relevant in therapy were detected in 14 out of 30 neuroblastoma patients. The result from this study show promising results and we predict that integration of WGS in clinical routine will lead to improved management of the therapeutically most challenged group.

8

Human Metallothionein-3 Up-Regulation in Neuroblastoma Cells Lines and its Impact on Cisplatin Susceptibility

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Metallothionein-3 (MT-3) is predominantly expressed in the central nervous system. MT-3 could participate in the processes of heavy metal detoxification, regulation of metabolism and protection against oxidative damage of free radicals in the central nervous system, thus it could play important neuromodulatory and neuroprotective roles. MT-3 had been postulated to be a multipurpose protein which could play important neuromodulatory and neuroprotective roles in CNS besides the common roles of MTs. However, the primary function of MT-3 and the mechanism underlying its multiple functions were not elucidated so far in neuroblastoma. In the present study we focused on up-regulation of MT-3 gene in different neuroblastoma cells. The up-regulation of different genes from biological pathways related to cellular senescence process were identified using electrochemical microarrays. Cisplatin or cis-diamminedichloroplatinum (CDDP) is one of the most commonly used drug in the treatment of Nbl. CDDP induces cytotoxic cell death mediated by activation of death receptor-mediated apoptotic signaling mechanisms as well as mitochondrial pathways. In general, multiple mechanisms have been identified for the acquisition of drug resistance by cancer cells. Further, the cytotoxicity of the CDDP was examined in hMT-3 and mock cells by MTT and clonogenic assays. We also provide a clear in vitro evidence that hMT-3 participates on a formation of chemoresistance against CDDP and its expression could be considered as potential prognostic biomarker. Our data strongly suggest that up-regulation MT-3 is involved in induction of chemoresistance of neuroblastoma cells chemotherapy.

9

A Rapid Prognostic Indicator of Neuroblastoma Disease Progression Using the Chick Embryo Transplant Model, Logic-Based Network and 5-color Immunofluorescence Predictor

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Correlative analyses of genomic information from profiles of pediatric neuroblastoma cancers and known patient outcomes have led to specific gene lists put forward as high risk for disease progression. However, this reliance on gene expression correlation patterns rather than mechanistic insight has shown limited predictive accuracy. This suggests a critical need for models that include molecular network interactions that would better predict neuroblastoma progression. In this study, we construct and simulate a logic-based model with a molecular network of tyrosine receptor kinases and downstream signals that are involved in sympathetic nervous system development, from which neuroblastoma pathogenesis is derived. We show that by using 6 input genes, our model predicts the probability of reaching steady state outcomes with distinct prognostic indicators of cell differentiation, proliferation, apoptosis, and angiogenesis. We validate the mechanistic model assumptions by using RNAseq of the SHSY5Y human neuroblastoma cell line to define the states of the 6 input genes and show the model predicts a steady state outcome that aligns with antibody staining. To further illustrate the predictive strength, we transplant YFP-labeled SHSY5Y cells into the trunk dorsal neural tube of a Stage 10-12 chick embryos and assess changes in cell position and state 48 hours later by 5-color antibody staining of our prognostic indicators. We find that SHSY5Y transplanted cells show a differentiated identity compared to non-transplanted, in vitro cultured SHSY5Y cells. This finding is indicative of an influence from the embryonic neural crest microenvironment to drive a favorable outcome. We propose the combination of our logic model and embryo transplant model with 5-color antibody staining represents a rapid prognostic indicator of neuroblastoma disease progression based on cell line and human patient derived tissues.

10

Inhibition of Fatty Acid Synthesis Induces Neuroblastoma Differentiation

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High MYC signaling or c-MYC/MYCN amplification are strongly correlated to poor prognosis and treatment failure in neuroblastoma patients. Survival for high-risk patients remains only around 40-50%, and survivors suffer long-term side effects from treatment. Alternative approaches, as differentiation-inducing therapies, are especially interesting for high-risk patients in which resistance to conventional chemotherapy arises. We previously demonstrated that silencing of MYCN or targeting the protein with small molecule inhibitors prompt changes in lipid metabolism due to reduced ß-oxidation and mitochondrial dysfunction (Zirath et al PNAS, 2013). Here our objective is to analyze the crosstalk between lipid metabolism and cell differentiation regulated by MYC(N), to identify new therapeutic targets with potential use for induction of differentiation.

Proteomic analysis of neuroblastoma cells shows that lipid metabolism and related signaling elements, as well as proteins involved in cell differentiation, are consistently affected by MYCN downregulation. Additionally, high expression of acetyl-CoA carboxylase (ACACA) and fatty acid synthase (FASN) correlates to poor survival in neuroblastoma patients, suggesting fatty acid synthesis as a promising target for treatment. Accordingly, the chemical inhibition of de novo fatty acid synthesis (targeting either ACACA or FASN) triggers MYC(N) protein downregulation and differentiation of both MYCN-amplified and non-amplified neuroblastoma cells.

Moreover, fatty acid synthesis inhibition has an impact in mitochondrial structure and function. The withdrawal of major nutrients as glucose and glutamine does not trigger neuroblastoma differentiation, while the incubation in delipidized cell culture medium induces neurite outgrowth. Consequently, cell membrane-permeable fatty acids prevent the phenotypes observed, suggesting a very specific relationship of lipid metabolism with induction of differentiation.

Our work aims to elucidate the impact of lipid metabolism on neuroblastoma biology, with potential implications for metabolism-targeted and differentiation-based therapies. Here we show that targeting fatty acid synthesis could be an interesting therapeutic strategy for neuroblastoma treatment, especially as a co-adjuvant in differentiation therapies.

12

Repetitive Retroelement Activity Is a Major Source of Genomic Instability and A Druggable Target Involved in Neuroblastoma Development and Progression

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Nearly half of the mammalian genome is occupied by interspersed repetitive elements belonging to LINE1 and SINE families. Their expansion has played an important role in the evolution of mammals, involving reverse transcription driven by a reverse transcriptase encoded by LINE1 elements (RTL1). This "retrobiome" is normally under strict epigenetic control that maintains transcriptional silencing and protects cells from genomic instability associated with new retrotranspositions. There is a growing body of evidence indicating that activation of expression and amplification of retroelements frequently occur in malignant tumours. However, the enormous complexity of the retrobiome has made it difficult to systematically analyse the specific contribution of retroelements to tumour initiation and progression.

We have recently solved this problem by developing new computational algorithms that enable us to detect, localize and quantify new retrotransposition events in Whole Genome Sequencing databases. We have applied these new bioinformatic tools to analyze the content of the retrobiome in tumours from TH-MYCN transgenic mice and in primary human neuroblastoma samples. This analysis has revealed massive acquisition of new LINE1, SINEs and processed pseudogenes (intronless, promoterless cDNA copies of mRNAs) in these tumours, by comparison with normal somatic tissues. The scale of these events exceeds many times the occurrence of point mutations or amplifications that have been detected so far in neuroblastomas and suggests that the activity of the retrobiome may be the major driver of tumour progression. Furthermore, treatment of homozygous TH-MYCN mice, immediately post-weaning, with Stavudine, an antiretroviral reverse transcriptase inhibitor, significantly delayed tumour development by comparison with control mice ($p<0.05$). When mice with an established tumour were treated with Stavudine in combination with cyclophosphamide/topotecan, survival was significantly increased by comparison with chemotherapy alone ($P<0.05$). These data support the involvement of retroelement activation in neuroblastoma tumorigenesis and progression and open up a new opportunity for pharmacological inhibition of neuroblastoma genomic instability, with the potential of improving treatment, slowing progression, and ultimately preventing this aggressive childhood disease.

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Combination of HDAC and Mitochondrial-Targeted Inhibitors Exhibit Strong Therapeutic Synergy In Vitro and In Vivo Against Neuroblastoma

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N-Myc overexpression in neuroblastoma has previously been associated with increased mitochondrial biogenesis and deregulation, resulting in apoptotic death resistance. Targeting deregulated mitochondria using specific inhibitors restores apoptotic pathways and drives cell death in neuroblastoma. Neuroblastoma tumorigenesis is characterized by ectopic histone deacetylase (HDAC) expression, resulting in transcriptional silencing of tumor suppressor genes. We have previously demonstrated the effectiveness of HDAC inhibitors in N-Myc-driven neuroblastoma, as there is a unique need for HDAC recruitment by the N-Myc oncogenic signal.

We have now shown that the expression of two mitochondrial adenine nucleotide transporters (SLC25A5 & SLC25A6) significantly correlated with poor patient prognosis and N-Myc expression in neuroblastoma. Directly targeting these transporters in neuroblastoma cells using the selective Phase II inhibitor, PENAO, resulted in significant cell death in vitro. We found that the clinically approved HDAC inhibitor, SAHA, synergistically enhanced the cytotoxicity of PENAO across a panel of neuroblastoma cell lines. Our in vivo studies indicated that PENAO + SAHA treatment of neuroblastoma cell xenografts in Balb/c nude mice, significantly prolonged survival and reduced tumor growth. Propidium iodide cell-cycle analysis of neuroblastoma cells treated with this combination revealed sub-G1 arrest, whilst TUNEL & Annexin-V/7AAD assays indicated a synergistic induction of apoptosis at clinically achievable concentrations. Further analysis through JC-1, MitoSox Red and Dihydroethidium assays, indicated that the combination induced mitochondrial superoxide formation and depolarization, suggesting that the combination induced an intrinsic mode of apoptosis. This was validated by the detection of a downregulated anti-apoptotic mediator, Bcl-2.

Expression microarray analysis of SH-SY5Y cells treated with the single agents or combination identified numerous genes to be modulated by this drug combination. Downstream analysis using GSEA suggested that the combination mediated its cytotoxicity by activating p53 and suppressing E2F signaling pathways. The preferential cytotoxicity of the combination to wild-type p53 over mutant p53 neuroblastoma cell lines further suggested that the overall mechanism of action of the combination was p53 dependent. Both agents are in clinical use, thus our promising data on this combination warrants further evaluation for an early phase clinical trial for advanced-stage neuroblastoma.

14

CHL1 Gene Acts as A Tumor Suppressor in Human Neuroblastoma

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A distal portion of human chromosome 3p is often deleted in neuroblastoma and other cancers and it is generally assumed that this region harbors one or more putative tumor suppressor genes. A 2.54 Mb region at 3p26.3 encompassing the smallest region of deletion pinpointed the locus for the neuronal cell adhesion molecule close homolog of L1 (CHL1). We evaluated the association of CHL1 gene expression with neuroblastoma patient outcomes, using online microarray data of neuroblastoma tumors obtained from the R2 Genomics Analysis and Visualization Platform (http://r2.amc.nl). We found that low CHL1 expression predicted poor outcome in neuroblastoma patients.

Then we used inducible cell models to analyze the impact of CHL1 on neuroblastoma growth. In vitro and in vivo assays were performed to investigate whether CHL1 affected cell proliferation and invasion of neuroblastoma cells. Three lentiviral small hairpin RNA plasmids, and the recombinant pCEFL-CHL1 over-expression vector were transfected into two neuroblastoma cell lines to down-regulate or up-regulate CHL1 expression. Over-expression of CHL1 halted tumor progression, inhibited anchorage-independent colony formation, induced neurite-like outgrowth and markers of neuronal differentiation in neuroblastoma cells, and suppressed the growth of human tumor xenografts. Our results demonstrated that over-expression of CHL1 inhibited the activation of Rho GTPases, of related p38/JNK MAPK pathways, and of p-Akt inducing cell apoptosis and differentiation. These findings demonstrate that CHL1 suppressed critical processes of malignancy in neuroblastoma cells.

Knock-down of CHL1 in HTLA-230 neuroblastoma cell line triggered colony formation and anchorage-independent growth, enhanced cell proliferation and migration, protected from p-Akt mediated apoptosis, induced activation of Rho GTPases, decreased expression of MAP2 and of the autophagy protein markers Beclin1 and LC3 inducing neurite retraction, and accelerated growth in orthotopic xenografts mouse model. CHL1 acts as a regulator of proliferation and differentiation of neuroblastoma cells through inhibition of the MAPKs and Akt pathways. Taken together, our data identify a novel unbalanced developmental network in neuroblastoma cells that may contribute to tumor aggressiveness. Thus, restoration of CHL1 expression or its associated pathways may represent a potential therapeutic strategy for neuroblastoma.

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Loss of Whole Chromosome X and Gain of Whole Chromosome 13 Predict Prognosis of Neuroblastoma Patients with Numerical Genomic Profile

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In neuroblastoma very few genetic mutations have been detected while chromosomal aberrations are common. Numerical whole-chromosomal aberrations (NCA) or recurrent patterns of segmental chromosomal alterations (SCA) suggest that neuroblastoma is driven by copy number chromosome aberrations rather than by specific gene mutations. SCA are associated with high-risk disease and with poor prognosis, whereas NCA are typically found in low-risk tumors with a much better prognosis; however, a small percentage of them (10-15%) subsequently relapse and/or die of disease. A cohort of 177 NB patients with NCA genomic profile was analyzed. Association between NCA and event free survival was investigated by the Kaplan-Meier estimator and prognostic Decision Tree. The chromosomes involved in whole chromosome gains and losses were the same included in SCA markers of poor prognosis for neuroblastoma. DT identified 65 patients with normal chromosome X and an excellent 5-year EFS (100%) independently from the stage at diagnosis. The poorest EFS (57%) was observed in 21 metastatic stages carrying abnormal X chromosomes (20 losses and 1 gain). Among the remaining patients with localized disease and abnormal X chromosome, those with a gain of chromosome 13 had the poorest survival (EFS=78%, n=40 vs. EFS=95%, n = 51). The association between EFS and NCA abnormalities of X and 13 chromosomes was confirmed after restriction to 126 patients with localized disease and age <18 months at diagnosis.

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Dysregulated Expression Levels of HIF-2α Affect Migration of Trunk Neural Crest-Derived Neuroblastoma Precursors

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Transcription factor hypoxia inducible factor (HIF)-2α is strongly expressed in a subset of stem cell- and neural crest-like neuroblastoma cells located in perivascular niches. In tumor specimens the presence of these HIF-2α positive cells correlates with distant metastasis and poor patient prognosis. Despite this, very little is known about the expression and action of HIF-2α during normal sympathetic nervous system development. We use the chick embryo model to show that HIF-2α is expressed during early embryogenesis, specifically in migrating trunk neural crest cells at time points consistent with when neuroblastoma precursor cells have been suggested to emerge. Imbalanced expression levels of HIF-2α, either by up- or down-regulation, leads to less migrating neural crest streams at the trunk axial level. Expression of gene sets associated with all neural crest populations (e.g. SOX10, TFAP2 and HNK1), and with gene sets recently linked to migrating trunk neural crest specifically (e.g. AGPAT4, MOXD1, HES6 and TAGLN3), decreased as a consequence of altered HIF-2α levels. These results might suggest that trunk neural crest cells with dysregulated HIF-2α fail to reach a predicted cellular state reflected by the established expression profile. Chick embryos were further treated with the HIF-2-specific antagonist PT2385, which inhibits binding between HIF-2α and its transcriptional binding partner ARNT. Interestingly, PT2385 treatment had virtually no effect on trunk neural crest-associated genes, suggesting that pure expression levels rather than transcriptional activity mediates the HIF-2-driven effect on trunk neural crest migration; possibly part of the recently proposed non-transcriptional functions of HIF-2α. In neuroblastoma as compared to other tumor forms, these identified migrating trunk neural crest-specific genes are enriched and thus reflect the cellular origin of neuroblastoma. In summary, we show that migration of trunk neural crest cells – presumed precursors of neuroblastoma – relies on neuroblastoma-related protein HIF-2α at embryonic time points previously associated with oncogenesis. Gene sets mapped to be specifically expressed in migrating trunk neural crest cells are expressed in neuroblastoma and correlate to patient outcome, outlining a gene network that together with HIF-2α is putatively important for neuroblastoma transformation.

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Analysis of The Biological Consequences of Targeting MYCN with Small Molecular Compounds in Neuroblastoma

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Neuroblastoma (NB) is an embryonic neural tumor and one of the most common extracranial solid tumors of childhood. Approximately 50% of all cases are patients with high-risk NB with a metastatic disease and only less than half of these patients can be cured. The MYCN oncogene is amplified in the 25% of the NB cases, and it is used as a genetic marker for poor outcome. Several small-molecule inhibitors of MYC have been identified to date, including 10058-F4, which targets MYC dimerization with MAX, leading to MYC degradation; and JQ1, a bromodomain and extraterminal (BET) domain protein inhibitor which has been shown to downregulate MYC transcription in some malignances.

Amplification of the MYCN oncogene suppresses differentiation of cancer cells. We have previously demonstrated that targeting MYCN by 10058-F4 induces lipid accumulation and neuronal differentiation in MYCN-amplified NB cells (Zirath et al., 2013). In addition, we found that JQ1 downregulates MYCN on protein level and induces metabolic alterations resulting in lipid droplet formation.

In order to explore the cellular effects of MYCN inhibition in neuroblastoma cell biology, we have now performed quantitative proteomics of untreated and JQ1-treated MYCN-amplified SK-N-BE (2) cells, based on high resolution isoelectric focusing of iTRAQ labelled peptides in a narrow pH gradient followed by LC-MS/MS analysis. For comparison, downregulation of MYCN expression using short hairpin RNA (shRNA) followed by proteomic analysis was performed. We identified 8351 proteins which will be used for studying novel pathways involved in NB carcinogenesis and for description of potential MYC related biomarkers. Our preliminary analysis shows that the largest numbers of affected proteins are involved in metabolic processes including protein, lipid and nucleic acid metabolism. Taken together, our findings establish proof of concept for the development of MYC-inhibition as a promising strategy to inhibit tumor progression.

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MYCN Negatively Regulates Starvation-Induced Autophagy Through miR-17/92 Family microRNAs

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Background: MYCN regulates expression of many microRNAs. However, how MYCN regulates cell growth and survival through microRNAs remains unclear. ULK1 is a serine/threonine kinase essential in autophagy, a key pathway for cell survival under starvation and other stressors. Here we reported that MYCN negatively regulated ULK1 expression and starvation-induced autophagy through miR-17/92 family member miR-19.

Methods: SHEP-Control, SHEP-MYCN, Kelly, IMR32, SMS-KCN-R, SY5Y, CHLA20 and SKNSH cells were used. Trypan blue, cell titer glo and flow cytometry assays were performed to measure cell growth and survival. ULK1 mRNA and protein levels were measured by western blotting and qRT-PCR. Survival of patients, and mRNA expression levels in human tumors were obtained from r2.amc.nl. For mouse experiments, TH-MYCN mice were treated twice a week, for two weeks with anti-miR19 (10mg/kg) or vehicle.

Results: Compared to SHEP-controls, SHEP-MYCN cells were sensitive to starvation-induced apoptosis, resistant to starvation-induced autophagy, and hypersensitive to inhibition of ULK1. Results were confirmed in additional MYCN-amplified and non-amplified cell lines. In neuroblastoma patient samples, levels of ULK1 and MYCN mRNA correlated inversely. Moreover, tumors from patients with MYCN amplification expressed significantly lower levels of ULK1. High levels of ULK1 correlated with improved patient survival. The ULK1 3'-UTR, showed binding sequences for miR-17/92 family microRNA miR-19, which was transcriptionally upregulated by MYCN. In SHEP-MYCN cells, ULK1 was downregulated at both RNA and protein levels. Anti-miR-19 treatment prolonged survival of TH-MYCN mice and induced autophagy. Finally, combination of anti-miR-19 and autophagy inhibition induced tumor apoptosis.

Conclusions: Our findings suggest that MYCN activation of miR-19 leads to downregulation of ULK1, and resistance to starvation induced autophagy. Blockade of MYCN thus drives autophagy as targetable pathway, contributing to resistance. Our study builds connections between ULK1 and MYCN and provides new therapeutic insights for patients.

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MYCN Mediated Metabolic Processes In MYCN-Amplified Neuroblastoma

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Neuroblastoma, arises from the developing sympathetic nervous system, is one of the most aggressive solid tumors in infants. Amplification of the MYCN oncogene can be found in around 30% of NB patients and it is associated with rapid tumor progression and poor prognosis. We demonstrated that inhibition of MYCN in NB cells results in metabolic changes including mitochondrial dysfunction.

In order to explore possible downstream effects of MYCN targeting therapy we have performed quantitative proteomics of MYCN-amplified stable transduced NB cells. We compared the protein profile generated from MNA NB cells with a gene expression data set of 649 patients. The analysis of protein and gene expression highlighted changes in key enzymes involved in metabolic pathways which are essential for cancer progression and are associated with poor patient outcome in NB.

To functionally access the metabolic alteration indicated by data analysis we used Agilent Seahorse XF technology, which provide the information about main energy production pathways and the metabolic phenotype of the cells. Importantly, the metabolic profiling performed using an extracellular flux analyzer showed that MNA NB cells display both enhanced glycolytic and respiratory capacity compared to NMNA NB cells. Thus, our functional data demonstrates that MYCN-amplified NB cells are characterized by a high energetic metabolic phenotype.

Based on this observation and our data analysis we hypothesized that OXPHOS is the main source of fuel in high energetic NB cells. We measured the oxygen consumption rate (OCR) in BE (2) shMYCN cells in the presence and absence of specific metabolic inhibitors. The drop in OCR in live cells upon acute etomoxir treatment suggests that 75 % of mitochondrial respiratory capacity or OXPHOS is fatty acid dependent in BE (2) shMYCN cells.

We next analyzed the level of the key enzyme of β-oxidation, CPT1 in a neuroblastoma patient data set and found that high expression of CPT1 correlates with reduced survival of NB patients with high MYCN level specifically. Additionally, the obtained data shows that CPT1 inhibition decreases survival of MNA NB cells. Furthermore, etomoxir treatment reduced tumor burden in vivo.

Taken together, our findings provide novel information about MYCN-mediated high-energetic metabolic phenotype in NB.

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A New Risk Score for Patients with First Recurrence from Stage 4 Neuroblastoma Aged ≥18 Months at First Diagnosis

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Background: For parents, the decision for a second therapeutic approach after recurrence from stage 4 neuroblastoma is heavily depending on the potential survival time and proportions. For clinical scientists, the reporting of phase 2/3 trials requires the comparison with equivalent risk groups. This study aims to support these tasks by a score system based on a large data set.

Patients and Methods: Inclusion criteria were first recurrence from neuroblastoma stage 4, age at first diagnosis ≥18 months-<21years and enrollment in first line trials between 1990 and 2010. Exclusion criteria were inadequate first line therapy (n=22), death from tumor in first line therapy without response (n=11), death from tumor <30 days after recurrence (n=2), death from toxicity (n=40), second malignancy (n=27), and insufficient information (n=2). Patients were stratified according to the variables MYCN amplification (yes/no), time from diagnosis to first recurrence (≤18/>18 months), number of recurrence organs (1/>1) and randomized into a training set (n=339) and a validation set (n=168). A multiple time dependent Cox regression analysis was performed to identify independent prognostic variables (prognostic index PI) adjusted for the individual treatment the patients received and to build a risk score system.

Results: 507 patients met the in- and exclusion criteria. More than one third of patients (n=179) had palliative treatment only, 328 were treated with curative intent. The median survival time for all 507 patients to a second recurrence was 4.4 months, to death 9.0 months. The prognostic index (PI) included time to recurrence (≤18/>18 months; HR=2.02), liver metastasis at recurrence (yes/no; HR=1.86), liver metastasis at diagnosis (yes/no; HR=1.65), number of recurrence organs (1/>1; HR=1.35), and age at diagnosis (≤42/>42 months; HR=1.28). Based on an optimal stratification of the PI, three risk groups were built which robustly discriminated patients in the validation set. The proportions for 2-year secondary EFS proportions were 21%/7%/3%, ($p<0.001$) and for 2-year secondary OS 36%/17%/6% ($p=0.001$). The scoring system was similarly useful for the subgroup of patients treated with curative intent.

Conclusion: A new and robust risk score system for patients with first recurrence from stage 4 neuroblastoma aged ≥18months at diagnosis is described.

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MYCN Interferes with Estrogen and NGF Signaling to Maintain an Undifferentiated and More Aggressive Phenotype in Neuroblastoma

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Background: Neuroblastoma (NB) is a highly heterogenic childhood tumor of the sympathetic nervous system with phenotypes ranging from spontaneous regression to poorly differentiated tumors and metastasis. Amplification of the MYCN gene is found in approximately 20-30% of all NB cases and is linked to an undifferentiated phenotype and a poor prognosis. Estrogen receptor alpha (ERα) and the nerve growth factor (NGF) receptors TrkA and p75NTR are involved in neuronal differentiation and survival. We have previously shown that MYCN, via miR-18a, downregulates ERα in NB cells, which in turn results in reduced neuronal differentiation. This study aims to investigate the effect of ectopic ERα expression on the morphology and functional response of NB cells with MYCN amplification.

Results: Here we show that interference with miR-18a or overexpression of ERα is sufficient to induce NGF signaling and to modulate both basal and NGF induced neuronal differentiation in MYCN-amplified NB cells. Proteomic analysis confirmed an increase of neuronal features and showed that processes linked to tumor initiation and progression were inhibited upon ERα overexpression. Indeed, ectopic ERα expression was sufficient to inhibit metabolic activity and tumorigenic processes, including glycolysis, oxidative phosphorylation, cell viability, migration, and anchorage independent growth. In line with our in vitro results, tumor growth was significantly decreased in ERα overexpressing cells in vivo when injected subcutaneous into nude mice. Interestingly, in addition to ERα, a majority of other nuclear hormone receptors (NHRs), including the glucocorticoid receptor and the retinoic acid receptor, correlated to clinical markers for favorable and low-stage NB disease.

Conclusion: In summary, our data suggest that MYCN targets ERα and thereby NGF signaling to maintain an undifferentiated and more aggressive phenotype in neuroblastoma. Notably, we identified the estrogen-NGF crosstalk and a set of other NHRs as potential targets for the development of new therapeutic strategies against NB.

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Combination of HDAC Inhibitors with Standard Chemotherapeutics in Zebrafish Xenografts Provides A Promising Approach for Neuroblastoma Therapy

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The majority of high-risk neuroblastoma patients respond poorly to multimodal chemotherapy, thus the development of novel drug combinations and improved preclinical drug testing models are necessary to effectively target the disease. Histone deacetylases (HDACs) play an important role in numerous cellular processes, and their aberrant expression has been associated with a variety of malignancies, including neuroblastoma. We have previously reported selective inhibition of HDACs as a promising approach to sensitize aggressive neuroblastomas to chemotherapy. In the current study we describe a zebrafish neuroblastoma xenograft model and use it to evaluate efficacy of combination treatments involving standard chemotherapy and selected HDAC inhibitors (HDACis).

A suspension of fluorescently labeled BE (2)-C, IMR-32 or patient-derived neuroblastoma cells, that have been briefly expanded in culture, is injected into the yolk sac or pericardium of zebrafish larvae. Cancer progression is monitored using confocal microscopy and cell proliferation by quantifying the expression of Ki67 and phospho-Histone H3, immunomarkers specific for cells undergoing mitosis. The efficacy of selective HDACis (tubastatin A) vs. pan-HDACis (panobinostat), alone and in a combination with doxorubicin, is assessed by comparing tumor volumes and numbers of migrating tumor cells. To validate in vivo class-selective on target activity of specific HDACis, acetylated tubulin and acetyl Histone H4 levels are quantified in paraffin embedded larvae using immunohistochemistry.

On average, 40%-50% of tumor cells remain mitotically active following injection, and we are able to monitor cancer progression, including tumor cell migration, with single-cell resolution in an intact environment. Importantly, tubastatin A and panobinostat significantly increase the expression of acetylated tubulin (up to five-fold), indicating that the compounds are acting on target in vivo. Furthermore, we observe a reduction in tumor volume following monotherapy with doxorubicin or tubastatin A, and future studies will inform on the efficacy of combination treatments with these and other drugs. The assessment of cleaved caspase-3 expression following the exposure to the compounds, suggests increased apoptosis as one potential mechanism involved. In summary, our findings indicate that engraftment of neuroblastoma cells into zebrafish can facilitate earlier results on drug responsiveness and toxicity, before moving on to more costly and time-consuming mammalian studies.

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Chemical Repression of USP5 Expression Targets MYCN Protein Stability in Neuroblastoma

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Vorinostat (SAHA) has showed clinical success in the treatment of cutaneous T-cell lymphoma but lacks efficacy as a single agent in many solid tumours. We screened 10,560 randomly selected compounds to identify a small enhancer molecule, SE486, which was synergistic with SAHA in neuroblastoma cells. Development of a SE486-focussed library identified a more potent analogue, SE486-11, which significantly improved the cytotoxicity of SAHA in combination treatment across a panel of three MYCN amplified or c-MYC overexpressing neuroblastoma cell lines by reducing cell viability and proliferation, with minimal cytotoxicity to normal fibroblast cell lines and MYCN non-amplified neuroblastoma cell lines. Further in vitro testing showed significant increase in mitochondrial depolarization of cells treated with the combination therapy of SAHA (1µM) + SE486-11 (5µM), as well as a significant decrease in MYCN protein levels through increased MYCN ubiquitination, and reduced protein half-life. Combination treatment completely inhibited tumour growth in TH-MYCN homozygous mice.

Using SILAC (Stable Isotope Labelling by Amino Acids in Cell Culture) analysis of neuroblastoma cells treated with or without combination therapy, we identified Ubiquitin-Specific Protease 5 (USP5) expression to be decreased by 70% in the presence of SAHA+SE486-11 treatment. siRNA knock-down of USP5 decreased MYCN protein stability and reduced neuroblastoma cell viability, whereas over-expression of MYCN in BE (2)-C and Kelly cells rescued combination therapy induced cell death. Co-immunoprecipitation studies have not shown any direct interaction between MYCN and USP5 which is supported by USP5's known substrate specificity for unanchored polyubiquitin chains. It is hypothesized that the combination therapy inhibits the isopeptidase activity of USP5, resulting in an increase in the pool of unanchored polyubiquitin chains which then bind to over-expressed MYCN, targeting it for proteasomal degradation. Taken together our data identifies a novel therapeutic target, USP5, and a combination therapy, SE486-11+SAHA, in MYCN-driven neuroblastoma.

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HIF-2 Inhibition Using PT2385 Is Effective Only in Cellular Systems Mainly Driven by the HIF-2 Transcriptional Complex

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Hypoxia inducible factor-2 (HIF-2) has been shown to possess oncogenic features in several human cancers and activating mutations in the HIF-2α subunit were recently demonstrated in paragangliomas and pheochromocytomas. In neuroblastoma, high expression of HIF-2α in tumor cells located in perivascular niches is associated with metastatic disease, aggressiveness and a more immature, neural crest- and stem like tumor cell phenotype. Thus, HIF-2 is a potential therapeutic target in neuroblastoma. A specific HIF-2α inhibitor was recently developed, PT2385, that inhibits the dimerization between HIF-2α and its binding partner ARNT. PT2385 has shown promising results in clear cell renal cell carcinoma (ccRCC) cell lines and ccRCC patient-derived xenograft (PDX) models.

To test the drug in a neuroblastoma setting, we had PT2385 synthesized and treated our established neuroblastoma PDX cells cultured in serum-free, stem cell promoting medium. We show that even though PT2385 prevents dimerization between HIF-2α and ARNT in our PDX cells, only limited, overall effects are observed on classical downstream HIF-2 targets in neuroblastoma cells. This could, in part, be explained by the presence of HIF-1α in these culture systems since additional data show that the HIF-2 transcriptional program is severely dampened by PT2385 in tumor settings were HIF-2 is the main driver of disease or where bona fide HIF-2 targets have been identified. Another explanation for this weak response to PT2385 treatment in the neuroblastoma setting could be due to the fact that HIF-2α possesses an additional role other than acting as a transcription factor. Indeed, in our PDX cells, we observe a high expression of cytoplasmic HIF-2α protein, in particular at oxygenated conditions, suggesting that HIF-2α could have oncogenic functions that are independent of ARNT. Thus, based on our findings, it will be of importance to unravel ARNT-independent, specific mechanisms of HIF-2α to inhibit these mechanisms as a treatment strategy for children with non-treatable neuroblastoma.

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Impact of HACA On Immunomodulation and Pain Following Ch14.18/CHO Long-Term Infusion with Interleukin-2: A SIOPEN Phase 2 Trial

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Background: Short-term infusions of the chimeric anti-GD2 antibody (Ab) ch14.18/CHO improve survival of high-risk neuroblastoma (NB) patients (pts). We initiated a European multi-center Phase II clinical trial evaluating long-term infusion (LTI) to reduce treatment toxicity with particular focus on pain which is an on-target side effect of anti-GD2 Ab-based immunotherapies. Pain has been suggested to be mediated by complement-dependent cytotoxicity (CDC). Here, we report impact of human anti-chimeric (HACA) on ch14.18/CHO-dependent immunomodulation and pain intensity.

Patients: 124 high-risk NB pts received up to 5 cycles (35d/cycle) of a 10 day (d) continuous LTI of 10 mg/m2/d ch14.18/CHO (d8-18) (100 mg/m2/cycle) combined with 6x106 IU/m2/d s.c. IL-2 (d1-5; 8-12) and 160 mg/m2/d oral 13-cis RA (d19-32).

Results: 2/124 eligible pts progressed prior to first Ab treatment and were excluded from the analysis. In HACA-negative pts (99/122 pts), evaluation of ch14.18/CHO showed at the end of LTI concentrations of 11.24±0.50, 11.44±0.56, 13.48±0.52, 13.67±0.56 and 14.79±0.64 µg/ml in cycle 1, 2, 3, 4 and 5, respectively. Trough levels were >1µg/ml allowing long lasting anti-tumor effects.

Analysis of HACA revealed 23/122 (19%) pts who developed either HACA with strong negative impact on ch14.18/CHO (non-neutralizing HACA, 5/122 (4%) pts) or HACA completely neutralizing ch14.18/CHO (18/122 (15%) pts). HACA occurred early in the majority of pts (20/23 pts: cycle 1 or 2; 3/23 pts: after cycle 2).

HACA-negative pts showed strong GD2-specific CDC- (>95%) and ADCC-responses (>10%) on d15 of cycle 1, 3 and 5. In contrast, CDC and ADCC were abrogated in HACA-positive pts.

Evaluation of i.v. morphine usage confirmed a steady reduction resulting in morphine-free treatment starting on d6 of LTI in the majority of pts and further decrease in subsequent cycles. Validated pain scores were in line with the results of morphine analysis.

Surprisingly, there were no differences in pain intensity and i.v. morphine usage in HACA-positive pts showing complete abrogation of CDC.

Conclusions: LTI of ch14.18/CHO in combination with s.c. IL-2 results in a strong activation of Ab effector functions. Importantly, HACA response abrogated CDC but did not affect pain intensity indicating CDC-independent pain induction.

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Development of New DNA- and Protein Vaccines for Active Immunotherapy Against MYCN-Expressing Neuroblastoma

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Introduction: MYCN oncogene is overexpressed in high-risk neuroblastoma (NB) and associated with aggressive and refractory disease. Interleukin-21 (IL-21) is known to promote anti-tumor effects by stimulating cytotoxic and inhibiting regulatory T cells. Here, we developed protein- and IL-21-based DNA vaccines containing minigenes that encode for highly antigenic T cell epitopes of MYCN for active immunotherapy against MYCN-positive NB. To increase proteasomal degradation and MHC class I presentation of MYCN epitopes to cytotoxic T lymphocytes, an upstream ubiquitin sequence was additionally integrated in the DNA vaccine. For evaluation of anti-NB effects in vivo, syngeneic mice will be immunized with protein vaccine in combination with the AddaVaxTM adjuvant and DNA vaccine using attenuated Salmonella typhimurium SL7207 as vehicle.

Methods and Results: For protein vaccine, a DNA sequence containing MYCN minigenes with high affinity to MHC class I molecules combined with an upstream leader sequence for in vitro secretion was synthesized and inserted into a plasmid encoding for human IgG1 constant heavy chain (fusion protein) using standard molecular biology techniques. To enable permanent production of the fusion protein, CHO cells will be stably transfected with the generated plasmid. Finally, the fusion protein will be isolated from supernatant for in vivo vaccination.

For DNA vaccine, MYCN minigenes linked to an upstream ubiquitin sequence and a DNA fragment encoding for IL-21 were inserted into the respective multiple cloning site of the bicistronic expression vector pIRES allowing simultaneous expression of both inserts. Correct plasmid assembly of protein and DNA vaccines was confirmed by gene-specific PCR, restriction and sequence analysis. MYCN epitope expression and IL-21 production was approved in vitro by ELISA and Western blot after transfection of CHO cells with the respective plasmid.

Conclusion: We generated and partly characterized new MYCN-based DNA and protein vaccines for active immunotherapy against MYCN-expressing NB.

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The Role of HDACs in the Transcriptional Regulation of Autophagy in Neuroblastoma

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Autophagy is an evolutionarily conserved intracellular degradation process that can either suppress tumorigenesis or promote tumor chemoresistance and survival, depending on context and tumor stage. Histone deacetylases (HDACs) have been described to modulate autophagy, however the exact mechanism of action is unclear.

In this project we aim to characterize the effects of HDAC inhibitors (HDACi) on the transcriptional regulation of autophagy in neuroblastoma cells. Using cell culture-based techniques (e.g. immunofluorescence, PCR and western blot), we are investigating the effects of various HDACi on autophagy transcription factor (e.g. FOXO) activity as well as on autophagic flux and cellular lysosome content. We show that treatment of neuroblastoma cells with the FDA-approved pan-HDACi vorinostat and panobinostat at clinically relevant concentrations alters the activation state of FOXO1 and induces nuclear translocation of the transcription factors FOXO1 and FOXO3a. Treatment of neuroblastoma cells with either HDACi upregulated the expression of the pro-autophagic FOXO1/3a target genes WIPI1, ATG16L2, MAPLC3a, GABARAPL1, as well as FOXO1 itself. In line with this observation, treatment with either HDACi increased the number of autophagic vesicles in combination with the late-stage autophagic flux inhibitor bafilomycin A, suggesting that vorinostat and panobinostat increase autophagic flux.

Our results indicate that pan HDACi treatment affects autophagy at the level of transcription. Unravelling the impact of targeted therapy, such as HDACi treatment, on autophagy-competent tumor cells enables the identification of rational, novel combination treatment strategies for children with tumors that depend on autophagy as a treatment resistance mechanism.

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Dual Role of HDAC10 in Lysosomal Exocytosis and DNA Repair Promotes Neuroblastoma Chemo Resistance

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Drug resistance is a leading cause for treatment failure in many cancers, including neuroblastoma, the most common solid extracranial childhood malignancy. Previous studies from our lab indicate that histone deacetylase 10 (HDAC10) is important for the homeostasis of lysosomes, i.e. acidic vesicular organelles involved in the degradation of various biomolecules. Here, we show that depleting or inhibiting HDAC10 results in accumulation of lysosomes in chemotherapy-resistant neuroblastoma cell lines, as well as in the intracellular accumulation of the weakly basic chemotherapeutic doxorubicin within lysosomes. Interference with HDAC10 does not block doxorubicin efflux from cells via P-glycoprotein inhibition, but rather via inhibition of lysosomal exocytosis. In particular, intracellular doxorubicin does not remain trapped in lysosomes but also accumulates in the nucleus, where it promotes neuroblastoma cell death. Our data suggest that inhibition of HDAC10 further interferes with DNA double strand break (DSB) repair, providing an additional mechanism of sensitizing neuroblastoma cells to doxorubicin. Taken together, we demonstrate that HDAC10 inhibition in combination with doxorubicin kills neuroblastoma, but not non-malignant cells, both by impeding drug efflux and enhancing DNA damage, providing a novel opportunity to target chemotherapy resistance.

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The Functions of Histone Demethylases in Neuroblastoma

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Methylation of lysine residues on histones is an epigenetic mark that is dynamically regulated by histone methyltransferases and histone lysine demethylases (KDMs), which play crucial roles in regulating gene expression, cell cycle progression, and genomic integrity. Dysregulation of KDM activity occurs in many diseases including cancer. Some pediatric cancers (e.g., neuroblastoma) have very low frequencies of somatic mutations, which suggests that deregulated epigenetics is involved in the pathogenesis of those diseases. Oncogenic MYCN amplification is the most important biological feature of high-risk neuroblastoma. We recently found that MYCN expression is correlated with the aberrant expression of several histone demethylases, including KDM4B, which is required for neuroblastoma growth and tumor maintenance. Here we further discuss the functions of other histone demethylases in neuroblastoma and the potential to develop small molecule inhibitors of KDMs to target neuroblastoma.

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Hypoxic Preconditioning Promotes Metastasis of Neuroblastoma Cells and Cyclin-Dependent Kinase Inhibitors Reverse This In Vivo

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Background: Hypoxia occurs in solid tumours such as neuroblastoma and has been suggested to promote reprogramming of neuroblastoma cells leading to metastatic dissemination and tumour aggressiveness. It has been reported that more than 60% of neuroblastoma cases are invasive and despite treatment advances in the last decade, metastasis is still one of the major obstacles to overcome. To seek a new treatment, we examined the effect of cyclin-dependent kinase inhibitors, as potential therapeutic agents, on tumour progression and metastasis of neuroblastoma cells in vivo.

Methods: GFP-labelled neuroblastoma cells, BE2C and SKNAS cells, preconditioned in 1% or 21% O2 were implanted on the chorioallantoic membrane (CAM) of the chick embryo at embryonic day 7 (E7) to form tumours. CDK4/6 inhibitor (Palbociclib) and CDK1 inhibitor (RO-3306) were injected into the allantois of the chicks between E10 to E13 at 20μM. Cell proliferation and cell death were analysed by immunocytochemistry and TUNEL respectively. Metastasis of neuroblastoma cells was monitored by dissection of the chick embryo organs using fluorescence microscopy. The effect of CDK inhibitors on the expression of genes previously shown to be sensitive to hypoxia was assayed by qPCR.

Results: CDK inhibitor treatment reduced cell proliferation more in normoxic tumours compared to hypoxic tumours although there was little change in the numbers of cells undergoing apoptosis. Interestingly, metastasis of neuroblastoma cells was reduced significantly by 50% - 60% following treatment with a single dose of CDK inhibitors at E10. At a molecular level, exposure to hypoxia upregulated the expression of several genes thought to be involved in adhesion to the vascular endothelium, extravasation and/or matrix degradation, however, the CDK inhibitors largely reversed the effect of hypoxia.

Conclusion: We showed that CDK inhibitors have a vital role in controlling hypoxia-promoted metastasis and are hence contributing to the emergence of a potential new treatment for metastatic tumours.

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JMJD6 Is a Tumorigenic Factor in Neuroblastoma with Chromosome 17q21-ter Gain

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Chromosome 17q21-ter gain has been shown to be an indicator of poor neuroblastoma patient prognosis, however it is unclear which gene in this region is driving tumorigenesis. Here we show that the JMJD6 gene, located at 17q21-ter was gained in >25% of human neuroblastoma samples in a microarray gene expression dataset of 341 patients. JMJD6 gene expression positively correlating to N-Myc and c-Myc gene expression in human neuroblastoma patient samples and JMJD6 gene over-expression was found to be an independent prognosis factor for poor patient outcome.

RT-PCR and chromatin immunoprecipitation sequencing revealed that knocking down JMJD6 significantly reduced E2F2, N-Myc and c-Myc expression. Gene set enrichment analysis of JMJD6 knockdown microarrays showed a reduction in E2F and Myc target gene transcription. Reducing JMJD6 expression lead to a reduction in neuroblastoma cell proliferation and induced apoptosis in vitro. Knocking down JMJD6 expression in a neuroblastoma xenograft mouse model delayed tumour progression.

Chromatin immunoprecipitation sequencing revealed super enhancer elements associated with high H3K27 acetylation and H3K4 mono-methylation, and low H3K4 tri-methylation near the JMJD6 gene locus. Treatment of neuroblastoma cell lines with THZ1, a CDK7 inhibitor, and panobinostat, a pan-histone deacetylase inhibitor, synergistically reduced N-Myc, c-Myc, E2F2 and JMJD6 gene expression. The THZ1-panobinostat inhibitor combination synergistically reduced neuroblastoma cell proliferation in vitro and tumour progression in vivo. Thus, we have identified JMJD6 as a gene amplified in chromosome 17q21-ter gain that increases neuroblastoma progression. An inhibitor combination targeting CDK7 and histone deacetylases is shown as a novel strategy to treat neuroblastoma featuring chromosome 17q21-ter gain.

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Synergistic Effects of ALK and STAT3 Inhibitors on ALK Mutated Neuroblastoma Cells

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The presence of ALK aberrations relatively frequently observed in patients with high-risk neuroblastoma. ALK inhibitors such as crizotinib has provided novel treatment opportunities for malignancies associated with ALK translocations. Crizotinib inhibited proliferation of cell lines expressing either R1275Q-mutated ALK or amplified wild-type ALK. In contrast, neuroblastoma cells harboring F1174L-mutated ALK were relatively resistant to crizotinib. Inhibition of F1174L-mutated ALK remains a therapeutic challenge in neuroblastoma. To identify compounds with the potential of inhibiting oncogenic activity of ALK in neuroblastoma, we implemented a high throughput chemical screen in 23 neuroblastoma-derived cell lines, using a curated library of ~450 compounds. In the drug screen, JAK-STAT kinase inhibitor (cucurbitacin I) was the most discriminatory with regard to sensitivity for ALK-mutated cell lines. In neuroblastoma cell lines harboring F1174L or R1275Q-mutated ALK, crizotinib combined with cucurbitacin I enhanced tumor responses and showed synergistic cytotoxicity. Although crizotinib and cucurbitacin I alone or combination therapy (cucurbitacin I + crizotinib) did not result in decreased viability over control (PC-12 cells) compared with vehicle, the combination therapy in all of 6 cell lines with ALK aberrations and 10 of 13 ALK wild-type cell lines with MYCN amplification or 11q LOH was more effective than vehicle, crizotinib alone, and cucurbitacin I alone. Analysis of downstream molecules through MAPK, AKT and STAT3 pathways showed that NIH3T3 cells stably expressed F1174L ALK or TGW, a cell line harboring R1275Q-mutated ALK, expressed lower levels of pERK, pAKT and pSTAT3 in combination therapy compared with cells treated with cucurbitacin I or crizotinib alone. These findings may provide an indication that the combination of low-dose ALK and STAT3 inhibitors may be beneficial for the treatment of neuroblastoma, by enhancing efficacy while reducing toxicity.

In conclusion, our studies suggest that the combination of ALK and JAK-STAT inhibitors could be a valuable therapeutic option for high-risk neuroblastoma with potential clinical application.

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The Novel HDAC6/8/10 Inhibitor TH34 Induces DNA Damage-Mediated Cell Death in High-Grade Neuroblastoma

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High expression of both histone deacetylase (HDAC) 8 and HDAC10 has been identified to predict exceptionally poor outcomes in neuroblastoma, the most common extracranial solid tumor in childhood. Inhibition of HDAC8 synergizes with retinoic acid treatment, inducing neuroblast maturation in cell culture and slowing down neuroblastoma xenograft growth in mice. HDAC10 inhibition increases intracellular accumulation of chemotherapeutics through interference with lysosomal homeostasis, ultimately leading to cell death in cultured neuroblastoma cells. So far, no HDAC inhibitor specifically covering HDAC8 and HDAC10 while sparing HDACs 1, 2 and 3 in its inhibitory profile has been described.

Here, we introduce TH34, a novel HDAC6/8/10 co-inhibitor for neuroblastoma therapy. TH34 is well-tolerated by non-malignant human foreskin fibroblasts but induces caspase-dependent programmed cell death in high-grade neuroblastoma cell lines. Moreover, it strongly interferes with colony formation of various high-grade neuroblastoma cell lines with varying genetic backgrounds (e.g. MYCN amplification, ALK mutation, p53 mutation), whereas medulloblastoma and embryonal rhabdomyosarcoma cell lines do not respond to TH34 treatment. In neuroblastoma, targeted HDAC6/8/10 inhibition induces DNA double strand breaks (DSB), mitotic aberrations and cell-cycle arrest. TH34 treatment also leads to elevated levels of neuronal differentiation markers (TH, NTRK1, CDKN1A), mirrored by formation of neurite-like protrusions under long-term treatment. The combination of TH34 with plasma-achievable concentrations of retinoic acid synergistically (CI<0.1) inhibits neuroblastoma colony formation, ultimately eliminating high-grade neuroblastoma cells.

In summary, our study supports the usage of selective HDAC inhibitors as targeted antineoplastic agents and underlines the therapeutic potential of selective HDAC6/8/10 inhibition in high-grade neuroblastoma as well as potential applications in combination therapy.

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ALYREF Is a Novel MYCN Binding Protein and Co-Factor For MYCN-Driven Oncogenesis

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MYCN amplification is a poor prognostic factor in neuroblastoma patients and a well-established driver of neuroblastoma tumourigenesis. Therapies that directly repress the MYCN oncogenic signal in neuroblastoma are limited. We and others have shown that MYCN requires multiple cofactors to increase its protein stability in neuroblastoma cells, so that the very high MYCN levels required to drive tumourigenesis can be achieved.

Here, we have identified ALYREF, a nuclear molecular chaperone protein, as a novel regulator of MYCN function in neuroblastoma. Using co-immunoprecipitation and mass spectrometry, we identified ALYREF, as a direct binding partner of nuclear MYCN protein. Notably, high expression of ALYREF predicted poor neuroblastoma patient survival and substantially correlated with MYCN levels in a large dataset (n=649) of human neuroblastoma tumour samples. ALYREF mRNA expression was also significantly increased in ganglia cells from the homozygous TH-MYCN neuroblastoma mouse in comparison to ganglia from wild-type littermates. Chromatin immunoprecipitation showed that MYCN bound the ALYREF gene promoter, and MYCN siRNA decreased ALYREF expression. A set of over-expression and knockdown experiments in MYCN-amplified neuroblastoma cells revealed that MYCN and ALYREF form a forward feedback expression loop. Overexpression of ALYREF further increased MYCN expression and protein stability in MYCN-amplified neuroblastoma cells. We found that ALYREF had a critical function in regulating the turnover of MYCN protein through transcriptional repression of the E3 protein ubiquitin ligase, NEDD4. Additionally, we demonstrated that ALYREF increased cell viability and proliferation of MYCN-amplified neuroblastoma cells.

Taken together, our findings demonstrate a crucial role for ALYREF in regulating MYCN function and suggest that ALYREF-mediated stabilization of MYCN protein contributes to the development of high risk disease. This function is critical for the growth of MYCN-amplified neuroblastoma cells thus inhibition of ALYREF activity using small molecules is a potential therapy for MYCN-amplified tumours.

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Coordinate MYCN Regulation of The Neuroblastoma Polyamine Pathway Can Be Effectively Targeted by Combined Inhibition of Polyamine Synthesis and Transport

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We previously demonstrated that the MYCN/MYC transcriptional target, ornithine decarboxylase (ODC1), the rate-limiting enzyme for polyamine biosynthesis, is an effective therapeutic target in preclinical models of neuroblastoma. An early phase clinical trial based on these findings is underway combining DFMO, celebrex (an inducer of catabolic SAT1) and combination chemotherapy. Although a promising therapeutic strategy, DFMO treatment can result in compensatory increases in polyamine uptake from the surrounding microenvironment, limiting its effectiveness in impeding cell growth. We have therefore focused on combining inhibition of polyamine biosynthesis with inhibition of cellular polyamine uptake, in order to optimise polyamine depletion as a promising new strategy for neuroblastoma treatment.

Using a 649-patient dataset and Kaplan-Meier analysis, we show that high expression of each of the 6 polyamine biosynthetic genes and low expression of each of the 5 catabolic genes in neuroblastoma tumors is associated with a poor event-free and overall survival. ChIP and luciferase reporter assays were used to demonstrate that each biosynthetic gene is positively and directly regulated by MYCN in neuroblastoma cells whereas each catabolic gene is negatively regulated. Furthermore, we have now identified SLC3A2 as a key transporter involved in the uptake of polyamines in neuroblastoma and demonstrate in a range of MYCN-amplified cell lines that siRNA-mediated knockdown of this gene leads to reduced radiolabelled spermidine uptake, while DFMO treatment increases SLC3A2 protein expression. We also show that SLC3A2 is directly and positively regulated by MYCN.

Using the polyamine transport inhibitor, AMXT1501, in combination with DFMO, we show that combined inhibition of polyamine uptake and synthesis is highly effective at abrogating tumor formation when given prophylactically to TH-MYCN mice, and significantly extends survival when given to mice with established tumors. Furthermore, the combination of DFMO, AMXT1501, cyclophosphamide and topotecan resulted in delayed tumor formation and doubled survival rates in TH-MYCN mice compared with those treated with the 4-drug protocol (DFMO, Celebrex, cyclophosphamide, topotecan) currently in clinical trial.

Our findings thus indicate that combining AMXT1501 and DFMO with standard chemotherapy for neuroblastoma represents a potentially valuable treatment strategy for future clinical trial.

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Inhibition of MYCN Transcription by M606, A Novel Small Molecule Inhibitor Identified Through Chemical Library Screening

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Background: Aberrant expression of Myc oncoproteins is a major causal factor in human cancer. The MYCN oncogene is one of the most powerful prognostic markers identified in neuroblastoma and a potentially valuable target for the development of novel therapeutics. We aimed to identify MYCN inhibitors using chemical library screening.

Methods: A chemical library of 34,000 diverse small molecules was screened using a cell-based assay. Signal transduction pathway analyses, PCR, Western blotting analysis, siRNA knockdown, luciferase reporter assay, ChIP assay, GC-MS based metabolomics were used to characterise hit compounds.

Results: Among a number of molecules identified, M606 was found to reduce MYCN protein levels and its downstream targets in MYCN-amplified BE(2)-C cells. Analysis of signalling pathways affected by M606 using FACTORIAL™ technology (Attagene Inc) indicated that this compound inhibited Myc-mediated transcription and activated the HIF1 pathway, while stability assays showed MYCN protein and mRNA stability were unaltered. siRNA-mediated knockdown of c-Myc/MYCN or HIF1A in HepG2 (hepatocellular carcinoma) and BE(2)-C cells followed by M606 treatment demonstrated that Myc downregulation and HIF1a upregulation by M606 are two independent events. Metabolomics analysis showed that the mechanism underlying the ability of M606 to inhibit MYCN involved its capacity to bind iron. Furthermore, the inhibition of MYCN promoter activity by M606 was reversed by the addition of iron. Iron also reversed the effect of M606 on the levels of HIF1a and MYCN protein. Luciferase deletion assays identified a minimal response region of M606 on the MYCN promoter. This region contains two E2F sites, the deletion of which resulted in an M606 unresponsive promoter. ChIP assays also confirmed the presence of E2F sites on the MYCN promoter and further analysis found iron restores the E2F-1 binding to the MYCN transcription start site following M606 treatment. Moreover, western blot analysis showed that the RB protein, which is required for E2F binding and transcriptional activation, becomes inactive due to hypophosphorylation following M606 treatment.

Conclusions: M606 is a novel Myc inhibitor that chelates iron to directly downregulate MYCN/Myc transcription; this mechanism of action represents a potentially valuable therapeutic approach in the treatment of cancers overexpressing Myc oncoproteins.

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The Ornithine Decarboxylase G316A Promoter Polymorphism Impacts Neuroblastoma Clinical Outcome and May Identify Patients Particularly Susceptible to Polyamine Inhibition

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Background: Polyamines are highly regulated essential cations that are elevated in rapidly proliferating cells. Ornithine decarboxylase (ODC1), the rate limiting enzyme in polyamine synthesis, is elevated in many cancer types including neuroblastoma, and is a direct transcriptional target of the MYCN oncoprotein. We have shown high ODC1 expression independently predicts poor clinical outcome in neuroblastoma, and that the suicide ODC1 inhibitor, difluoromethylornithine (DFMO), delays neuroblastoma development and progression in multiple preclinical models. A phase I clinical trial combining DFMO with chemotherapy is currently underway. An ODC1 single nucleotide polymorphism (SNP), G316A, has been shown to influence outcome in several cancer types. Here, we examine the clinical significance of this SNP in neuroblastoma.

Methods: 839 primary neuroblastomas were genotyped for the G316A SNP, and Kaplan-Meier analysis performed. Cell proliferation, histone acetylation, EMSA, qRT-PCR, promoter activity and colony assays were used to characterise neuroblastoma cell clones with differing endogenous or CRISPR-cas9-induced SNP genotypes.

Results: The GG genotype occurred at a frequency of 60.3%, AG 32.4% and AA 7.3%. In contrast to reports with other cancers, the G allele was associated with worse outcome, with AG/GG genotypes associated with adverse outcome in patients with MYCN non-amplified tumors, while in amplified tumors the homozygous GG genotype identified a subset of patients with dismal outcome. CRISPR/Cas9-derived SK-N-BE (2)-C cell clones with the AG genotype demonstrated reduced cell proliferation, ODC1 expression, and histone acetylation within the SNP region, compared to parental cells with the GG genotype. Additionally, AG clones were also more sensitive to DFMO treatment ($P<0.005$). Furthermore, increased promoter activity was driven from constructs with the GG genotype compared to those with AA, and EMSA showed that MYCN preferentially bound the G allele over the A allele. Induced expression of MYCN in SH-EP-TET-21/N cells had a greater stimulatory effect on promoters containing the G allele.

Conclusions: The ODC1 G316A polymorphism has both prognostic and functional significance in neuroblastoma. The GG genotype predicts poor clinical outcome and is associated with increased MYCN

binding and increased ODC1 expression. Our findings suggest that ODC1 SNP genotyping will identify subsets of patients particularly responsive to polyamine inhibition therapy.

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Combined RNA Sequencing and Mutation Analysis Classified High-Risk Neuroblastoma Into 3 Groups in Terms of Differentiation and Expression Pattern

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Introduction: Recent genome-wide studies have revealed that there were few recurrent somatic mutations in high-risk Neuroblastoma (NBL), thought to originate from undifferentiated neural crest cells (NCC), and that the clinical course was strongly influenced by the presence of chromosomal aberrations rather than gene mutations. Furthermore, based on the landscape of super-enhancer, NBL was divided into two subtypes, Noradrenergic (ADRN) and Mesenchymal (MES), which showed distinct expression pattern in core regulatory circuitry related genes. However, these genetic changes do not explain entire genetic mechanisms of high-risk NBL, and our knowledge about molecular basis of high-risk NBL is still limited.

Methods: To unravel genetic basis of high-risk NBL, we performed RNA sequencing and targeted capture sequencing in 30 specimens with stage 4 NBL.

Results: Unsupervised consensus clustering combined with 4 samples of normal adrenal gland from open database identified two expression clusters, ADRN-cluster and MES-cluster. These clusters showed distinct expression patterns validated by the same gene set which used for classification of ADRN-cluster and MES-cluster in a previous report. ADRN-cluster was consisted of 25 NBL cases with high expression of HAND2 and GATA3 and remaining 5 NBL cases with high ETV6 expression were classified into MES-cluster with normal adrenal gland samples. Pathway analysis revealed that pathways related to neuron and synapse were enriched in ADRN-cluster which is consistent with the feature of early type in NCC differentiation. In contrast, secretion and vesicles related pathways were enriched in MES-cluster, consistent with chromaffin cell-like phenotype, which could be considered as the late type of NCC differentiation originating from schwann cell precursors newly described recently. ADRN-cluster was further divided into 2 clusters, ATRX-cluster and MYCN-cluster, by second unsupervised consensus clustering. ATRX-cluster contained all cases with ATRX abnormality and showed high NTRK1 expression. MYCN-cluster had all cases with MYCN amplification.

Conclusion: In total, NBL was classified into 3 clusters, 2 ADRN clusters (MYCN-cluster and ATRX-cluster) and MES-cluster. Our results revealed that NBL is heterogeneous disease in terms of differentiation. To improve prognosis of high-risk NBL, considering of origins and expression pattern might be helpful for development a new therapeutic strategy for high-risk NBL.

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Therapeutic Inhibition of MYCN by Targeting AKT Activity in High-Risk Neuroblastoma

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Neuroblastoma is the most common paediatric solid tumor. Approximately half of all neuroblastoma patients are diagnosed with high-risk disease. Amplification of the MYCN oncogene is highly predictive of poor prognosis and occurs in 45% of high-risk neuroblastoma cases. Currently there are no direct inhibitors of MYCN, and outcomes remain below 50% in this patient population.

One possible strategy to treat MYCN-amplified neuroblastoma patients is antagonizing proteins involved in stabilizing MYCN protein. The AKT/GSK3β pathway directly regulates stabilization of MYCN, providing a therapeutic rational for using AKT inhibitors. In this study, we addressed the question of how AKT can play a pivotal role in neuroblastoma and whether the AKT inhibitor perifosine can be a promising anti-cancer drug in children diagnosed with MYCN-amplified neuroblastoma.

Our data revealed that high gene expression of AKT1 and AKT2 was correlated with MYCN amplification and was significantly associated with poor outcome in neuroblastoma patients. By RNAi-mediated depletion of AKT isoforms, we demonstrated that the total AKT activity rather than expression of particular isoforms was necessary to cause a significant decrease in neuroblastoma cell proliferation. These results demonstrated the potential of targeting AKT activity in neuroblastoma. Using perifosine, one of the most clinically promising AKT inhibitors, we showed that perifosine exerted anti-tumoral activity as a single agent against all neuroblastoma cell tested. Interestingly, combining perifosine with conventional cytotoxic drugs currently used in neuroblastoma treatment led to a synergistic interaction more importantly in MYCN-amplified neuroblastoma cell lines. Mechanistically, inhibition of AKT activity using either RNA interference or pharmacological inhibition through perifosine, led to significantly downregulate MYCN expression through GSK3β regulation.

Collectively, these promising results unveil the existence of a cross talk between MYCN and AKT and provide evidence that targeted inhibition of AKT in combination with conventional drugs could be a novel therapeutic strategy to indirectly target MYCN in neuroblastoma. Focusing on perifosine, already in clinical trials, and anticancer drugs currently used in the clinic, we hope to identify novel therapeutic combinations, which can rapidly impact on outcome of children with high-risk neuroblastoma.

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Growth Factor Receptor Trafficking in Neuroblastoma Differentiation

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Background/Objectives: UBE4B is an E3/E4 ubiquitin ligase involved in growth factor receptor (GFR) trafficking. The UBE4B gene is located in the chromosome 1p36 region commonly deleted in high-risk neuroblastoma tumors. We have previously identified associations of UBE4B expression with neuroblastoma patient outcomes. However, the functional roles of UBE4B in neuroblastoma tumor differentiation are not known.

Design/Methods: We analyzed the association of UBE4B gene expression with expression of known markers of neuroblastoma tumor differentiation using publicly available databases and screened cell lines and neuroblastoma tumor samples for UBE4B protein expression using Western blot and quantitative immunohistochemistry. We measured UBE4B expression by Western blot in cell lines before and after induction of differentiation with 13-cis-retinoic acid treatment and determined the effects of UBE4B overexpression and depletion on retinoic acid-induced differentiation using continuous live-cell imaging of neurite morphology, immunohistochemistry, and Western blot for markers of differentiation. Effects on signaling through the Ras/MAPK pathway were measured using Western blots.

Results: UBE4B expression was associated with neuroblastoma differentiation in patient tumors, and UBE4B gene and protein expression were associated with expression levels of known markers of neuroblastoma differentiation. Retinoic acid treatment resulted in increased UBE4B expression in retinoic acid-sensitive, but not -resistant, neuroblastoma cells, and UBE4B depletion was associated with increased ERK phosphorylation, increased proliferation, and inhibition of retinoic acid-induced neuroblastoma differentiation.

Conclusion: We have demonstrated associations between UBE4B expression and tumor differentiation in gene expression databases and in neuroblastoma tumor samples, and our data suggests that UBE4B expression is required for retinoic acid-induced differentiation, potentially through effects on activation of the Ras/MAPK pathway. These associations between UBE4B and neuroblastoma differentiation combined with the established role of UBE4B in GFR trafficking suggest that UBE4B-mediated receptor trafficking may contribute to the responses of neuroblastoma tumors to therapy and to the outcomes of patients with neuroblastoma.

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EZH2 Regulates Neuroblastoma Cell Differentiation Via NTRK1 Promoter Epigenetic Modifications

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The polycomb repressor complex 2 molecule EZH2 is now known to play a role in essential cellular processes, namely, cell fate decisions, cell cycle regulation, senescence, cell differentiation, and cancer development/progression. EZH2 inhibitors have recently been developed; however, their effectiveness and underlying molecular mechanisms in many malignancies have not yet been elucidated in detail. Although the functional role of EZH2 in tumorigenesis in neuroblastoma (NB) has been investigated, mutations of EZH2 have not been reported. A Kaplan-Meier analysis on the event free- and overall survival of NB patients indicated that the high expression of EZH2 correlated with an unfavorable prognosis. In order to elucidate the functional roles of EZH2 in NB tumorigenesis and its aggressiveness, we knocked down EZH2 in NB cell lines using lentivirus systems. The knockdown of EZH2 significantly induced NB cell differentiation, e.g. neurite extension, and the neuronal differentiation markers, NF68 and GAP43. EZH2 inhibitors also induced NB cell differentiation. We performed a comprehensive transcriptome analysis using Human Gene Expression Microarrays and found that NTRK1 (TrkA) is one of the EZH2-related suppression targets. The depletion of NTRK1 canceled EZH2 knockdown-induced NB cell differentiation. Our integrative methylome, transcriptome, and chromatin immunoprecipitation assays using NB cell lines and clinical samples clarified that the NTRK1 P1 and P2 promoter regions were regulated differently by DNA methylation and EZH2-related histone modifications. The NTRK1 transcript variants 1/2, which were regulated by EZH2-related H3K27me3 modifications at the P1 promoter region, were strongly expressed in favorable, but not unfavorable NB. The depletion and inhibition of EZH2 successfully induced NTRK1 transcripts and functional proteins. Collectively, these results indicate that EZH2 plays important roles in preventing the differentiation of NB cells and also that EZH2-related NTRK1 transcriptional regulation may be the key pathway for NB cell differentiation. ONCOGENE, in press.

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Long-Term Outcome of The GPOH NB97 Trial for Children with High-Risk Neuroblastoma Comparing Myeloablative Therapy and Oral Chemotherapy as Consolidation

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Background: A metanalysis of the three published randomized trials with additional follow-up data on 739 patients concluded that myeloablative therapy (ASCT) seems to work in terms of event free survival (EFS), but not for overall survival (OS) (Cochrane Database Syst Rev, 2015(10): p. CD006301). The purpose of this study is the long-term outcome analysis of the NB97 trial after 12 years more of observation.

Patients and Methods: 295 patients with neuroblastoma stage 4 ≥1year or with MYCN amplified tumors and stages 1/2/3/4S were randomly assigned to myeloablative therapy (melphalan, etoposide, carboplatin) with ASCT or to maintenance therapy (MT) with oral cyclophosphamide cycles for 3 months. The outcome data of the randomized treatment groups were updated to 15-SEP-2017 and analyzed according to intent-to-treat (ITT), as treated (AT), and treated as randomized (TAR).

Results: The 10-year EFS proportions were superior for the patients receiving ASCT compared those treated with MT in all three cohorts (ITT: 36% for ASCT vs. 27% for MT, n=149/146, log rank p=0.022; AT: 43 vs. 26%, n=110/102; p=0.001; TAR: 46 vs. 25%, n=75/70, p=0.001). The 10-year OS proportions were also in favor of the ASCT group (ITT 41 vs. 35%, p=0.075); AT: 46 vs. 32%, p=0.017; TAR: 49 vs. 31%, p=0.005). The remission status (CR/VGPR vs. PR/MR/SD) before the intervention, stage (4 vs. 1/2/3/4S) and further treatment (antibodies vs. isotretinoin) had no influence on EFS/OS within the ASCT or MT groups, while MYCN amplification was more important for the MT and LDH elevation important for both groups. Late sequelae as hearing, renal, thyroid, hepatic, cardiac, neurological impairment and growth retardation were substantial. Focal nodular hyperplasia was more frequent in the MT cohorts (ITT/AT/TAR) and hearing impairment more in the ASCT group (TAR).

Conclusion: The long-term results confirmed an outcome advantage of myeloablative therapy over maintenance therapy as well for EFS as for OS. The late sequelae of the survivors were substantial, but not much different between the groups.

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The Utility of Phosphorylated ERK Immunohistochemical Staining for Selecting MEK Inhibitor-Sensitive Neuroblastoma Patients

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Background: Several MEK inhibitors have been reported to be effective against neuroblastoma with MAPK activation. Selecting appropriate patients with MEK inhibitor-sensitive neuroblastomas is necessary for the clinical application. We evaluated two MEK inhibitors' therapeutic effects and analyzed the ERK phosphorylation in vitro and in vivo. We also analyzed the phosphorylated ERK (pERK) immunohistochemical staining (IHC) in clinical neuroblastoma samples as a candidate biomarker for MEK inhibitor treatment.

Materials & Methods: We used Trametinib (MEK inhibitor) and CH5126766 (RAF/MEK inhibitor). The in vitro study included a cell viability assay, Western blotting of pERK and MYCN, immunocytochemical staining of pERK in five neuroblastoma cell lines. The in vivo study was performed using neuroblastoma xenograft mice in which three neuroblastoma cell lines had been transplanted. Either Trametinib or CH5126766 was administered for two or eight weeks. The tumor sizes were measured, and the tumors were then harvested at the endpoint for pathological analyses (H&E, pERK and Ki67). Clinical neuroblastoma samples from 38 patients (35 primary samples, 16 post-chemotherapy samples, 3 relapsed samples) were stained with pERK and analyzed for the correlation with the prognosis.

Results: Both MEK inhibitors inhibited the proliferation of cells with ERK phosphorylation in vitro. pERK IHC-positive xenograft tumors were sensitive for MEK inhibitors during the first four weeks and started to regrow from five weeks. Regarding the clinical neuroblastoma samples, 9 of 35 (26%) primary tumors were nuclear pERK-positive. However, only 1 of 9 (11%) pERK-positive primary tumors relapsed, whereas 6 of 16 (38%) post-chemotherapeutic tumors were nuclear pERK-positive, and 4 (67%) of 6 pERK-positive post-chemotherapeutic tumors relapsed.

Discussion: Consistent with previous reports, MEK inhibitors were preclinically effective against MAPK-activated neuroblastomas that could be identified by pERK IHC in this study. Regarding the clinical samples, one-quarter of primary tumors were pERK-positive, although only a small percentage of primary neuroblastomas have been reported to have MAPK pathway mutations. However, most of these pERK-positive primary tumors were well-treated with conventional chemotherapy. In contrast, post-chemotherapeutic pERK-positive tumors had a relatively high frequency of relapse. MEK inhibitors may be useful for treating post-chemotherapeutic pERK-positive tumors.

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Upregulation of The PD1/PD-L1 Immune Checkpoint Pathway in Neuroblastoma by Ch14.18/CHO and Hu14.18-IL-2 and Effects of PD-1 Blockade in Vivo

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Background: Antibody (Ab)-based immunotherapies directed against GD2 are effective against neuroblastoma (NB), but strategies to further improve treatment efficacy are needed. Recently, ligand expression of programmed death 1 inhibitory receptor (PD-L1) on NB cells has been reported. Here, we show impact of GD2-specific ADCC and IL-2 on the PD-1/PD-L1 immune checkpoint pathway and efficacy of ch14.18/CHO-based immunotherapy in combination with PD-1 blockade in vivo.

Methods: LAN-1 NB cells were cultured with effector cells (E/T 10:1, 24h) and subtherapeutic concentrations of ch14.18/CHO or hu14.18-IL-2 immunocytokine (IC). Expression of PD-L1 was analyzed by flow cytometry. Rituximab and rituximab-IL-2 served as negative controls. Mice (n=10) were treated with ch14.18/CHO (5x300 µg, i.p.) alone, anti-PD-1 (8x250 µg, i.p.) alone or ch14.18/CHO in combination with anti-PD-1.

Results: ADCC with ch14.18/CHO (10 and 50 ng/ml) induced a strong PD-L1 expression on both NB and effector cells (2-fold increase for NB and 5- and 7-fold for granulocytes and monocytes, respectively). These effects were further increased by IL-2 (100 IU/ml; 3-, 7- and 9-fold, respectively). IL-2 alone induced PD-L1 only on granulocytes and monocytes (2-fold). Pre-incubation with the anti-idiotype Ab ganglidiomab completely abrogated ch14.18/CHO-dependent PD-L1 induction showing GD2-specificity of the observed effects.

PD-L1 analysis performed with the equivalent IC concentrations (12.5 and 62.5 ng/ml) revealed stronger effects compared to ch14.18/CHO on both NB (2- and 3-fold increase) and effector cells (7- and 7-, 9- and 16-, and 2- and 2-fold increase for granulocytes, monocytes and lymphocytes, respectively). Incubation of both NB and effector cells with only IC or corresponding IL-2 concentrations (37.5 and 187 IU/ml) revealed induction of PD-L1 only by granulocytes and monocytes (3-, 3-, 3- and 3-fold for granulocytes and 4-, 3-, 5- and 4-fold for monocytes, respectively). Again, ganglidiomab completely abrogated IC mediated upregulation of PD-L1.

Finally, compared to controls treatment of mice with ch14.18/CHO in combination with anti-PD-1 showed reduced tumor growth and prolonged survival.

Conclusions: ADCC with ch14.18/CHO and hu14.18-IL-2 upregulate PD-L1/PD1 checkpoints and PD-1 blockade in vivo augments anti-neuroblastoma immune response induced by anti-GD2 Ab suggesting a rationale to combine anti-PD1 with anti-GD2 treatment in clinical trials.

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Optimizing GD2 Directed T Cells for The Treatment of Neuroblastoma

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Antibodies targeting GD2 have significantly increased survival in patients with high risk neuroblastoma. However, despite the combination of GD2 antibodies with high intensity chemotherapy, many children with high risk disease continue to have poor outcomes. Unlike antibodies, T cells are long-lived, actively patrol the body and can infiltrate tumor tissues. Therefore, we have evaluated T cells redirected to GD2 using chimeric antigen receptors (CARs) that combine a GD2 antibody-derived ScFv (14g2a) with the ζ chain of the T cell receptor (GD2.CARTs).

In our first study of GD2.CARTs for neuroblastoma, using a construct without costimulatory endodomains, 3 of 11 patients achieved complete tumor responses. In a second study we evaluated a 3rd generation GD2.CAR encoding costimulatory domains from CD28 and OX40. 2 of 8 patients who received GD2.CARTs in combination with lymphodepletion entered CR after salvage chemotherapy.

To improve on these results, we have further optimized our approach in preclinical studies by determining the best costimulatory domain and employing an innovative approach to provide necessary pro-survival cytokines to GD2.CARTs. We modified the hinge region of the GD2.CAR and replaced the CD28 and OX40 endodomains with a 4-1BB endodomain that promoted the development of a central memory phenotype and produced higher antitumor activity. We next incorporated a constitutively active IL-7 receptor (C7R) into GD2.CARTs to supply cytokine without affecting bystander cells and without causing cytokine-induced toxicity. We chose the IL-7 receptor, since IL-7 not only induces T cell proliferation, but also improves T cell survival. In preclinical testing, C7R enabled the expansion of CAR-stimulated T-cells in the absence of cytokines and prevented early T cell exhaustion and apoptosis but did not produce autonomous proliferation of mature T cells in the absence of cognate tumor antigen or costimulation. The combination of the optimized GD2.CAR with the constitutive C7R vector improved tumor cell killing in serial co-culture assays and in mouse xenograft models of both subcutaneously engrafted and metastatic neuroblastoma. In summary, optimization of costimulation and cytokine signal lead to improved anti-tumor efficacy of GD2.CARTs in preclinical neuroblastoma models and a clinical trial evaluating this approach in high-risk neuroblastoma will open this year.

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IND-Enabling Preclinical Evaluation of NKT Cells Expressing A GD2-Specific Chimeric Antigen Receptor and IL-15 For Treating Neuroblastoma

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Vα24-invariant natural killer T cells (NKTs) are attractive effector cells for expression of tumor-specific chimeric antigen receptors (CARs) due to their natural anti-tumor properties and ability to preferentially localize to the tumor site in neuroblastoma (NB) and other solid tumors. We previously demonstrated that adoptively transferred human NKTs expressing GD2-specific CARs (CAR.GD2) more effectively localize to the tumor site than T cells and mediate anti-tumor activity in a xenogenic model of NB in NOD/SCID/IL-2Rγnull mice without xenogenic graft-versus-host disease (GvHD).

In this study, we explore whether inclusion of IL-15, the main homeostatic cytokine for NKTs, within the CAR.GD2 construct further enhances NKT cell in vivo persistence and therapeutic efficacy. To that end, we synthesized CAR.GD2 constructs with a costimulatory CD28 or 41BB endodomain with or without IL-15. NKTs transduced with CD28/IL-15 and 41BB/IL-15 CARs secreted similar levels of IL-15 and significantly improved NKT cell in vitro expansion compared with CARs lacking IL-15 in response to repeated stimulation with NB cells. After transfer to mice with human NB xenografts, NKTs expressing IL-15-containing CARs persisted significantly longer compared with those expressing CARs lacking IL-15. In particular, CD28/IL-15 CAR NKTs underwent progressive in vivo expansion at sites of NB metastases, with the frequency of CD28/IL-15 CAR NKTs reaching 30% of bone marrow cells two months after a single injection. Treatment with CD28/IL-15 CAR NKTs resulted in a 70-day median survival compared with 42 - 53 days in NKTs expressing any other CAR construct or unmodified NKTs ($p < 0.001$). Despite robust in vivo expansion and potent anti-tumor activity, CD28/IL-15 CAR NKTs did not produce significant toxicity or induce GvHD as determined by detailed pathological analysis of mouse tissues.

Thus, combined use of the CD28 costimulatory endodomain and IL-15 in the CAR.GD2 construct promotes enhanced in vivo expansion and anti-tumor activity of CAR.GD2 NKTs cells without evident toxicity in a xenogenic NB model. These results have enabled an investigational new drug application for GD2-specific CAR and IL-15 expressing autologous NKT cells for treatment of children with relapsed or refractory neuroblastoma in a phase I clinical trial (NCT03294954).

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Metabolism of Catecholamine and Metanephrines in Ectopic Xenograft Models of Neuroblastoma

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Background: Metabolism of catecholamine and metanephrines (MNs; normetanephrine, metanephrine and methoxytyramine, NMN, MN, and MT respectively) remains enigmatic in patients affected by neuroblastoma (NB). Source of plasma MNs in patients are suspected to directly arise from tumor, as extensively described for pheochromocytoma and paraganglioma two other neuroendocrine tumors secreting MNs. However, NB biopsy availability is a limitation, precluding large scale molecular and cellular studies to decipher catecholamine metabolism in these tumors.

Aim: To validate the use of murine NB xenografts as a model to study catecholamine metabolism in NB.

Methods: NB xenografts were generated by subcutaneous injection of primary and established NB cell lines into athymic nude mice (Crl:NU(NCr)-Foxn1nu). MNs (free, sulfated and glucuronated forms) were measured in plasma and tumors by HPLC MS/MS and mRNA expression levels of genes involved in catecholamine metabolism were quantified by qPCR.

Results: Sum of total plasma MNs (MN, NMN and MT) was quantified at a geo. mean of 246 nmole/l in NB-bearing mice (n=4), representing a 2.5-fold increase compared with control mice (102 nmole/l, $p<0.0095$ n=7). These values in NB-bearing mice are highly comparable with those recorded in a cohort of patients (n=10), and the relative amount of each MNs (ie: NMN>MT>MN) were also similar. Interestingly, we observed in murine plasma an elevated fraction of glucuronated forms of MNs and virtually no sulfated MNs in contrast to human plasma where sulfated forms prevail. Surprisingly, intratumoral concentration of MNs were found to be very low (less than 1 nmol/g of tissue) compared with plasma values in both patients and xenograft mice models, contrasting with MNs values commonly observed for pheochromocytoma and paraganglioma (>100 nmol/g of tissue). Besides, as observed in human NB, norepinephrine and dopamine are found in large excess compared to epinephrine in NB xenografts. Moreover, mRNA expression levels of the main genes involved in catecholamine metabolism, ie TH, DBH, MAOA, COMT, PNMT, NET, VMAT1, and VMAT2 were also similar between biopsies from patients and NB xenografts.

Conclusion: Our results demonstrate that murine subcutaneous NB xenograft models are highly suitable for studying catecholamine and MNs metabolism in NB tumor and blood.

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Phase I Study of OKT3 x hu3F8 Bispecific Antibody (GD2Bi) Armed T cells (GD2BATs) in Recurrent High-Risk Neuroblastoma (NB)

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Background: Despite the initial success of anti-GD2 monoclonal antibodies in eradicating minimal residual disease (MRD) in NB, there is a need for a further development of anti-GD2 strategy to improve outcomes in patients with more than MRD. We hypothesized that arming of ex vivo expanded and activated T cells (ATC) with chemically conjugated GD2Bi would redirect ATC to NB and result in enhanced cytotoxicity. ATC coated with small amounts (50 ng per 10e6 cells) of GD2Bi exhibited significant specific killing of NB cell lines and secreted high levels of Th1 cytokines.

Methods: In a phase I study (NCT02173093, opened 11/2013), patients with GD2-positive tumors including recurrent NB, osteosarcoma and desmoplastic small round cell tumor (DSRCT) received 8 biweekly infusions of GD2BATs + daily low-dose IL-2 and biweekly GM-CSF. ATC were produced as described in Lum LG et al. Biol Blood Marrow Transplant 2013;19:255-33. The study followed the standard 3+3 design with dose levels of 40, 80, and 160 x 10e6 cells/kg/infusion. Immune monitoring was performed at pre and post GD2BATs infusions for anti-NB responses by IFNgamma EliSpots and direct cytotoxicity in peripheral blood lymphocytes.

Results: Eleven children and young patients with NB (6), osteosarcoma (3), DSRCT (2) were enrolled and 9 patients treated up to date. Infusions were given in outpatient settings and no unexpected toxicities were observed across all dose levels. Almost all patients developed relatively mild and manageable form of cytokine release syndrome with grades 2-3 fevers/chills, headaches and occasional hypotension for up to 48 hours after infusion. No patients developed pain. MTD has not been reached. Out of 6 NB patients, one patient failed to grow cells, 3 patients completed infusions, and 2 patients are still on therapy. One patient with NB had complete clearance of bone marrow disease and had remained progression free for 2.5 years after completion of infusions.

Conclusions: Large numbers of GD2BATs were feasible to obtain and grow in heavily pretreated patients and infusions of GD2BATs were well tolerated with manageable toxicities. Ongoing phase II portion of the trial will focus on evaluation of clinical activity of GD2BATs in patients with NB.

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Efficacy of MIBG131 and 5-azaC in Patients with 4 Stage Neuroblastoma (NB)

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Background: 5-azaC is an inhibitor of DNA methylation used for chemotherapy of high-risk leukemias and MDS. Therefore, we tested a combination of 5-azaC with MIBG131 and high-dose chemotherapy as a consolidation regimen for stage 4 NB.

Methods: From November 2014 to April 2017, 7 pts MIBG positive (M/F 4/3) with stage 4 NB with bone involvement, older than 2 years of age at the time of diagnosis were included in our pilot study. The median age was 4,5 (2-19). A course of high-dose MIBG131 at 440 MBk/kg was followed by high-dose chemotherapy (HDCT) with autologous PBSC rescue. HDCT schedule was as follows: 5-azaC D-9,-8,-7,-6,-5 (total dose 375 mg/m2); Treosulfan D-6,-5,-4 (total dose 30000 mg/m2); Melphalan D-3,-2 (total dose 140 mg/m2).

Results: No unexpected toxicities were observed. Four pts achieved CR and 2 VGPR, and 1 SR prior to receiving HDCT. Two pts 2 pts relapsed at 7 and 24 months (1 died, 1 alive). All others showed disease of MIBG uptake during the of observation (median 20 months). 2-year EFS-75,4%, OS-85%.

Conclusions: Combination of MIBG131 and 5-azaC with HDCT is an effective consolidation regimen in patients with stage 4 NB pts.

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Keywords: MIBG131, 5-azaC, neuroblastoma, HD chemotherapy

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PUMA Mediates the BDNF/TrkB Protection of Neuroblastoma Cells from Etoposide-Induced Cell Death

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Expressions of brain-derived neurotrophic factor (BDNF) and its tyrosine kinase receptor TrkB in the tumor tissues often indicate a poor prognosis in neuroblastoma (NB) patients. We previously identified that BDNF/TrkB induced chemo-resistance in NB cells through activation of phosphoinositied-3-kinase (PI3K)/AKT pathway. In this study, we investigated the role of P53 and its downstream targets, proteins of BCL2 (B cell lymphoma gene 2) family, in the protective effect of BDNF/TrkB in etoposide-induced NB cells death. TB3 and TB8, two tetracycline-regulated TrkB-expressing NB cell lines, were utilized. Our results showed that the expression of P53 was significantly and time-dependently increased in NB cells after treatment with etoposide. Pretreatment with BDNF (100ng/ml) blocked the etoposide-induced expression of P53, as well as the etoposide-induced cell death. Knockdown of P53 expression by siRNA could also block the etoposide-induced cell death. We examined the expressions of Bcl-2 family members (both anti-apoptotic members and pro-apoptotic members) and found that etoposide treatment increased the PUMA(p53 up-regulated modulator of apoptosis) expression at both protein and RNA levels, and pretreatment with BDNF blocked the etoposide-induced increase of PUMA. Knockdown of PUMA by siRNA could also protected NB cells from etoposide-induced cell death. Overexpression of PUMA induced NB cell death that could be attenuated by BDNF pretreatment. This data indicated that BDNF/TrkB protected NB cells from etoposide-induced cell death through the down-regulation of P53/PUMA, which provides new insights on the function of BDNF/TrkB.

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DNA Vaccine Conjugated with Polyethylenimine Induces More Effective Immune Response Against Neuroblastoma

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Introduction: DNA vaccination is a promising immunotherapeutic approach for solid tumors including neuroblastoma. The effectiveness of given method is limited by low transfection efficiency of plasmid DNA. Therefore, additional physical means or chemical carriers are required to enhance the delivery of DNA vaccine into cells. Polyethylenimine (PEI) is a cationic polymer which can bind negatively charged plasmid DNA. DNA-PEI nanoparticles facilitate the delivery of DNA through the membrane into the nucleus and enhance expression of the antigen.

Aim: To compare the immunogenicity of the 'naked' DNA vaccine and complex with PEI on the neuroblastoma animal model.

Materials and Methods: A/J mice were used as an animal model (n=45), males (n=12) and females (n=33), 8-10 weeks aged. Mice (n=36) were engrafted with 106 NB41A3 cell line in total volume of 100 µL. Plasmid DNA encoding the fragment of tyrosine hydroxylase as the antigen and PVXCP as the immune-enhancing gene based on the pING vector was used for immunization. Empty vector pING was used as a control. Intramuscular injection with 50 µg of DNA vaccine was performed three times (day 5, 10, 15 after tumor engraftment). Mice were sacrificed after one month of monitoring or according to indications.

Results: It was found that the tumor progressed much more slowly, or it did not form at all in the group of mice receiving vaccine complexed with PEI compared to the placebo group ($p=0.015$) and to 'naked' plasmid ($p=0.03$). According to the data of the cytotoxicity test specific killing activity in 10:1 ratio (effectors: tumor cells) of splenocytes from mice vaccinated with DNA+PEI conjugate was in 1.6 times higher than in a group with DNA alone (median - 38.3% vs 23.1%) ($p<0.05$). Production of IFNγ by ELISPOT was three times higher than in the other two groups ($p<0.05$).

Conclusion: Conjugation of plasmid DNA with PEI helps to overcome the insufficient transfection and protect DNA from degradation in intracellular matrix thereby increasing the level of immune response and the effectiveness of the vaccination.

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Impact of Different Intensity Induction Chemotherapy Protocols on Outcome of High Risk Neuroblastoma

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Background: Neuroblastoma high risk (HR-NB) patients are known to have poor prognosis. High-dose intensity induction chemotherapy might improve outcome.

Aim: The following is a retrospective study that aimed to determine difference in response and survival outcome of HR-NB patients, at Children Cancer Hospital Egypt 57357 (CCHE), receiving different induction regimens.

Methods: HR-NB patients presenting to CCHE from 2007 till 2016 were included. Patients were treated with high dose induction chemotherapy followed by BMT, radiotherapy and Roaccutane. Induction 1 (Ind1) patients received 8 cycles of systemic chemotherapy, in the form of etoposide (200 mg/m2/day, days 1 to 3) and carboplatin (450 mg/m2/day, day 1 only) alternating with cyclophosphamide (300 mg/m2/day, days 1 to 5), doxorubicin (60 mg/m2/day, day 5 only) and vincristine (1.5 mg/m2/day, days 1 and 5), VP16-Carbo/CAdO.

Induction 2 (Ind2) patients received 2 cycles of etoposide (200 mg/m2/day, days 1 to 3) and cisplatin (50 mg/m2/day, days 1 to 4) and 4 cycles of cyclophosphamide (2100 mg/m2/day, day 1 and 2), doxorubicin (25 mg/m2/day, days 1 to 3) and vincristine (2 mg/m2/day, day 1 only), CiE/CDV.

Cycles, for both groups, were administered at 3-week intervals. Other than induction, both groups were matching in allocation for inclusion and treatment. Target was complete or very good partial response after induction.

Results: Study included 764 HR-NB patients; 241 on Ind1, 523 on Ind2 chemotherapy. Among Ind2 patients 31.1% reached targeted response, while only 9.6% of Ind1 patients did.

3-year overall survival (OS) by regimen was 49.7±2.6% for Ind2 vs. 41.6±3.3% for Ind1 patients; 3-year event-free survival (EFS) of Ind2 and Ind1 were 24.9±2.3% and 25.6±2.9%, respectively.

Multivariate Cox regression analysis of OS and regimen, after accounting for age, gender, pathology, INPC, MYCN, stage, surgery, BMT and induction response, showed significantly better OS for Ind2 compared to Ind1 patients (Hazard Ratio=1.36); meanwhile, no significant impact of Ind1 was found on patients' EFS. During induction, 25 deaths (4.8%) occurred among Ind2 patients and 11 deaths (4.6%) among Ind1 patients.

Conclusion: Results show that using the more intensive Ind2 regimen for HR-NB induction improves OS, but not EFS compared to the less intensive Ind1 regimen.

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Inflammatory Response and Treatment Tolerance of Long-Term Infusion of The Anti-GD2 Antibody Ch14.18/CHO in Combination with Interleukin-2 in High-Risk Neuroblastoma

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Background: The monoclonal anti-GD2 antibody (Ab) ch14.18/CHO in combination with IL-2 is active and effective in high-risk NB patients. Here, we investigated the inflammatory response and treatment tolerance of long-term infusion (LTI) of ch14.18/CHO (10 x 10 mg/m2; 24h) in combination with s.c. IL-2 in a single center program.

Methods: Fifty-three NB patients received up to 6 cycles of 100 mg/m2 ch14.18/CHO (d8-18) as LTI combined with 6x10E6 IU/m2 s.c. IL-2 (d1-5; 8-12) and 160 mg/m2 oral RA (d19-32). Side effects of ch14.18/CHO and IL-2 treatment require hospitalization of patients on d8. Treatment tolerance was evaluated daily with clinical parameters (body temperature, vital signs, Lansky performance status, requirement of i.v. concomitant medication) to define an outpatient candidate status. sIL-2-R and CRP values were determined to assess the inflammatory response.

Results: LTI of ch14.18/CHO (d8–18) in combination with s.c.IL-2 (d8-12) showed an acceptable treatment tolerance that allowed all patients to receive part of the treatment as an outpatient (median time point of discharge: d15 for all cycles). The treatment tolerance improved from cycle to cycle and the time to become an outpatient candidate decreased from d15 to d13 in subsequent cycles.
Clinical and laboratory parameters indicate a maximum inflammatory response at d11 of each cycle. Interestingly, the sIL-2R remained increased at base-line of the next cycle indicating immune activation over the entire treatment period of 6 months.

Conclusions: LTI of ch14.18/CHO combined with s.c.IL-2 shows an improving tolerance in subsequent cycles allowing outpatient treatment.

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Inhibition of A Novel Long Noncoding RNA In Neuroblastoma Causes Complete Tumor Regression

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Background: Neuroblastoma is the most common solid tumor in early childhood. N-Myc gene amplification occurs in a quarter of human neuroblastoma tissues and is a marker for poor patient prognosis. Long noncoding RNAs (lncRNAs) play important roles in cancer development.

Aim: To identify novel lncRNAs and study their functional roles in N-Myc induced oncogenesis.

Methods: RNA sequencing was performed to identify novel lncRNAs that are differentially expressed in N-Myc amplified and N-Myc non-amplified neuroblastoma cell lines. Genes modulated by the novel lncRNA were examined by Affymetrix microarray. Modulation of gene promoter activity was examined by chromatin immunoprecipitation. Neuroblastoma cell proliferation and colony formation were determined by Alamar blue and Clonogenic assays. siRNAs or doxycycline-inducible shRNAs were utilized to knock-down RP1X and DEPDC1B gene expression. RP1X in neuroblastoma progression was determined in mice xenografted with doxycycline-inducible RP1X shRNAs. Correlation of RP1X and DEPDC1B gene expression in neuroblastoma patients was examined using the publically available gene expression databases (http://r2.amc.nl).

Results: Five novel lncRNAs, including RP1X were most differentially expressed between N-Myc gene amplified and non-amplified human neuroblastoma cell lines. DEPDC1B was one of the few genes considerably down-regulated in neuroblastoma cells after RP1X depletion. Knocked-down RP1X expression reduced histone H3 lysine-4 trimethylation at the DEPDC1B gene promoter and decreased its activity. Depletion of RP1X or DEPDC1B in neuroblastoma cells significantly reduced ERK protein phosphorylation, N-Myc protein phosphorylation at Serine 62, N-Myc protein stabilization, cell proliferation and abolished colony formation capacity. Importantly, treatment with doxycycline in mice xenografted with neuroblastoma cells stably transfected with doxycycline-inducible RP1X shRNA led to tumor regression or eradication. In 498 human neuroblastoma patients, high levels of RP1X gene expression correlated with DEPDC1B gene expression and poor patient prognosis.

Conclusion: This study identifies the novel lncRNA RP1X as an important regulator of N-Myc protein stability and neuroblastoma tumorigenesis.

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Targeting Copper Homeostasis as A Therapeutic Strategy for Neuroblastoma

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Background: Neuroblastoma is an aggressive childhood cancer. Despite intense therapy, survival rates are poor, and survivors experience long term side effects from the treatment. Targeted therapies are therefore urgently required. We demonstrated that intracellular copper levels are ~50% higher in neuroblastoma cell lines compared to normal astrocytes, and this is associated with high expression of the major copper transporter (CTR1). Given clinical evidence that copper is elevated in many tumours, copper homeostasis is considered an emerging target for anticancer drug design. We developed a polyphenol sugar conjugate called Dextran-Catechin that exerts its anticancer properties by targeting copper in neuroblastoma.

Material and Methods: Cell viability was tested in neuroblastoma cell lines SH-SY5Y, IMR-32, BE(2)C, and doxorubicin-resistant BE(2)C-ADR using the Alamar Blue assay and apoptosis was assessed via PARP cleavage. Gene and protein expression of CTR1 was determined using qRT-PCR and western blotting, respectively. Intracellular copper was measured by spectrophotometric analysis. NADH/NAD+ ratio was used to determine induction of oxidative stress. Cellular levels of GSH was examined using colorimetric assay. PET imaging with 64Cu was performed in a xenograft neuroblastoma model to monitor copper uptake in tumors. In vivo anticancer activity was assessed in a neuroblastoma xenograft model.

Results: Neuroblastoma cell lines SH-SY5Y, IMR-32, BE(2)C, and BE(2)C-ADR were sensitive to Dextran-Catechin (IC50 9.7µg/ml, 17.83µg/ml, 16µg/ml and 18.2µg/ml, respectively) at concentrations not toxic to normal astrocytes. A direct correlation was observed between higher intracellular copper levels and sensitivity to Dextran-Catechin. We demonstrated that Dextran-Catechin reacts with copper generating reactive oxygen species and inducing cancer cell death. Dextran-Catechin treatment caused a decrease of NADH/NAD+ ratio and GSH levels, confirming oxidative stress. PET imaging analysis of the neuroblastoma xenograft model revealed high accumulation of copper in the tumor mass. The high levels of copper were maintained in the tumor mass, while 64Cu was cleared by the other organs. Importantly, we showed that Dextran-Catechin significantly reduced Cu uptake and tumor growth in human neuroblastoma xenografts.

Conclusion: Dextran-Catechin targets neuroblastoma cells containing high levels of copper and inhibits tumour growth. This study has revealed a potential therapeutic strategy for targeting copper-dependent cancers such as neuroblastoma.

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A Safety and Feasibility Study of 131I-MIBG In Newly Diagnosed High-Risk Neuroblastoma: A Children's Oncology Group (COG) Pilot

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Background: Metastatic neuroblastoma continues to be a therapeutic challenge. 131I-metaiodobenzylguanidine (131I-MIBG) is highly effective in relapsed neuroblastoma, but rarely tested in newly diagnosed patients. The Children's Oncology Group (COG) designed a pilot study for newly-diagnosed high-risk neuroblastoma to assess the tolerability and feasibility of induction chemotherapy ending with a block of 131I-MIBG, followed by consolidation with busulfan/melphalan (Bu/Mel) and autologous stem cell rescue (ASCR).

Methods: Patients with stage 4 neuroblastoma and MIBG avid tumors were diagnosed at one of 23 participating institutions (12 had 131I-MIBG administration capability). The first two patients to receive 131I-MIBG and Bu/Mel developed Grade 4/5 toxicity with sinusoidal obstruction syndrome (SOS). The trial was suspended and re-designed to treat cohorts at 12, 15, and 18 mCi/kg 131I-MIBG with ASCR and a required minimum 10 weeks rest before Bu/Mel. Patients who completed induction chemotherapy were evaluable for the feasibility of 131I-MIBG; those who completed 131I-MIBG therapy were evaluable for the feasibility of administering 131I-MIBG and Bu/Mel. Tolerability was based on pre-defined unacceptable Grade 4/5 toxicities.

Results: Fifty-nine of 68 subjects (87%) who completed the induction therapy received 131I-MIBG. Thirty-seven of 45 subjects (82.2%) were evaluable for 131I-MIBG plus Bu/Mel feasibility. Of the 35 subjects enrolled post-suspension who received Bu/Mel after 12 mCi/kg (n=6), 15 mCi/kg (n=17), or 18 mCi/kg (n=12) of 131I-MIBG, 3 developed unacceptable severe SOS; one at each dose level. Overall rates of SOS occurrence at 12 mCi/kg, 15 mCi/kg, and 18 mCi/kg doses were 33%, 23.5%, and 25%, respectively. No other unacceptable toxicities occurred. The 131I-MIBG and 131I-MIBG plus Bu/Mel feasibility rates at the dose level of 15 mCi/kg slated for further study on ANBL1531 were 96.7% (29/30) [95% CI: (83.3%, 99.4%)] and 81% (17/21) [95% CI: (60%, 92.3%)].

Conclusion: This trial demonstrated the feasibility of administering 131I-MIBG therapy during induction chemotherapy followed by myeloablative therapy in a cooperative group setting. While stopping rules outlined in the protocol were not met, the potential for SOS with 131I-MIBG in close proximity to Bu/Mel led to selection of 15 mCi/kg 131I-MIBG administered earlier during induction for investigation in an upcoming Phase 3 COG clinical trial.

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Combination of Ch14.18/CHO and GPOH Induction Chemotherapy Cycles in Refractory Relapsed or Progressing High Risk Neuroblastoma Patients

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Background: Since a response rate (RR) of 53% (9/17patients) was reported in relapsed/refractory neuroblastoma when combining ch14.18/SP0/2 with irinotecan-temozolomide, we explored the GPOH induction (N5/N6 cycles) in combination with continuous infusion of ch14.18/CHO (dinutuximab beta; DB) for future use in front-line treatments. Here we report feasibility, toxicity and RR of this compassionate use program.

Patients: 16 high risk NB patients (15 refractory relapsed/progressing: 5 patients 1 event; 5 patients 2 events, 5 patients 3 events; 1 refractory frontline patient) were treated (median age at diagnosis: 3.4y; initial stage: 14 M, 1 MS, 1 L2 MYCN amplified (MNA), 18.7% MNA, median time from diagnosis to relapse/progression: 1.8y (range, 0.35 – 8.89y), type of relapse/progression: 92% disseminated; 8% local). All patients failed at least one second line therapy and 10/16 previously received N5/N6 cycles.

Patients were treated with alternating cycles of N5 (cisplatin, etoposid, vindesin; d1-5) and N6 (vincristine, dacarbacine, ifosfamide, doxorubicine; d1-7) combined with ch14.18/CHO continuous infusion (50 mg/m2 over 5 days) (start: d5+6 in N5; d6+7 of N6). Grade 3/4 toxicity was evaluated (CTCAEv4.3) and response was determined after 2 (mid evaluation) and after 4 cycles (end evaluation) (bone marrow smears, 123I-mIBG SPECT, CT/MRI, urine catecholamine metabolites).

Results: 43 cycles were applied (21xN5/DB 22xN6/DB). Response assessments revealed a best overall RR rate of 50% (2/16 CR, 6/16 PR). The end of treatment RR was 38% (2/16 CR, 4/16 PR). Frequencies of grade 3/4 toxicity were 100% hematological (any kind), 25% renal function, 25% pain, 12% fever, 12% central neurotoxicity (somnolence, dizziness, disorientation) 8% cardiac function (decreased fraction shortening), 6% capillary leak. No grade 3/4 cytokine release or allergy/hypersensitivity was observed. No treatment related death occurred. The median cumulative morphine doses in cycle 1, 2, 3 and 4 were 1.45, 0.56, 0.68 and 0.43 mg/kg/cycle, indicating a steady decrease in pain intensity from cycle to cycle comparable to previous experience.

Conclusions: The combination of dinutuximab beta on day 5 or later of N5/N6 chemotherapy cycles is feasible leading to an encouraging response rate in refractory relapsed patients who failed at least one second line therapy.

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Blinding the CYCLOPS – Neuroblastoma Vulnerabilities Unveiled by Genomic Loss

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Heterozygous deletions within distal 1p are observed in 30% of neuroblastomas. So far, several potential 1p tumor suppressor genes have been identified. However, in this study we are focusing on 1p genes whose inactivation is not necessarily linked to tumor development, but which mediate cell-essential functions, rendering cells with copy number loss vulnerable to further impairment. These genes are candidate therapeutic targets according to the concept of CYCLOPS (copy number alterations yielding cancer liabilities owing to partial loss).

To identify genes for which heterozygous loss may be tolerated but further reduction leads to cell death, we performed siRNA screens mediating the systematic knock-down of distal 1p genes in five 1p-deleted versus five non-1p-deleted neuroblastoma cell lines. Forward liquid transfections were done using three independent siRNAs per gene. Hoechst stained cell nuclei were count 96h post transfection. Target gene validation was done by viability and colony formation assays, cell cycle analysis and rescue experiments with cDNA over expression.

We identified many potential CYCLOPS candidates, among them EphB2 which is required for embryonic and neuronal development. Knock-down of EphB2 reduced cell viability and colony formation in 1p-deleted cell lines but did not in 1p-non-deleted cells. G1/G0 phase arrest with corresponding S phase decrease was observed in both 1p-deleted and 1p-non-deleted cells. Additionally, neurite-like outgrowth was shown in 1p-non-deleted cells indicating an induction of differentiation.

This study identified EphB2 as candidate CYCLOPS gene in neuroblastoma. Heterozygous deletions of chromosome arm 1p are also frequently observed in other cancers including melanoma, colorectal and breast cancer. We hypothesize that this proof-of-principle opens a new therapeutic window for tumors harboring a heterozygous deletion of EphB2 or other cell essential genes on chromosome arm 1p.

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Polo-like Kinase 4 Mediates Epithelial-Mesenchymal Transition in Neuroblastoma Via PI3K/Akt Signaling Pathway

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Neuroblastoma (NB) is the most common malignant tumor in infancy and most common extracranial solid tumor in childhood. With the improvement of diagnosis and treatment, the survival rate of patients with low-risk and intermediate-risk NB can reach up to 90%. In contrast, for high-risk NBs, the long-term survival rate is still less than 40% because of heterogeneity of this tumor. The pathogenesis of NB is still not explicit; therefore, it is of great significance to explore the mechanism of NB tumorigenesis and discover new therapeutic targets for NB. Polo-like kinase 4 (PLK4), one of the polo-like kinase family members, is an important regulator of centriole replication. The aberrant expression of PLK4 was found in several cancers and a recent study has unraveled a novel function of PLK4 as a mediator of invasion and metastasis in Hela and U2OS cells. However, the function of PLK4 in NB development and progression remains to be elucidated. The study showed the expression level of PLK4 in NB tissues was remarkably up-regulated and high expression of PLK4 was negatively correlated with clinical features and survival, which suggested that PLK4 could be a potential tumor promoting factor of NB. Functional studies indicated down-regulation of PLK4 suppressed migration and invasion and promoted apoptosis in NB cells. Further experiments showed that down-regulation of PLK4 in NB cells inhibited EMT through the PI3K/Akt signaling pathway. Animal experiments demonstrated that the down-regulation of PLK4 in SK-N-BE(2) cells dramatically suppressed tumorigenesis and metastasis. PLK4 may be a promising therapeutic target for NB.

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Combined T-SNE and ARACNE Analyses Identify Core Regulatory Circuitries Underlying Subgroups of Neuroblastoma

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Many mRNA profiling studies have highlighted the strong expression differences between low- and high-risk neuroblastoma. Nonetheless, both disease entities are histopathologically very similar and are both thought to arise from the sympatho-adrenergic lineage. We therefore analyzed neuroblastoma mRNA profiles not only for differences, but also for commonalities between both low- and high-risk tumours to identify key transcriptional networks underlying different molecular subtypes of neuroblastoma. To achieve this, we performed gene-based T-SNE dimension reduction analysis in combination with gene regulatory network reconstruction (ARACNE) on mRNA profiling datasets of NB. Interestingly, we identify 3 key dimensions of the disease, consisting of gene-networks regulating epithelial to mesenchymal transition (EMT), immune cell/inflammatory response and finally cell cycle/MYCN. These core networks were validated and remained stable across 3 independent NB datasets consisting of over 900 tumours. Interestingly, these networks were identifiable independent of patient stage, risk-status or MYCN-amplification. Clustering of patients based on these 3 core networks identified sub-division of patients within the each of the clinically relevant NB subgroups (low-risk, high-risk and MYCN-amplified). This suggests underlying biological subtypes of NB that differ based on root biological networks independent of the aggressiveness of the disease. In addition, by combining core-network description with high-throughput transcription factor binding site data generated both in house and available through ENCODE we identify transcription factors that are enriched and bind targets within the underlying core networks of NB. These findings may provide insight into identifying key therapeutic networks and targets within both high-risk and MYCN-amplified patients that may have been previously overlooked.

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The R2 Platform as Epigenome Resource for Neuroblastoma Core Transcriptional Regulatory Circuitry (CRC) Analysis and Visualization

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Over the past decade, the R2 platform has proven to be a useful and ever-expanding resource for the Neuroblastoma community. As science progresses, so does the development of the R2 platform.

Methods: The R2 program, rooted in neuroblastoma research, has a user-friendly interface enabling a wide range of interconnected analyses, which can be harnessed by users with limited or no bioinformatics training. The web-based genomics analysis and visualization platform has been greatly extended over the past period. Novel analyses, easier workflows, and new types of omics data have been introduced.

Results: Aside from mRNA expression, aCGH, SNP, Exome and whole genome sequencing data we have greatly extended R2 with intuitive tools for the analysis of epigenome and ChIPseq analyses. Various different technologies can be combined in integrative analyses and visualizations.

We have added many histone modification profiles of neuroblastoma cell lines and tumor samples. On these profiles peak calling has been performed as well as super enhancer analyses if applicable. Interactive plots, like ROSE SE visualizations, genome browser views and heatmaps of aligned peaks provide accessible entry points for further exploration of one, or a series of profiles. In addition, integration with other genomic features, such as chromatin states, transcription factor binding sites, gene expression patterns help to test hypotheses. Genome reconstruction has also been added to the repertoire. Finally, Tools to work with core regulatory circuitries (CRC) are now being implemented.

Other additions: The popular tSNE dimensionality reduction algorithm has been added and allows for interactive definition of patient subgroups on the basis of the underlying genomics data. Improved expression signature generation, coupled to various downstream analyses now allows the users to get a better understanding of their cohorts or experiments. The introduction of data scopes, creates easier workflows and allows for more focused analyses within R2.

Conclusions: R2 remains a valuable resource for high throughput data of Neuroblastoma. The R2 program and database has been used in more than 630 PubMed listed publications and is publicly accessible at http://r2.amc.nl. R2 assists researchers in the identification of important genes and biological processes in neuroblastoma.

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Plasticity of Transcriptional and Epigenetic Cellular States in Neuroblastoma Is Driven by Core Lineage Transcription Factors

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Background: Core Regulatory Circuitries (CRCs) of lineage transcription factors associated with super-enhancers are the central drivers of lineage identity and differentiation stage of cells. We recently showed that most neuroblastomas include two types of tumor cells with divergent gene expression profiles. Undifferentiated mesenchymal (MES) cells and lineage-committed adrenergic (ADRN) cells resemble cells from developmental differentiation stages. MES and ADRN cells of isogenic origin were found to spontaneously interconvert. For each cell type, we identified the unique super-enhancer landscape using H3K27ac ChIP-sequencing. Super-enhancers associated with lineage transcription factors identified the Core Regulatory Circuitries (CRC) for MES and ADRN cells. Clinically important, MES cells were resistant to chemotherapy.

Results: We studied plasticity of the MES and ADRN CRCs in cellular transdifferentiation. The MES transcription factors PRRX1 or NOTCH were expressed as transgenes in ADRN cells. Each gene induced a step-wise reprogramming of the ADRN transcriptome towards a MES state. The transcriptional switch was accompanied by genome-wide remodeling of the epigenome to a MES enhancer state. Both transgenes repressed super-enhancers of ADRN core transcription factors, leading to transcriptional downregulation of the ADRN CRC. This process was associated with Polycomb repression. The transdifferentiation was initially reversible but became stabilized over time. Expression of NOTCH induced an endogenous feed-forward loop at the epigenetic, transcriptional and signaling levels and included ligands, receptors and co-factors from the NOTCH signaling route. This endogenous NOTCH-cascade maintained a transgene-independent MES state. Consistently, NOTCH receptors and co-factors in stable MES cell lines were associated with super-enhancers. Both NOTCH-induced MES cells and ADRN cells were tumorigenic in vivo. Finally, we validated expression of CRC transcription factors in pre- and post-chemotherapy tumor pairs of nine patients and identified a strong expression of MES transcription factors in post-treatment biopsies.

Conclusions: Our results demonstrate that single transcription factors from the MES CRC impose transdifferentiation via remodeling of the epigenetic and transcriptional landscape of ADRN cells, mimicking spontaneous interconversion. Plasticity of CRCs and lineage identity may have profound implications for treatment strategies in neuroblastoma.

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Neurocan, A Chondroitin Sulfate Proteoglycan, Is A Crucial Extracellular Component in Neuroblastoma Tissues, Which Promotes Malignant Phenotypes

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Neurocan (NCAN), an extracellular chondroitin sulfate proteoglycan, is known as the major inhibitor of axon regeneration in nervous injury. Here we investigated its involvement in neuroblastoma (NB). Firstly, we found that NCAN was highly expressed in the NB patients with unfavorable outcome. We also examined its expression in TH-MYCN mice, a NB model, and found that NCAN protein was strongly positive at the extracellular regions in both early lesions and terminal tumor tissues. The results of in situ hybridization, which detected the expression of NCAN mRNA, indicated that the NB cells expressed and secreted NCAN protein. Interestingly, exogenous NCAN including overexpression, recombinant protein and conditioned medium transformed adherent NB cells into spheres. Those NCAN-induced sphere cells showed the higher malignancies both in vitro (anchorage-independent colony formation and chemoresistance against cisplatin) and in vivo (xenograft tumor formation) compared with parental adherent ones. We showed that both chondroitin sulfate sugar chains and core protein of NCAN were essential for the induction of sphere formation. Among the core protein, the CSG3 domain was revealed to be essential for its function. Next, we comprehensively compared the mRNA expression patterns between NCAN-induced sphere cells and parental adherent ones. As a result, it was suggested that NCAN promoted cell division, and maintained the undifferentiated state of NB cells. Finally, the knockdown of NCAN in tumor sphere cells cultured from TH-MYCN mice suppressed their growth both in vitro and in vivo. Taken together, these results suggest that NCAN should be a crucial component of extracellular matrix in NB tissues, which provides growth advantage.

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Longitudinal Monitoring of Cell-Free DNA Biomarkers in Blood and Bone Marrow Plasma from Patients with Neuroblastoma

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The invasive nature of surgical biopsies prevent sequential application for patient monitoring, and single biopsies often fail to reflect changing tumor dynamics, intratumor heterogeneity and drug sensitivities occurring during tumor evolution and treatment. Implementing molecular characterization of cell-free neuroblastoma-derived DNA isolated from blood and bone marrow plasma could improve outcome prediction, patient monitoring and treatment selection for high-risk patients. We established droplet digital PCR (ddPCR) protocols for MYCN and ALK copy number status as well as ALKF1174L and ALKR1275Q hotspot mutations from patient plasma samples. We collected 137 samples from 24 patients (>2 samples from 16 patients, 20 per patient maximally) with neuroblastoma. Cell-free DNA (cfDNA) was isolated from blood and bone marrow plasma and analyzed for MYCN and ALK status using ddPCR. Longitudinal monitoring revealed a highly individual course for each patient. For a patient with metastasized high-risk neuroblastoma, we identified MYCN amplification and wildtype ALK in blood plasma collected at second biopsy, while the initial biopsy indicated an ALKF1174L mutation and diploid MYCN. The tumor, resected 250 days after second biopsy, harbored a MYCN amplification and the ALKF1174L mutation, while the corresponding plasma sample indicated diploid MYCN status and ALK mutation, underlining the potential of cfDNA analysis for detecting subclonal populations and tumor heterogeneity. ALK gains or ALKR1275Q mutations were detected in the plasma throughout or almost throughout complete treatment course (8 plasma samples from 2 patients receiving different treatment blocks), indicating that at least some tumor cells are not sufficiently targeted. Blood plasma and tumor biopsy at diagnosis of another patient agreed for MYCN amplification and an ALKR1275Q mutation, and the total cfDNA concentration in the plasma was high. Plasma samples taken after each of the 9 treatment blocks indicated diploid MYCN, wildtype ALK and decreasing total cfDNA concentration, which likely reflects treatment success. This patient is currently alive, which is in concordance with our liquid biopsy data. Taken together, these data indicate that molecular disease characterization using cfDNA from blood and bone marrow plasma can reflect tumor dynamics, intratumor heterogeneity and therapy success or failure in high-risk neuroblastoma patients.

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Event-free Survival of Infants and Toddlers Enrolled in the HR-NBL-1/SIOPEN Trial Is Associated with Level of Neuroblastoma mRNAs at Diagnosis

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Background: To evaluate whether levels of neuroblastoma mRNAs in bone marrow and peripheral blood from stage M infants (≤12 months of age at diagnosis, MYCN amplified) and toddlers (between 12 and 18 months, any MYCN status) predict event-free survival (EFS).

Methods: Bone marrow aspirates and peripheral blood samples from 97 infant-toddlers enrolled in the HR-NBL-1/SIOPEN trial were collected at diagnosis in PAXgene™ blood RNA tubes. Samples were analyzed by RTqPCR according to standardized procedures.

Results: Bone marrow TH or PHOX2B levels in the highest tertile associated with worse EFS; hazard ratios, adjusted for age and MYCN status, were 1.5 and 1.8 respectively. Expression of both TH and PHOX2B in the highest tertile predicted for worse outcome (p=0.015), identifying 20 (23%) infant-toddlers with 5-year EFS of 20% (95%CI: 4%–44%). Prognostic significance was maintained after adjusting for over-fitting bias (p=0.038), age and MYCN status. In peripheral blood, PHOX2B levels in the highest tertile predicted a two-fold increased risk of an event (p=0.032), identifying 23 (34%) infant-toddlers with 5-year EFS of 29% (95%CI: 12%–48%). Time-dependent ROC analysis confirmed the prognostic value of combined TH and PHOX2B in bone marrow and of PHOX2B in peripheral blood during the first year of follow-up.

Conclusions: High levels of bone marrow TH and PHOX2B and of peripheral blood PHOX2B at diagnosis allow early identification of a group of high-risk infant and toddlers with neuroblastoma who may be candidates for alternative treatments. Integration with additional biomarkers, as well as validation in additional international trials is warranted.

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Updated Clinical and Biological Information from The Two-Stage Phase II Study of Imatinib Mesylate In Subjects with Relapsed/Refractory Neuroblastoma

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Background: Several studies support the notion that the kinase inhibitor Imatinib mesylate exert off-target effects on cells of the immune system. After our first report of continuous daily oral administration in subjects with relapsed/refractory neuroblastoma (NB, EudraCT: 2005-005778-63), we report additional information on potential surrogate markers for prediction of efficacy in these subjects.

Subjects and Methods: Peripheral blood (PB) samples collected at study entry and after the second cycle of Imatinib mesylate treatment were tested for IFN-α, TNF-α, TGF-β, IL-10, CXCL12 and soluble (s) B7-H6 plasma levels, whereas paired PB and bone marrow (BM) samples were evaluated for CXCL12, CXCR4 and NKp30 isoform mRNA levels. Correlation between level of each parameter and response/outcome was then evaluated.

Results: Out of the six subjects still alive at the time of the first report, three are presently alive ten years after the last Imatinib cycle. One of the three subjects achieved complete response (CR) during Imatinib treatment and never relapsed, whereas the other two subjects underwent additional treatments and are presently in CR. Response to Imatinib was confirmed to be associated with low Imatinib exposure. Moreover, a significant increase in NKp30 isoform mRNA levels occurred in BM samples after Imatinib treatment, indicating the off-target effect of Imatinib mesylate on NK cells in vivo.

Conclusions: Imatinib mesylate efficacy in relapsed/refractory NB has been confirmed at a longer follow-up. Therefore, a Phase II study should be performed, envisaging the collection of samples at study entry and after two cycles to evaluate in a large cohort of subjects the predictive value of potential surrogate markers of efficacy.

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Helper- and Regulatory T Cells Adversely Affect Survival of Neuroblastoma Patients Treated with Long-Term Infusion of ch14.18/CHO Combined with interleukin-2

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Background: Treatment of high-risk neuroblastoma patients (pts) with long-term infusion (LTI) of the anti-GD2 antibody (Ab) ch14.18/CHO in combination with interleukin-2 (IL-2) is effective against neuroblastoma. Here, we report results of immunophenotyping and impact of effector cells on functional immune- and survival-parameters.

Methods: 53 pts received 5 cycles (35 days (d)) of 6x106 IU/m2 subcutaneous IL-2 (d1-5; 8-12), LTI of 100 mg/m2 ch14.18/CHO (d8-18) and 160 mg/m2/d oral 13-cis RA (d22-35) in a closed single center program (APN311-303). The counts of cytotoxic NK cells (CD16++/CD56dim), helper T cells (CD3+/CD4+), cytotoxic T cells (CD3+/CD8+), regulatory T cells (Treg; CD4+/CD25+/CD127-) and granulocytes (CD64+) were determined by flow cytometry (cycle 1; d1, 8 and 15). Ab-dependent cellular cytotoxicity (ADCC) was determined by a cytotoxicity assay. Correlation between cell counts and progression-free survival (PFS) was analyzed.

Results: Compared to the baseline (d1), IL-2 treatment resulted in a strong increase of cytotoxic NK cells, cytotoxic- and helper T cells and Treg on d8 (2.4-, 3.9-, 2.6- and 15.0-fold increase, respectively). Interestingly, subsequent combined treatment with IL-2 and ch14.18/CHO did not further increase lymphocyte count on d15. In contrast, elevation of granulocytes occurred during the combined treatment. Unexpectedly, we did not observe any correlation between cell counts and ADCC on d15 as well as between cytotoxic NK and cytotoxic T cells and PFS. Similar observations were made for granulocytes.

In contrast, pts with low Treg (≤ 138 cells/µl) on d15 (n = 11) showed a better PFS compared to high Treg pts (n= 31) (P = 0.072). The 5-year PFS was 44% (95% CI [0.13, 0.74]) and 19% (95% CI [0.05, 0.33]) for low and high Treg count, respectively. On d15, pts with low helper T cells (n = 11, ≤ 365 cells/µl) also showed an improved PFS compared to those who had high helper T cells (n = 31; P = 0.013). The 5-year PFS was 53% (95% CI [0.23, 0.83]) and 16% (95% CI [0.03, 0.29]) for low and high helper T cell count, respectively.

Conclusions: IL-2-dependent helper T and Treg cells negatively affect efficacy of a combined treatment with ch14.18/CHO and IL-2.

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Decoding the Metabolic Program of Deregulated MYCN Expression in Neuroblastoma

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The dynamics of central metabolic pathways are altered in malignant cells in comparison to nontransformed cells. Malignant cells meet the increased energy demands caused by rapid proliferation and invasion into surrounding tissue by increasing aerobic glycolysis, known as the Warburg effect, and sustained mitochondrial activity. Oncogenic MYC influences the metabolism in many human cancers making them sensitive for both glucose and glutamine utilization to fuel their central metabolic pathways. MYC- or MYCN-driven tumors show enhanced glycolysis by activating the transcription of glycolytic genes. Since MYCN resides in macromolecular complexes and induces or represses the transcription of multiple genes involved in fundamental cellular processes including proliferation and differentiation, we hypothesized that MYCN has a regulatory effect on the cellular metabolism in neuroblastoma cells. We analyzed different neuroblastoma cell lines with high and low MYCN expression levels as well as the SHEP cell line and its genetically engineered clone SHEP-Tet21/N with adjustable MYCN levels to investigate the relationship between metabolism and MYCN expression. Individual modes of carbon usage were tracked within the TCA cycle and glycolysis, pentose-phosphate, glutaminolysis and amino acid synthesis pathways for 2, 5, 10, 20 and 40 min using pulsed stable isotope-resolved metabolomics (pSIRM) and absolute quantitative GC-MS analysis. We integrated further parameters such as cell growth and the expression of key enzymes in the central carbon metabolism obtained by mass spectrometry-based proteomics coupled to liquid chromatography (LC-MS/MS) to produce an overview of metabolic performance in the analyzed neuroblastoma cell lines and cell models with adjustable MYCN levels. In total for each cell type, absolute quantification was carried out on ~25-30 metabolites within central carbon metabolism and ~2000-4000 proteins identified with the LC-MS/MS approach. Current data analysis is focused on identifying predictive markers for MYCN activity and druggable nodes to bypass MYCN overfunction.

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Suppression of TERT Expression in Neuroblastoma Is A Coordinated Regulation of ARID1A Co-occupancy with SIN3A/HDAC

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Although high-risk neuroblastoma (NB) clinically associates with induction and rearrangement of hTERT, the regulation of hTERT remains poorly understood. Here our ChIP-seq analysis show that AT-Rich Interaction Domain 1A (ARID1A), a subunit of SWI/SNF chromatin remodelers, occupies and restructures chromatin in the hTERT promoter region. We further demonstrate that ARID1A is essential for the recruitment of SIN3A/HDAC complex to suppress telomerase activity and expression. This mechanism is inactivated by truncation of its ARID domain or siRNA targeted silencing. Moreover, mutation in -124C>T in hTERT promoter enhances the ARID1A- bound by in vitro luciferase activities. Finally, our NB cohorts that show loss or low expression of ARID1A are relatively linked to elevated expression of TERT, an eminent feature of aggressive NB. These results indicate ARID1A plays a tumor suppressive role for TERT expression by recruiting SIN3A/HDAC complex via its modifying chromatin access.

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Phosphorylation of ASCL1 Controls Proliferation Versus Differentiation in Neuroblastoma

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Neuroblastoma is associated with stalling of normal differentiation, resulting in excessive proliferation of neuroblastic precursors. Here we show that CDK-dependent phosphorylation of the transcriptional master regulator ASCL1 plays a critical role in controlling the balance between cell proliferation and differentiation and can be modulated to reactivate differentiation of neuroblastoma cells.

We show that ASCL1 is phosphorylated by Cyclin-Dependent Kinases and that phospho-ASCL1 supports the pro-proliferative programme in neuroblastoma cells. However, preventing CDK-dependent phosphorylation of ASCL1 results in changes in the genome-wide transcriptional programme of neuroblastoma cells, leading to suppression of pro-proliferative targets and simultaneous activation of genes that drive cell cycle exit and differentiation. Mechanistically, ASCL1 ChIPSeq reveals enhanced binding of un(der)phosphorylated ASCL1 at sites associated with pro-differentiation targets. Moreover, PHOX protein binding at key downstream regulatory elements is also modulated by ASCL1 phosphorylation. Finally, we also show that chemical CDK inhibition is sufficient to drive differentiation of neuroblastoma cells in a manner dependent on endogenous ASCL1.

Therefore, we conclude that CDK-dependent phosphorylation of ASCL1 acts as a critical fulcrum controlling the balance between proliferation and differentiation and thus offers a novel therapeutic opportunity for neuroblastoma.

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Low Protein Content of Ketogenic Diet is Not Responsible for The Effect on Neuroblastoma Growth

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Neuroblastoma (NB) is a pediatric malignancy characterized by alterations of the OXPHOS system and high dependency on glucose and glutamine metabolism. The metabolic phenotype of NB provides opportunities to target NB cells specifically by dietary intervention. Our previous preclinical studies showed that a high-fat, low-carbohydrate/protein, ketogenic diet (KD), enhances the anti-proliferative effect of low dose chemotherapy in NB xenograft models. In the present study, we investigated whether dietary protein restriction per se can sensitize NB xenografts to low dose of chemotherapy to a similar extend as a KD.

We established NB xenografts with SH-SY5Y (MYCN-non- amplified) and SKNBE(2) (MYCN- amplified) cell lines in CD-1 nu/nu mice. The NB-bearing mice were treated with low doses of cyclophosphamide in combination with a standard diet matching the low protein content of the KD (8% protein) (SDLP) or a standard diet containing essential amino acids only (8% - no protein) (SDEA), a KD and standard diet (16% protein) (SD). The effect of low dose cyclophosphamide in combination with different diets on tumor growth, survival, body weight and plasma amino acid levels was evaluated.

In contrast to KD, reduction of dietary protein to 8% in SDLP did not affect growth of SH-SY5Y and SKNBE(2) xenografts. Interestingly, SDEA caused a significant growth reduction only of SH-SY5Y xenografts. All mice were able to tolerate the modified diets. Surprisingly, plasma amino acid levels of certain non-essential amino acids, like glutamine, serine, proline and alanine were elevated in the SDEA-fed mice compared to the SD group.

Our data indicate that a lower protein content in KD is not the reason for NB growth inhibition. Furthermore, the metabolic stress induced by a diet containing only essential amino acid containing diet but no protein seems to sensitize MYCN-non- amplified to low dose chemotherapy.

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The Genetic Basis of Favorable Outcome and Fatal Tumor Progression in Neuroblastoma

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Background: Recent genomic and transcriptomic profiling studies of neuroblastoma have shed light on cancer gene mutations frequencies, structural genomic rearrangements and copy number alterations in this tumor type. However, none of these had been able to convert the data into a coherent model that explains the pathogenesis of the divergent subtypes and can separate those cases in which spontaneous regression occurs from those whose disease progresses.

Methods: To determine the molecular basis of favorable and adverse disease courses, we performed massively parallel sequencing of 416 untreated neuroblastomas (whole-genome or whole-exome sequencing, n=218; targeted sequencing, n=198).

Results: We found that clinical high-risk and non-high-risk tumors differed in their mutation numbers, rates, and signatures. Furthermore, we detected genomic alterations of 17 genes related to the RAS and p53 pathways in 74/416 patients. The presence of these mutations was strongly associated with dismal outcome in the entire cohort, as well as in the high-risk and non-high-risk subgroups. We noticed, however, that the prognostic effect of RAS/p53 pathway mutations was strictly dependent on the occurrence of telomere maintenance mechanisms. Survival of patients whose tumors were telomere maintenance-positive was dramatically inferior when additional RAS/p53 pathway mutations were present as compared to those without such alterations. By contrast, patients whose tumors lacked telomere maintenance mechanisms had excellent outcome, and spontaneous regression or differentiation occurred both in the presence and absence of RAS/p53 pathway mutations.

Conclusions: Together, our data suggest a precise mechanistic definition of clinical neuroblastoma phenotypes that is based on the presence or absence of telomere maintenance and RAS or p53 pathway mutations and provide a starting point for improving diagnostic and therapeutic management of neuroblastoma patients.

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Targeting A Synthetic Lethal PGBD5-Induced DNA Repair Dependency in Neuroblastoma

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We recently identified the DNA recombinase, PGBD5, which promotes site-specific genomic rearrangements in human cells and is active in the majority of pediatric solid tumors including neuroblastoma. Murine and human cells deficient in non-homologous end joining (NHEJ) DNA repair cannot tolerate the expression of PGBD5, suggesting a synthetic lethal relationship. In a chemical screen of DNA damage signaling inhibitors, we identified the ATR inhibitor, AZD6738, as a specific sensitizer of PGBD5-dependent DNA damage and apoptosis. Expression of PGBD5, but not a mutant deficient in nuclease activity, was sufficient to induce hypersensitivity to AZD6738 treatment in untransformed human cells. Depletion of endogenous PGBD5 using shRNA conferred resistance to AZD6738 in neuroblastoma cell lines. PGBD5-expressing neuroblastoma cells accumulated unrepaired DNA damage induced by AZD6738 treatment, and underwent apoptosis predominantly in G1 phase in the absence of immediate DNA replication stress. AZD6738 exhibited nanomolar potency in vitro against the majority of neuroblastoma cell lines tested, while sparing non-transformed human and mouse embryonic fibroblasts. AZD6738 treatment of mice harboring subcutaneous xenografts derived from neuroblastoma cell lines caused tumor regression and induced apoptosis and DNA damage in the xenograft tumor cells. This anti-tumor effect was (i) potentiated by combining AZD6738 treatment with cisplatin in mice harboring patient-derived neuroblastoma xenografts derived from two primary high-risk neuroblastomas, (ii) independent of MYCN status and (iii) correlated with PGBD5 expression levels. Our findings delineate a therapeutically actionable synthetic dependency induced by PGBD5 in neuroblastomas.

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CDX1 Regulates Neuroblastoma Stemness Through MYC Pathway Modulation and Reprogramming Gene Activation

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Background: Cancer stem cells (CSCs) are regarded as essential for tumor maintenance, recurrence and distant metastasis. However, CSC or tumor-initiating cells were not identified in neuroblastoma (NB). CSCs have the capacity for self-renewal and cell differentiation. The regulation mechanism to maintain NB-CSC populations in NB tissues are still unclear.

Methods: Gene expression profiling of CDX1-expressing or sphere-forming CDX1 knockdown and over-expression were performed by lentiviral system. Cells were subjected to proliferation, nude mice tumorigenic and sphere formation assays. Chromatin immunoprecipitation (ChIP) was used to determine direct binding of CDX1 on transcriptional targets. NB cell lines as well as primary NB cells were conducted by Agilent microarray. Gene set enrichment pathway analysis was conducted with WikiPathway and MSigDB.

Results: We previously reported that stem cell related gene CD133 was expressed in neuroblastoma (NB) spheres (Takenobu et al., Oncogene, 2010). To identify the responsible transcription factors for CD133 transcription, we analyzed the promoter region by in Silico and wet-lab analysis and identified CDX1 expressed specifically in sphere-forming NB cells. CDX1 regulates sphere formation efficiency in NB cells as well as CD133. In NB sample analysis by expression microarray, CDX1 high expression related to the unfavorable prognosis. We conducted microarray-based expression analysis of NBs; Pathway analysis of microarray data indicated that genes in transcriptional regulation of pluripotent stem cells pathway (WP2821) such as OCT4 and NANOG, were induced both in CDX1-expressing NB cells and NB spheres. Intriguingly, cell proliferation related gene set, MYC module (Kim et al., Cell, 2010) was significantly downregulated. MYC downstream cell cycle regulator genes such as CDK4, MCM-family replication licensing factors were suppressed in CDX1-expressing or sphere-forming NB cells. These results indicate that CDX1 plays an important role in NB stemness by suppression of MYCN-related cell cycle progression and makes CSCs dormant having undifferentiated properties.

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KIF1Bβ Increases ROS to Mediate Apoptosis and Reinforces Its Protein Expression Through O2− In A Positive Feedback Mechanism in Neuroblastoma

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Background: Relapse-prone, poor prognosis neuroblastoma is frequently characterized by deletion of chromosomal region 1p36 where tumor suppressor gene KIF1Bβ resides. Interestingly, many 1p36-positive patients failed to express KIF1Bβ protein. Since altered cellular redox status has been reported to be involved in tumor suppression and protein modification, we investigated the potential relationship between reactive oxygen species (ROS) and KIF1Bβ.

Methods: Overexpression of KIF1Bβ wild-type or apoptotic loss-of-function mutants and knockdown of KIF1Bβ in human neuroblastoma cell lines with different KIF1Bβ genotypic profiles based on 1p36 status, were achieved by transient transfection and lentiviral shRNA transduction respectively. Cells were subjected to treatment with ROS scavengers/inhibitors or compounds that alter ROS, followed by immunoblot analysis, intracellular superoxide (O2−) detection using lucigenin-based chemiluminescence assay, proteasome activity assay, flow cytometric analysis of intracellular ROS using DCFDA, and cell death assays via Annexin V/PI staining, detection of caspase cleavage and colony formation. PC12 cells were used to assess the role of ROS during NGF withdrawal-induced KIF1Bβ-mediated apoptosis.

Results: Here, we showed that wild-type KIF1Bβ protein expression positively correlates with O2− and total ROS levels in neuroblastoma cells, unlike apoptotic loss-of-function KIF1Bβ mutants. Overexpression of KIF1Bβ apoptotic domain variants increases total ROS and, specifically O2−, whereas knockdown of endogenous KIF1Bβ decreases ROS and O2−. Interestingly, O2− increases KIF1Bβ protein expression, independent of the proteasomal degradation pathway. Scavenging O2− or ROS decreases KIF1Bβ protein expression and subsequent apoptosis. Moreover, treatment with investigational redox compound Gliotoxin increases O2−, KIF1Bβ protein expression, apoptosis and colony formation inhibition.

Conclusion: Overall, our findings suggest that ROS and O2− may be important downstream effectors of KIF1Bβ-mediated apoptosis. Subsequently, O2− produced may increase KIF1Bβ protein expression in a positive feedback mechanism. Therefore, ROS and, specifically O2−, may be critical regulators of KIF1Bβ-mediated apoptosis and its protein expression in neuroblastoma.

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New Synthetic Lethal Genes In MYCN-Amplified Neuroblastoma Cells and Their Potential for New Therapeutic Approaches

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MYCN gene amplification clearly correlates with poor prognosis in patients with neuroblastoma and some types of cancer. Basically, transcriptional factors, including N-Myc, are thought to be “un-druggable” targets, and therefore, alternative approaches are required to develop new therapies of MYCN-amplified neuroblastoma. For instance, synthetic lethal (SL) approaches are emerging as a promising strategy for cancer therapy. Previous studies have revealed that aurora kinases and some cyclin-dependent kinases are SL genes in MYCN-amplified neuroblastoma cells. In order to identify new SL genes, we performed a genome-wide shRNA library screening. The commercial library that consists of over 80,000 shRNA constructs targeting about 16,000 human genes was used. IMR-32 cells (MYCN-amplified) and SH-SY5Y cells (MYCN single copy) were transduced with lentiviruses carrying the shRNA sequences. The relative abundance of shRNA constructs in each cell line is then quantified by next-generation sequencing. shRNA sequences with a false discovery rate <0.1 from an exact test analysis in edgeR software were selected. In addition to already known SL genes (e.g. SMC2, CSNK1E), about 130 genes were identified as new candidates. Based on our experimental validations using siRNA and chemical compounds, some mitotic kinases and enzymes involved in nucleic acid metabolism were proposed to be new SL targets. In accordance with the previous reports, the importance of mitotic kinases was further confirmed by our results. Elucidation of the molecular mechanisms underlying the lethality and development of molecularly targeted drugs will be required. On the contrary, chemotherapeutic agents targeting nucleic acid metabolism are widely used in the treatment of various types of cancer; however, their potential use in patients with MYCN-amplified neuroblastoma has not been well examined. Repositioning of existing agents should be worth considering as a new therapeutic strategy.

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Inhibition of Phosphoglycerate Dehydrogenase Inhibits Neuroblastoma Growth and Arginine Deiminase Reinforces the Effect by Altering Tumor Metabolism

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Background: In recent years, tumor metabolism has been received great attention. L-asparaginase for acute lymphoblastic leukemia is one of the successful examples. However, tumor metabolism in neuroblastoma (NBL) is not fully understood.

Methods: To extract candidate therapeutic targets in NBL, we performed RNA sequencing and DNA methylation analysis in 34 NBL specimens. In addition, the open data of "TARGET NBL" was also analyzed. Since phosphoglycerate dehydrogenase (PHGDH) showed significantly high expression in NBL with worse prognosis and has been shown to be involved in cancer-specific metabolism, functional analyses were further conducted. The effects of si-RNA mediated knock down of PHGDH and its inhibitor, CBR-5884, combined with cisplatin on NBL cell lines were assessed. We also analyzed the effect of arginine depletion by recombinant arginine deiminase (rADI) to the sensitivity to CBR-5884.

Results: In both TARGET cohort and our cohort, high expression with hypermethylation of gene body regions of PHGDH were observed in all samples with MYCN amplification and around half of samples with 11q deletion. Intriguingly, cases with 11q deletion showed significant association with high PHGDH expression and worse prognosis.

Inhibition of PHGDH on NBL cells with high PHGDH expression by si-RNA mediated knock down or CBR-5884 exhibited significant suppression of cell growth. It also significantly enhanced effects of cisplatin.

NBL cells with low PHGDH expression were less affected by knock down of PHGDH or CBR-5884.

Administration of rADI reinforced the expression of PHGDH and enhanced the cytotoxic effect of CBR-5884 on the NBL cells with low expression of PHGDH.

Discussion: PHGDH is the essential enzyme in serine biosynthesis, and low expression of PHGDH has been reported as the key to trigger p53-mediated apoptosis. Thus, high expression of PHGDH would be involved in the aggressiveness, and thus, it would be the promising target for high-risk NBL. Serine biosynthesis has been reported to be reinforced by ADI in several tumors. ADI might induce the addiction to PHGDH and serine metabolism in the NBL cells with low expression of PHGDH and resolved the resistance against CBR-5884. Our results showed that tumor metabolism is a promising therapeutic target in NBL.

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Manufacture of Neuroblastoma-specific CAR-T Cells from Mobilized Cyropreserved Peripheral Blood Stem Cell Units Depends on Monocyte Depletion

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Cytotoxic chemotherapy and radiation can render lymphocyte repertoires qualitatively and quantitatively defective. Thus, heavily treated patients are often poor candidates for the manufacture of autologous CAR-T cell products. In the USA and Europe, children with high-risk neuroblastoma undergo apheresis early in the course of their treatment to collect peripheral blood stem cells (PBSCs) for cryopreservation in preparation for high-dose chemotherapy followed by autologous stem cell rescue. Here, we investigated whether these cryopreserved chemotherapy and G-CSF mobilized PBSCs can be utilized as the starting material for CAR-T cell manufacture. We evaluated T cell precursor subsets in cryopreserved PBSC units from 8 patients with neuroblastoma using FACS-based analysis. Every cryopreserved unit collected early in treatment contained both CD4 and CD8 precursors with significant numbers of naïve and central memory precursors. Significant numbers of Ki67+/PDCD1+ T cells were also detected that presumably resulted from lymphopenia and subsequent homeostatic proliferation induced by early chemotherapy. Cryopreserved PBSC units containing at least 56-112x106 T cells were amenable to immunomagnetic selection, CD3x28 bead activation, lentiviral transduction and cytokine-driven expansion, provided that CD14 monocytes were depleted prior to the initiation of cultures. CD171-CAR+ CD8 effector cells derived from cryopreserved units displayed anti-neuroblastoma lytic potency and cytokine secretion comparable to those derived from healthy donors. These CD171-targeting CAR-T cells also mediated the regression of xenograft tumors grown intracranially from the human SK-N-DZ neuroblastoma cell line in NSG mice. Cryopreserved PBSCs procured during standard early neuroblastoma treatment can serve as an alternative starting source for CAR-T cell manufacturing, extending the options for heavily treated patients with relapsed neuroblastoma.

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A High-Throughput Drug Screen Identifies Compounds Selectively Targeting Neuroblastoma

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We have previously established neuroblastoma orthotopic patient-derived xenografts (PDXs) from high-risk patient tumors. The PDXs retain the histological hallmarks and genomic aberrations of their original patient tumors and they have the ability to metastasize to distant organs (Braekeveldt et al, 2015 & 2016). Further, we have showed that PDX-derived cells can be grown under serum-free conditions, where they maintain tumorigenic and metastatic capacities, and more accurately represent the disease as compared to conventional cell lines (Persson et al, 2017). Here, we use the PDX-derived cells in a high-throughput drug screen to identify novel compounds targeting aggressive neuroblastoma.

The FIMM set of clinically approved and emerging investigational oncology compounds was used. PDX cells were seeded in 384-well plates pre-plated with drugs in 5 concentrations in 10-fold dilutions. PDX cells from three high-risk MYCN amplified patient tumors were tested at both 21 and 5% O2. After 72 hours, cell viability was measured using the CellTiter-Glo luminescent assay. For analysis, each plate was normalized to negative and positive controls and dose-response curves were calculated for each compound. The curve was subsequently used to determine a drug sensitivity score (DSS), defined as the area under the dose-response curve compared to the total graph area, above a 10% threshold.

The screen identified drugs with potent effect in all three PDX models, under both high and low oxygen tensions. Neuroblastoma-selective compounds were identified by comparing the DSS of each drug to the results from a counter screen with normal bone marrow-derived cells. Among the selectively active compounds, we found conventional chemotherapies, apoptotic modulators, metabolic modifiers and various kinase inhibitors. Many of these compounds are already tested in neuroblastoma while several others are not previously investigated and may thus serve as novel therapeutic strategies.

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The Mesenchymal Differentiation State of Neuroblastoma: Consequences for Norepinephrine Transporter Expression and MIBG Uptake

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Background: Meta-iodobenzylguanidine (MIBG) is used as imaging agent and targeted radiotherapeutic in neuroblastoma. Approximately 10% of neuroblastomas are non-avid at diagnosis, meaning that these tumors do not accumulate or retain MIBG, leading to false-negative imaging interpretations. The Norepinephrine Transporter (NET) is involved in the uptake and retention of MIBG in neuroblastoma cells. However, the biological mechanism underlying MIBG avid and non-avid tumors remains as yet unkown. Recently, the existence of two types of neuroblastoma cells with highly diverging gene expression profiles was demonstrated. In addition to classic neuroblastoma cells with an adrenergic lineage identity, undifferentiated mesenchymal cells exist, leading to intratumoral heterogeneity. These mesenchymal cells are enriched in post-therapy and relapsed tumors. The aim of our study was to investigate whether these mesenchymal neuroblastoma cells show differences in NET expression and consequently in MIBG uptake.

Methods: Using Real-time quantitative PCR, 13 human neuroblastoma cell lines (SK-N-BE(2)C, SK-N-SH, LAN-6, GIMEN, NGP, N206, IMR32, SJNB-1, SJNB-8), including two isogenic cell line pairs (SH-SY5Y/SHEP2, and 691-B/691-T) were analyzed for NET mRNA expression. NET protein expression was determined in human neuroblastoma cell lines using Western Blotting. Two transgenic adrenergic cell lines were generated containing an inducible regulator of mesenchymal transformation: SK-N-BE(2)C with inducible PRRX1, and SH-SY5Y with inducible NOTCH3. In a time series, NET mRNA expression was measured using RT qPCR after induction of mesenchymal transformation.

Results: NET mRNA expression in mesenchymal cell lines (691-T, SHEP2, GIMEN) is either very low or undetectable in comparison with the classic adrenergic cell lines. The mesenchymal cell lines showed no protein expression in our Western Blot analysis. Inducible expression of PRRX1 and NOTCH3 in the adrenergic cell lines SK-N-BE(2)C and SH-SY5Y, respectively, resulted in a motile and mesenchymal phenotype, accompanied by a dramatic decrease in NET mRNA expression over time.

Conclusion: The mesenchymal differentiation state of neuroblastoma seems to be accompanied by the absence of NET expression. [125-iodine]-labeled MIBG internalization studies and immunohistochemistry on patient tumor material to further elucidate the relation between the mesenchymal differentiation state and NET expression and MIBG avidity are in progress.

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To Excrete or Not to Excrete - A Catecholamine Phenotypic Duality in Neuroblastoma

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Introduction: Urinary catecholamines can be used to diagnose patients with neuroblastoma. However, even when a panel of 8 catecholamine metabolites is used (Verly et al. Eur J Cancer 2017), 5% of the patients have no elevated catecholamines at diagnosis (catecholamine negative). Recently, two cell types, the adrenergic (ADRN) and mesenchymal (MES), were identified in neuroblastoma. Each type with its unique set of super-enhancers associated transcription factors (TFs) such as TFAP2B and PRRX1, respectively. This cell type duality might affect catecholamine excretion. In this study we investigated the biology of catecholamine excretion in vitro and in vivo.

Material and Methods: Catecholamine metabolites were measured in culture medium of 26 neuroblastoma cell lines. The presence of the catecholamine enzymes in neuroblastoma cell lines and primary tumors was examined with qRT-PCR, western blot and immunohistochemistry (IHC), respectively. Finally, catecholamine excretion in 3 matched ADRN- and MES neuroblastoma cell lines was examined. Furthermore, catecholamine excretion was tested after overexpressing PRRX1 and TFAP2B.

Results: Catecholamine metabolites were detected in the medium of 8/26 of the tested cell lines, which was related to the presence of the enzyme tyrosine hydroxylase (TH). TH protein was present in all the excreting and absent in all the non-excreting cell lines. IHC staining for TH in primary tumors showed an identical pattern with all catecholamine positive patients being TH positive (n = 70), whereas all catecholamine negative patients were TH negative (n = 3). Finally, catecholamine excretion was observed in all ADRN cell lines (3/3), but absent in all MES cell lines (0/3). Overexpression of PRRX1 induced mesenchymal transition and stopped catecholamine excretion. TFAP2B overexpression restored catecholamine excretion in ADRN non-excreting cell line, but not in a MES cell line.

Conclusion: In patients with neuroblastoma, catecholamine excretion is determined by the presence of TH protein in the tumor. Only ADRN cells can excrete catecholamines, which seems to depend on TFAP2B expression. MES cells do not excrete catecholamines, probably due to downregulation of ADRN TFs such as TFAP2B as well as catecholamine enzymes. Acknowledgements: this study was supported by a grant from the Villa Joep Foundation and SKOCA.

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Results of A Prospective Clinical Trial JN-L-10 Using IDRF-Based Surgical Decision for Children with Low-Risk Neuroblastoma Disease in Japan

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Background: In our previous studies on low-risk neuroblastoma (#9405 and #9805), 9.1% to 16.7% of patients had surgical complications. It is desirable to reduce the incidence of surgical complications while maintaining a good prognosis.

Purpose: We aim to reduce the incidence of treatment complications and improve the outcomes of low-risk neuroblastoma patients.

Patients and Methods: We conducted the JN-L-10 clinical trial from 2010 to 2013, with the aim of reducing the incidence of surgical complications in low-risk neuroblastoma patients. The eligible patients had low-risk neuroblastoma without MYCN amplification; these patients included children (<18 years of age) who had INSS stage 1 or 2 disease, children (<365 days of age) who had stage 3 tumors, and infants who had stage 4S disease with hyperdiploidy or a favorable histology. Patients underwent tumor resection based on the Image Defined Risk Factors (IDRF) determination. In IDRF-negative cases, treatment was completed with surgical resection alone. In IDRF-positive cases, the timing of surgery was determined based on IDRF and low-dose chemotherapy consisting of which regimen, A (vincristine/cyclophosphamide), regimen B (vincristine/cyclophosphamide/pirarubicin) or regimen C (vincristine/cyclophosphamide/carboplatin) was administered. The endpoints were overall survival, progression-free survival, adverse events and surgical complications.

Results: A total of 58 eligible patients were eligible in this trial. The median age was 0.5 years (range 0–14 years). Thirty-two patients were identified as IDRF-negative at the onset of disease and underwent primary surgical resection, 26 patients who were identified as IDRF-positive and underwent induction chemotherapy. The 3-year over-all and progression-free survival rates of the 58 patients were 100%, and 82.8% (95%CI: 70.3-90.3), respectively. Neutropenia was the most frequently reported grade 3 or 4 chemotherapy-related toxicity (41.7%). With regard to surgical complications, 2.5% of all patients developed pleural effusion and ascites as early complications after surgery, while only 2.5% developed renal atrophy as a long-term complication of surgery. There were no fatal toxicities.

Conclusion: Our treatment strategy using IDRF-based surgical decision-making for the low-risk neuroblastoma achieved a good prognosis and reduced the incidence of long-term complications.

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Prognostic and Therapeutic Implications of TERT Activation in Neuroblastoma

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Background: TERT-rearrangements have been recently discovered in high-risk neuroblastoma. Both TERT-rearrangements and MYCN-amplification lead to massive telomerase activation. We therefore investigated the potential prognostic and therapeutic relevance of telomerase activation in neuroblastoma.

Methods: The genomic status of the TERT locus was assessed in 454 primary neuroblastomas by break-apart FISH analysis. Alternative lengthening of telomeres (ALT) was examined by detection of ALT-associated PML bodies in 264/454 tumor samples, and TERT expression was analyzed in 214/264 samples by microarrays. Viable cell count upon treatment with the telomerase-interacting compounds BIBR-1532, costunolide or 6-thio- 2'- deoxyguanosine was assessed in neuroblastoma cell lines in vitro, and tumor growth was examined in neuroblastoma xenografts treated with 6-thio-2'-deoxyguanosine.

Results: We detected TERT-rearrangements in 47/454 cases (10.4%; high-risk, n=42, non-high-risk, n=5) and ALT activation in 47/264 cases (17.8%; high-risk, n=32, non-high-risk, n=15). MYCN-amplification was found in 91/454 cases (20.0%) All three alterations were associated with unfavorable prognostic markers but occurred in nearly mutually exclusive fashion. Additionally, we identified a small subgroup of tumors (9/214; 4.2%) mainly derived from patients ≥18 months with stage 4 disease that showed increased TERT expression without harboring a TERT-rearrangement or MYCN-amplification. Patients whose tumors were positive for telomerase activation (i.e., TERT-rearrangement, MYCN-amplification or elevated TERT expression) had significantly worse overall survival than patients positive for ALT activation or patients whose tumors lacked telomere maintenance (OS at 5 years, 0.491±0.068 vs. 0.750±0.083 vs. 1.0, p=0.027 and p<0.001, respectively). In multivariable analyses, telomerase activation predicted both EFS and OS independently of established clinical variables (p<0.001 each). In vitro analysis showed that treatment of neuroblastoma cell lines bearing TERT-rearrangements or MYCN-amplification with 6 thio-2'-deoxyguanosine, costunolide or BIBR-1532 inhibited cell growth at significantly lower concentrations than in cell lines with ALT activation (p<0.001, p=0.016, p=0.004, respectively). In addition, treatment with 6-thio-2'-deoxyguanosine impaired tumor growth of neuroblastoma xenografts bearing TERT-rearrangements or MYCN-amplification resulting in significantly improved overall survival compared to vehicle-treated controls.

Conclusion: We here demonstrate that telomerase activation defines a clinical neuroblastoma subgroup of poor outcome, and that telomerase may represent a promising therapeutic target in these patients.

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The Randomised Induction for High-Risk Neuroblastoma Comparing COJEC and the N5-MSKCC Regimen. Early Results from the HR-NBL1.5/SIOPEN Trial

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Aim: From 2013 the HRNBL1.5/SIOPEN trial tested the hypothesis that the N5-MSKCC induction (Kushner, J Clin Oncol.2004;22(24):4888-92) would improve the metastatic complete response (mCR) or event free survival (EFS) rate as compared to Rapid COJEC (Ladenstein, J Clin Oncol.2010;20;3516–24).

Methods: Eligible patients all had stage 4(4s) high-risk neuroblastoma with MYCN amplification (MNA) any age <21 years or without MNA in age group 12-18 months with proven segmental chromosomal alterations and >18 months with any genomic profile. Randomisation was stratified according to national group and metastatic sites. Further treatments included addition of 2 TVD if <mCR, attempted gross resection of primary tumour, BuMel/SCT, 21Gy radiotherapy to the primary site and ch14.18/CHO antibody ± IL2 in addition to 13-cis-RA. Two primary endpoints were investigated: improving EFS by 12.5 % with N5-MSKCC (2-years EFS: 52.5%) and the mCR rate to 45% (33% for Rapid COJEC).

Results: 630 patients were randomised (313 Rapid COJEC/317 N5; 56% male; 99% stage 4; median follow-up 1.7years). The median age was 3.2years (0.2-20.4), only 16 were infants (3%) and 56 (9%) between 12-18mo. No primary endpoint differences were observed: 2y- EFS was 53% ±4 for Rapid COJEC vs 51%±4 for N5-MSKCC and the mCR rate after induction was 33% for COJEC and 37% for N5-MSKCC. There was no difference in secondary endpoints: Overall Survival was 72%±3 for Rapid COJEC vs 69%±3 for N5-MSKCC, non-relapse mortality (2y-NRM) was 8%±2 with Rapid COJEC vs 6±2 with N5-MSKCC and the cumulative incidence of relapse/progression (2y-CIR) was 39%±3 for Rapid COJEC and 43%±4 for N5-MSKCC. A comparison of CTC Grade 3&4 toxicities showed the following significant differences favouring Rapid COJEC over N5-MSKCC: non-haematological 47% vs. 68% (p=0.000), general condition 12% vs 19% (p=0.055); WBC 94% vs 98% (p=0.020); platelets 91% vs96% (p=0.031); infection24% vs35% (p=0.005); stomatitis 3% vs 26% (p=0.000); nausea/vomiting 7% vs 16% (p=0.001); diarrhoea 3% vs 7% (p=0.033); hypertension 10% vs 6% (p=0.050); central neurotoxicity 0% vs 1% (p=0.049).

Conclusion: No differences in primary or secondary outcomes were identified between the two arms. Rapid COJEC is the preferred SIOPEN standard induction due to significantly higher toxicities observed with N5-MSKCC.

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Targeting Aberrant Mitochondrial Metabolism for Neuroblastoma Combination Therapy with 131I-MIBG

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Maximal therapeutic benefit from the neuroblastoma-targeting radiopharmaceutical 131I-MIBG may be obtained by its combination with chemotherapy. Aberrant mitochondrial metabolism is a common trait among cancers. The synthetic glucose analogue 2-deoxyglucose (2-DG) and the anti-diabetic drug metformin both target mitochondrial metabolism following inhibition of glycolysis and the electron transport chain, respectively. Both 2-DG and metformin have shown potential as radio- and chemosensitisers. Our purpose was to investigate the potential of 2-DG and metformin to enhance the efficacy of experimental therapy of neuroblastoma.

Clonogenic assay was used to determine the cytotoxicity of 2-DG and metformin on SK-N-BE(2c) neuroblastoma cells and UVW glioma cells transfected with the noradrenaline transporter, NAT (UVW/NAT cells). NAT facilitates the intracellular transport of the noradrenaline analogue, 131I-MIBG. Flow cytometry was used to determine the effect of 2-DG and metformin on cell cycle redistribution.

2-DG acted as a radiosensitiser when administered at 10 mM. This was exemplified by the significant reduction in radiation dose required to sterilise 50% of SK-N-BE(2c) clonogens (from 2.23 Gy to 1.32 Gy; $p<0.01$) or UVW/NAT clonogens (from 2.66 Gy to 1.69 Gy; $p<0.01$). Metformin failed to radiosensitise SK-N-BE(2c) or UVW/NAT cells when administered at concentrations ≤3 mM. However, triple agent therapy consisting of metformin, 2-DG and X-irradiation enhanced the cell kill achieved by either single agent modality administered alone to UVW/NAT cells. Furthermore, we observed that irradiating cells 6 h prior to treatment with 2-DG and metformin was the most effective treatment regimen. This schedule produced the greatest G2/M cell cycle arrest and also killed the greatest number of UVW/NAT clonogens.

The glycolytic inhibitor 2-DG sensitised neuroblastoma and glioma cells to external beam ionising radiation. Moreover, the efficacy of 2-DG was further enhanced by its combination with the anti-diabetic drug, metformin. We hypothesise that the mechanism of 2-DG radiosensitisation entails the onset of apoptosis following the prolonged accumulation of cells in G2/M of the cell cycle. Disruption of cancer cell metabolism using glycolytic inhibitors is expected to improve the treatment of neuroblastoma using 131I-MIBG targeted radiotherapy.

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Success of the HR-NBL1/SIOPEN Trial Local Control Strategy in High-Risk Neuroblastoma Patients Receiving High-Dose Therapy and Stem Cell Transplantation

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Aim: Investigation of the local relapse rate (LRR) and outcomes in high-risk neuroblastoma.

Methods: HR-NBL1/SIOPEN treatments included Rapid COJEC or N5-MSKCC induction, plus two TVD courses if unsatisfactory metastatic response, attempted primary tumour gross resection, HDT/SCT (BuMel or CEM) and 21Gy radiotherapy to the primary site followed by 13-cis-RA with addition of ch14.18/CHO antibody after 2009. Analysis included patients starting treatment prior to 2016, with HDT/SCT, alive without event 30 days after SCT and information on radiotherapy. The cumulative incidence of local relapse/progression (CILR/P) either isolated or combined with other sites was calculated considering competing risks from other causes including isolated distant relapse/progression (R/P), R/P at unknown sites, death or secondary malignancy without prior R/P.

Results: Radiotherapy data was available in 1210/1297 (93%) of eligible patients (median follow-up 4.9years): 807/888 (91%) were in local CR (LCR) and 290/334 (90%) had <LCR. For LCR patients the isolated CILR (29/807) was 0.03±0.01 whilst the combined (local and distant) CIR was (77/807) 0.13±0.01 at 5 years. LCR patients without radiotherapy had an isolated CILR (6/81) of 0.08±0.03 whilst the combined CIR (10/81) was 0.22±0.05. Patients with <LCR receiving radiotherapy had an isolated CILR (17/290) of 0.06±0.02 whilst the combined CIR (39/290) was 0.21±0.03. In patients without radiotherapy and <LCR, the isolated CILR/P (3/32) was 0.09±0.05 whilst the combined CIR/P was (9/32) 0.38±0.09. The 5-year event-free survival (5yr-EFS) was 0.55±0.02 in LCR patient receiving radiotherapy but was only 0.38±0.06 for those without. The 5yr-EFS was 0.47±0.03 for <LCR patients receiving radiotherapy but was only 0.10±0.06 for those without. Decision not to irradiate, was primarily related to large radiotherapy fields based on pre-operative volumes and/or very young age, in 112 patients. In stage4>1.5yrs the 5-yr EFS pre-2009 was 0.36±0.03 in LCR and 26±0.05 in <LCR patients but post-2009 was 0.46±0.03 in LCR and 0.36±0.02 in <LCR.

Conclusion: Local radiotherapy with 21Gy is an important component lowering the local relapse rate in LCR and <LCR patients compared with European historical data of a 40% LRR. Further research to optimise the

use of radiotherapy is needed. Most importantly both, local radiotherapy and immunotherapy contributed to improved outcomes.

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Co-treatment with MYCN Inhibitors Can Partially Overcome the Negative Influence of High-Level MYCN on CD171-directed CAR-T Cell Therapy In Vitro

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Current treatment protocols have only limited success in patients with neuroblastomas harboring amplifications of the central oncogenic driver, MYCN. The success achieved by immunotherapies against lymphomas and leukemias have not yet been reached for solid tumors, but immunotherapy has great potential to improve cure rates in high-risk patients. We aimed to investigate the influence of MYCN amplification on tumor cell response to neuroblastoma-specific CAR-T cell therapy. As a starting point, we used the neuroblastoma cell line, SK-N-AS, with the normal diploid MYCN complement (MYCNnon-amp) and overexpressed MYCN (MYCNamp) in these cells using a tetracycline-inducible system. Neuroblastoma cells with the different MYCN backgrounds were co-cultured with second-generation CD171-targeting CAR-T cells harboring either CD28 or 4-1BB as a costimulatory domain. CD171-CAR-T cell function was assessed in assays for activation/inhibitory receptor expression, cytokine release and cytotoxicity. To investigate whether indirect MYCN inhibition could enhance the efficacy of CAR-T cell therapy, we co-treated our co-culture model with the AURKA inhibitor, MLN8237, or the bromodomain inhibitor, TEN010. Co-culture with MYCNamp SK-N-AS cells reduced expression of activation markers (CD25 by 38% and CD137 by 28% compared to parental cell co-culture) on CD171-CAR-T cells. Expression of the inhibitory Tim3 receptor was increased by almost two-fold on CD171-CAR-T cells in co-culture with MYCNamp SK-N-AS cells in comparison to the parental cell line. CD171-CAR-T cells released up to 10-fold less IFNγ and IL-2 during co-culture with the MYCNamp SK-N-AS cells and demonstrated 33% less killing potential against the MYCNamp SK-N-AS cells (in comparison to the parental cell line). All effects were observed for CD171-CAR-T cells harboring the CD28, but not 4-1BB, costimulatory domain. Co-treatment with targeted MYCN inhibitors increased CD171-CAR-T cell-directed neuroblastoma cell killing by up to 40%, regardless of MYCN background. MLN8237 yielded a superior synergistic response with CD171-CAR-T cells to TEN010. High-level MYCN expression in neuroblastoma cells negatively influences CD171-directed CAR-T cell therapy in vitro. This negative influence can be partially overcome by combination of immunotherapy with inhibitors of MYCN function, with AURKA inhibition achieving a stronger effect than bromodomain inhibition.

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The p53 Activator PRIMA-1MET Induces Apoptotic Cell Death in Neuroblastoma and Synergizes with Etoposide and Cisplatin

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Introduction: Neuroblastoma (NB) is the most common extracranial solid tumor in infants and children with low survival for high-risk patients despite dose-intensive multimodal therapy. In contrast to other cancers, NB has low frequency of TP53 mutations and its downstream pathway is usually intact. In this study, we assessed the efficacy of the p53 activator, PRIMA-1MET (P-1M) in inducing NB cell death and investigated possible synergies with currently used chemotherapeutics.

Methods: LD50 was used to study susceptibility and specificity of eight NB cell lines (3 with TP53 mutation) to P-1M and to test for synergistic effects with cisplatin and etoposide. Real-time PCR and in-cell western blot were used to assess gene and protein expression of the most common p53 transactivation targets. Flow cytometry was used to analyze cell cycle phase and induction of apoptosis, reactive oxygen species, collapse of mitochondrial membrane potential, and autophagy.

Results: NB cell lines are at least 4 times more susceptible than primary cell lines to P-1M. P-1M induces cell death rapidly and in all phases of the cell cycle, independent of the p53/ATM axis. Noxa is consistently upregulated in the seven susceptible NB cell lines and its induction correlates well with LD50. Whole exome sequencing of the resistant BE-2C cell line identified the homozygous TP53 mutation C135F. This mutation could be important in the development of resistance. P-1M significantly impacts the intracellular concentration of glutathione and synergizes with the glutathione inhibitor BSO. The effect of P-1M is not altered by thioredoxin inhibition, and in most cases P-1M does not induce oxidative stress. In addition, we observed clinically important synergy of P-1M with cisplatin and etoposide, as well as induction of autophagy and collapse of mitochondrial membrane potential.

Conclusions: P-1M could be a promising new agent used to treat NB tumors. It has good antitumor action and synergizes well with the currently-used chemotherapeutics cisplatin and etoposide by helping to overcome resistance. In addition, it's fast action, its ability to kill irrespective of cell cycle phase, the augmentation of its efficacy after glutathione depletion, and its inhibition of thioredoxin reductase could present additional opportunities for P-1M utility.

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N-Myc Interactome Assay Identified KAT2A As A Novel Druggable Target In High-Risk Neuroblastoma

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N-Myc, a transcription factor encoded by MYCN gene is a neuroblastoma oncogenic driver, however, pharmacological strategies that directly suppress N-Myc activity in cancer remain limited. To identify druggable N-Myc protein partners that are essential for N-Myc transcriptional and oncogenic activity, we performed co-immunoprecipitation and mass-spectrometry assays. By using two different anti-N-Myc antibodies to pull down N-Myc in IMR32 cells, we identified >300 common novel N-Myc partners. DAVID analysis revealed N-Myc partners are significantly enriched in transcription regulation, DNA repair, DNA replication, spliceosome and chromatin remodeling, such as the components of the STAGA, SWI/SNF and NuRD complex. STAGA (SPT3-TAFII31-GCN5L acetylase) is a chromatin-acetylating transcriptional coactivator and recent studies show that KAT2A (GCN5L) acetylates and stabilizes a number of proteins. Thus, KAT2A could potentially modulate both N-Myc stability and N-Myc mediated chromatin modification. Co-transfection of KAT2A and MYCN constructs in 293T cells, resulted in increased N-Myc acetylation and protein half-life. N-Myc's increased stability is potentially mediated via abrogation of proteasome degradation since decreased HERC2 and FBXW7 (ubiquitin protein ligases) binding was found in acetylated vs non-acetylated N-Myc co-IP products. Genetic knockdown of KAT2A in neuroblastoma cells led to a 2.5-fold decrease of N-Myc protein levels. Pharmaceutical inhibition of KAT2A using KAT2A inhibitors (MB3 and CTPH2) also led to a decrease in N-Myc protein (2~3-fold). Moreover, pharmacologic and genetic KAT2A inhibition reduced expression of N-Myc target genes and cell viability. We further treated nine neuroblastoma cell lines differing in MYCN or c-Myc status with MB3. Western blots show inhibition of KAT2A destabilizes N-Myc or c-Myc. Growth rate inhibition (GR) assay showed that N-Myc/c-Myc high neuroblastoma cells are more sensitive to the pharmaceutical inhibition of KAT2A than N-Myc/c-Myc low cell lines and immortalized normal cell line (~2-fold lower GR50, $p<0.05$). The inhibition of KAT2A led to elevated cleavage of caspase3 and PARP1 in N-Myc/c-Myc-high cell lines, indicating that inhibition of KAT2A preferentially induces apoptosis in N-Myc or c-Myc-high cell lines. Taken together, this is the first study to show that the inhibition of KAT2A destabilizes N-Myc or c-Myc proteins, making KAT2A a promising therapeutic target in high-risk neuroblastoma as well as other Myc-driven cancers.

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Combined Immunotherapy with Anti-PDL-1/PD-1 and Anti-CD4 Antibodies Cures Syngeneic Disseminated Neuroblastoma

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Background: Anti-PD-1 or anti-PD-L1 blocking monoclonal antibodies (mAbs) have shown potent anti-tumor effects in adult cancer patients and clinical studies have recently been started in pediatric cancers, including high-risk/relapsing Neuroblastoma (NB). IL-21, an immune enhancing cytokine produced by T helper cells, showed promising results in pre-clinical and clinical cancer immunotherapy. We previously observed that the administration of anti-CD4 cell-depleting antibody strongly enhanced the anti-tumor effects of recombinant (r)IL-21immunotherapy in a syngeneic model of disseminated NB. The removal of pre-existing and NB-induced CD4+CD25high Treg cells by the anti-CD4 mAb synergized with IL-21 in the induction of anti-NB CD8+ T cell responses.

Methods: We studied the effects of anti-PD-1/PD-L1 mAbs in two syngeneic models of disseminated NB generated by the injection of either Neuro2a or NXS2 cells in A/J mice. Both murine NB cell lines express PD-L1. In addition, we tested the combination of these agents with the immune-enhancing cytokine IL-21, the Ecto-NTPDase inhibitor POM-1, an anti-CD25 mAb targeting Treg cells, or an anti-CD4 mAb.

Results: Here we show that mono-therapy with anti-PD-1/PD-L1 mAbs had no effect on systemic NB progression in vivo, and also their combination with IL-21, POM-1 or anti-CD25 mAb was ineffective. The combined use of anti-PD-1 with an anti-CD4 mAb mediated a very potent, CD8-dependent, synergistic effect leading to significant elongation of tumor-free survival of mice, complete tumor regression and durable anti-NB immunity. Similar results were obtained by combining anti-PD-L1 and anti-CD4 mAbs.
Conclusion: These findings indicate that both PD-1/PD-L1 and CD4+ T cell-related immune-regulatory mechanisms must be simultaneously blocked to mediate therapeutic effects in these models.

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NKT Cells Control Tumor Associated Macrophages and Metastatic Growth in Neuroblastoma

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Vα24-invariant NKT cells (NKTs) control tumor growth via poorly understood interactions with CD1d-positive tumor-associated macrophages (TAMs). TAMs comprise M1- and M2-like subsets, but only CD163high M2-like TAMs are associated with poor outcome in neuroblastoma (NB) patients. Here, we demonstrate that NKTs selectively target M2-like TAMs via contact-dependent and independent mechanisms. Upon direct contact with antigen-pulsed M1 or M2, NKTs selectively kill the latter. Additionally, we found that antigen-activated NKTs could reprogram M2 into functional M1-like macrophages via GM-CSF production. Furthermore, adoptive transfer of human NKTs resulted in M1-like polarization of TAMs in metastatic NB xenografts in humanized NSG mice. To further explore the role of NKT–TAM axis in tumor immune surveillance, we examined the effect of NKT deficiency on tumor progression and TAM accumulation in NB-Tag transgenic model of NB. Mice lacking either type I (Jα18-/-NB-Tag) or all (CD1d-/-NB-Tag) NKTs had shortened survival compared with NB-Tag mice ($P < 0.0002$). At four month of age we observed an increase of CD11b+Ly6G-Ly6C-F4/80+ TAMs in primary tumors of NKT deficient groups compared with NB-Tag mice. Despite no difference in the size of primary adrenal tumors between groups, the increase of TAM frequency coincided with metastatic spread in NKT deficient groups as detected by CT imaging and confirmed by pathological analysis. By five months, nearly all mice in NKT deficient groups had distant metastases in liver and lungs but none of the NKT replete mice had detectable distant metastases. Thus, our results reveal a novel mechanism of immune regulation, in which NKTs selectively control M2-like TAMs and suppress tumor metastasis.

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MGMT Inhibitor O6-Benzylguanine Enhanced Temozolomide + Irinotecan Activity Against In Vitro And In Vivo Models of Progressive Disease High-Risk Neuroblastoma

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Introduction: Patients with high-risk neuroblastoma (NB) treated with DNA-damaging chemotherapy often relapse with treatment-refractory disease. Temozolomide (TMZ; DNA-methylating agent) and irinotecan (IRN; topoisomerase I inhibitor) are well-tolerated and have clinical activity in relapse/refractory NB. We hypothesized that DNA repair genes with increased expression in alkylator-resistant NB models would provide potential therapeutic targets for enhancing chemotherapy.

Methods: TaqMan Low Density Arrays (TLDA) were used to screen mRNA expression of 62 DNA repair genes in 9 alkylator-resistant and 4 alkylator-sensitive cell lines. Differential gene expression was validated in an expanded NB cell line panel (n=26) by qRT-PCR. Gene overexpression was by lentiviral transduction of pLenti plasmid constructs. In vitro cytotoxicity was assayed using a digital imaging microscopy system (DIMSCAN). DNA double-strand breaks, apoptosis, and DNA fragmentation were assessed using phospo-histone H2AX, cleaved caspase-3, and TUNEL, respectively. In vitro testing used the SN38 active metabolite of IRN. Subcutaneous patient-derived xenografts (PDXs) in nu/nu mice were treated with TMZ, IRN, +/- O6-benzylguanine (O6BG).

Results: Relative to drug-sensitive NB cell lines, alkylator-resistant NB cell lines showed increased mRNA expression of O6-methylguanine-DNA methyltransferase (MGMT), glutamate-cysteine ligase modifier subunit (GCLM), proliferating cell nuclear antigen (PCNA), and single-strand-selective monofunctional uracil-DNA glycosylase 1 (SMUG1) ($p<0.05$) by TLDA screen. MGMT, GCLM, and SMUG1 were expressed significantly higher in alkylator-resistant compared to alkylator-sensitive models ($n=26$, $p<0.05$) by qRT-PCR. MGMT was selected for further focus due to existence of a clinical-stage inhibitor and relevance to TMZ+IRN. MGMT expression positively correlated with in vitro TMZ+SN38 IC50 ($n=14$, $p<0.05$). Overexpression of MGMT in non-expressing progressive disease NB cell lines significantly decreased TMZ+SN38 cytotoxicity ($p<0.05$). In MGMT-expressing cell lines O6BG enhanced TMZ+SN38 cytotoxicity, H2AX phosphorylation, caspase-3 cleavage, and apoptosis by TUNEL. TMZ+IRN+O6BG increased median event-free survival ($p<0.05$) relative to TMZ+IRN in 2 of 5 MGMT-expressing progressive disease PDX models.

Conclusions: High MGMT expression is associated with in vitro drug resistance. The MGMT inhibitor O6BG synergistically enhanced the activity of TMZ+IRN in a subset of MGMT-expressing NB models both in vitro and in vivo at clinically achievable drug concentrations. Further studies evaluating MGMT as a therapeutic target in recurrent high-risk neuroblastoma are warranted.

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CNS Relapses of High-Risk Neuroblastoma: Analysis of Risk Factors and Clinical Outcomes

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Background: CNS relapses in patients with high-risk neuroblastoma are an extremely unfavorable clinical event. The aim of the study was to analyze the frequency of occurrence and the factors predisposing to the development of the CNS involvement in patients with high-risk neuroblastoma at the time of the first relapse.

Materials and Methods: Patients with high-risk neuroblastoma who received treatment for the period 01.2012 - 12.2015 were included in the analysis. Patients were stratified to risk groups and treated according to German NB2004 protocol. High-dose preparative regimens included melphalan/etoposid/carboplatin (MEC) (till June 2013) and treosulfan/melphalan (TreoMel) (since July 2013).

Results: 111 patients were analyzed. 58/111 (52.3%) had progression/relapse, CNS involvement was observed in 10/58 (17.2%). Isolated CNS involvement was noted in 7/10 (70%). Median age at the time of the neuroblastoma diagnosis in patients with CNS involvement was 27.3 months (range 1,8-43,5). Male: female ratio was 1.5:1. The primary tumor located in the abdomen in 9/10 cases, unknown primary in 1/10. All 10 patients had stage 4 disease. MYCN amplification was detected in 8/10 (80%). Median time from diagnosis till progression/relapse was 15.2 months (range 5.9-20.0). Comparison between two groups of relapsed patients (CNS involvement/ lack of CNS involvement) by sex, age, site of the primary tumor, MYCN gene status, skull metastases, bone marrow involvement at the onset of the disease, the number of metastatic compartments, response to induction therapy before auto-HSCT, type of preparative regimen (CEM vs Treo/Mel) showed no statistically significant difference. 2-year overall survival after the development of CNS-relapse in the study group was 13.1 ± 12.1%.

Conclusions: CNS involvement at the time of relapse is a frequent event in high-risk neuroblastoma. The introduction of CNS MRI into the standard work-up at the time of relapse/progression will allow timely detection of metastases to optimize the therapeutic strategy.

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Treatment of High-Risk Neuroblastoma: Experience of Russian Federal Centers

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Aim: The aim of the study was to analyze the results of therapy of high-risk neuroblastoma in three federal centers in Russia.

Methods: 270 patients with NB were treated for the period 01.2012-06.2015 (42 months). Patients were stratified and treated according to the German NB2004 protocol. 94 (34,8%) patients were stratified for the high-risk group. High-dose preparative regimens included carboplatin/etoposide/melphalan (CEM) (till June 2013) and treosulfan/melphalan (TreoMel) (since July 2013). Since July 2014 patients with clear MIBG-positive residual primary tumor and/or metastases prior to hematopoietic stem cell transplantation (HSCT) received 131I-MIBG-therapy. Database was locked on 01.09.2017.

Results: Male: female ratio was - 1.18:1. The median age at the diagnosis was 32.0 months (range 1.3-128.4). MYCN amplification was observed in 43 (45.7%). 82 (87.2%) patients had stage 4 NB. Induction therapy was completed in 90 (95.7%). Median number of chemotherapy cycles prior to transplantation was 6 (range 6-10). Surgery was done in 89 patients, resection was macroscopically complete in 39 (43.8%). Complete, very good partial response and partial response were achieved in 80 (85.1%) cases. 9 (9.5%) patients, all stage 4, progressed during the induction. 14/82 (17.0%) stage 4 patients with MIBG uptake after the induction received 131I-MIBG-therapy. 78/94 (82.9%) received high-dose chemotherapy: 28 - CEM, 50 - TreoMel. Contraindications for HSCT included tumor progression (n=9), therapy complications (n=7). Transplant-related mortality was 3/78 (3.8%), all in CEM group. 26/94 (27.6%) patients received radiation therapy to primary tumor and/or solitary metastatic site.

Median follow-up time for all patients was 33.1 months (range 3.4-70.7), for alive patients 42.9 months (range 19.0-70.7). 3-year and 5-year EFS was 38.7±5.0% and 36.7±5.1%, OS - 60.5±5.2% and 53.8±5.6%. Age, MYCN status, extent of surgery, type of preparative regimen didn't impact EFS in univariate analysis. Patients with stages 1-3/4S had significantly better EFS (83.3% versus 29.6%, p=0.002) and OS (91.6% versus 48.6%, p= 0.01) than stage 4 patients.

Conclusion: Our results are consistent with other research groups. Intensive therapy allows achieving satisfactory results of therapy in stages 1-3/4S high-risk NB patients. The introduction of novel therapies are urgently required to improve prognosis in stage 4 NB.

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Association of Anti-Tumor Activity in Neuroblastoma Patient-Derived Xenografts with Levels of GD2 Expression

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Introduction: Maintenance therapy with a chimeric anti-GD2 antibody, ch14.18 (dinutuximab) combined with cytokines and isotretinoin improves outcome for high-risk neuroblastoma (NB). A phase II clinical trial combining dinutuximab with temozolomide + irinotecan (TMZ + IRN) showed promising activity in recurrent NB (Lancet Oncol, 18(7): 946-957, 2017), but a report that ~ 12% of NB patients have low or negative GD2 expression (Pediatr Blood Cancer 64(1): 46-56, 2017) indicates the need to assess GD2 expression on NB and to define expression levels necessary for activity.

Methods: Using dinutuximab and flow cytometry we quantified GD2 surface expression from patient-derived NB cell lines and xenografts (PDXs) established at diagnosis (DX, n = 12) or at progressive disease (PD, n = 19). Activity of dinutuximab in combination with TMZ + IRN was assessed in PDXs in nu/nu mice established from late-stage PD patients in terms of tumor response and mouse event-free survival (EFS).

Results: GD2 expression was low or negative on 16% of 31 NB cells lines. Low GD2 (≤ 50% of median fluorescence intensity) was seen in 5 of 19 (26%) PD NB cell lines but not in any of the 12 DX NB cell lines. In a GD2-positive PDX, dinutuximab enhanced mouse event-free survival (EFS) when combined with TMZ + IRN. However, in a PDX with low GD2 expression, dinutuximab did not significantly increase EFS over TMZ + IRN alone. Using two-color flow cytometry with directly-labeled dinutuximab + directly-labeled HSAN 1.2, (neuroblastoma specific, marrow negative) we have demonstrated low GD2 expression on neuroblastoma in blood and marrow.

Conclusion: Our data with patient-derived NB cell lines and PDXs are consistent with a prior report and indicate that low GD2 expression can occur in NB and may be more frequent in PD patients. NB PDXs in nu/nu mice provide a preclinical model to assess dinutuximab activity when combined with chemotherapy. Dinutuximab enhanced activity of temozolomide + irinotecan in a GD2-positive NB PDX but not a PDX with low GD2 expression. Quantifying GD2 expression in NB is a potential biomarker of activity that warrants evaluation in patients treated with dinutuximab combined with temozolomide + irinotecan.

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Intestinal Microbiota in Pediatric Neuroblastoma Treatment

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Malnutrition and illness at an early age has been associated with disruptions in intestinal microbiota maturation, resulting in an immature composition of gut commensals for age. Such microbiota immaturity has been correlated with poor growth and abnormal immunity. Children undergoing treatment for neuroblastoma often experience frequent infections, malnutrition and prolonged hospitalizations, all conditions that have been associated with dysbiosis. Therefore, we have undertaken a study characterizing the intestinal microbiota of children undergoing treatment for neuroblastoma.

Microbiota composition in stool samples from patients with neuroblastoma was analyzed using 16S ribosomal RNA gene sequencing. Stool collection was undertaken during every phase of therapy including, induction chemotherapy, surgical resection, consolidation with antibody therapy and post-treatment immunization with anti-GD2 vaccine. The microbiota composition in this cohort was compared to a previously analyzed population of healthy twin pairs using t-Distributed Stochastic Neighbor Embedding (tSNE) visualization. Diversity was analyzed using the Simpson's Diversity Index. Microbiota maturity was determined using a Random Forest model approach previously described (1).

Twenty-three patients, ages 0-9 years, were included in the study. Pre-treatment samples demonstrated no significant dysbiosis, with predicted microbiota maturity falling within six months of chronologic age. Dysbiosis developed in all patients receiving induction chemotherapy, with both loss of diversity and domination by Enterococcus faecium observed in 70% (7/10) of patients. Microbiota immaturity was observed in all patient samples during induction and consolidation, with predicted microbiota age below 12 months, independent of chronologic age. In 10 children analyzed after completion of standard therapy for high-risk neuroblastoma, gut microbiota continued to be immature, despite overall improvement in intestinal diversity. In this group, predicted microbiota age ranged 8-18 months, for chronologic ages of 3-9 years. Therefore, treatment for intermediate and high-risk neuroblastoma in young children results in intestinal dysbiosis and delayed microbiota maturation for age.

1. Planer JD, et al. Nature. 2016 Jun 9;534:263-266.

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Recapitulating Neuroblastoma in An Avian Embryo: A Novel Model to Decipher the Etiology and Dissemination Programs of The Disease

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Neuroblastoma (NB) is a childhood cancer whose particularity is to arise from a transitory embryonic cell population, neural crest cells (NCCs) of the sympatho-adrenal lineage. Disseminated forms have high frequency of multiple tumoral foci at diagnosis whose etiology remains unknown. The latter fact is mainly due to the NB embryonic origin that limits access to early tumorigenesis events in patients and current models. Inspired by early inter-species grafting experimental paradigms in the avian embryo (LeDouarin and Dieterlen-Lievre, 2013), we developed a novel in vivo embryonic model, driving human NB tumorigenesis in tissues homolog to those of patients (Delloye-Bourgeois et al, 2017). It consists in micrografting human NB cells or patient samples in an avian embryo, at the place where their cells of origin, the sympatho-adrenal NCCs, are generated. Our model allowed us to unambiguously show that aggressive NBs have a metastatic dissemination mode, forming primary tumors first, and secondary disseminating at multiple foci via peripheral nerves and dorsal aorta.

To characterize the gene programs specifically active in NB cells preparing for dissemination, we conducted comparative transcriptomic analysis of primary tumors prior to metastatic onset and and of equivalent ungrafted NB cells. This revealed a unique picture of gene activities in NB cells, reflecting the impact of the embryonic sympatho-adrenal microenvironment. We exploited these data to uncover a pro-metastatic switch engaging NB cells in the primary tumor towards dissemination. This switch turned out to result from the decline in NB cells of a Semaphorin3C / Neuropilin / PlexinA4 NCC signaling normally dedicated to the regulation of collective migration and cell-cell cohesion.

Future projects conducted in the team aim at exploiting our new NB model to provide an extensive and detailed view of the morphogenetic programs of the embryo that contribute to the NB metastatic disease, and the nature of dysfunctional and intact molecular signaling acting in NB cells to support their metastatic dissemination and selection of secondary tumor foci. Deciphering NB interplays with the embryonic environment in which they emerge should open new therapeutic perspectives taking into account the pediatric status of the disease.

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Disrupted Endoplasmic Reticulum-Mitochondria Contact Sites Promote Neuroblastoma Multidrug Resistance

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Background: Many neuroblastoma patients succumb to multidrug resistant disease. Resistance is attributed to insensitivity to stress-induced apoptosis, however, its mechanisms remain obscure. Mitochondria (mito) provide platforms for integrating stress signals to determine cell survival or death. Mito interact with endoplasmic reticulum (ER) at contact sites that regulate calcium and lipid transfer to modulate apoptotic sensitivity. These ubiquitous ER-mito contacts are maintained by tethering complexes that include MFN2 and PACS2. We show that disruption of ER-mito tethering in therapy sensitive neuroblastomas, as seen in resistant tumors, induces apoptotic insensitivity and multidrug resistance.

Methods and Results: We studied neuroblastomas from diagnosis (DX) and relapse (REL) from the same patients treated with high-risk therapy, and neuroblastoma cell lines with in vitro induced resistance from the Resistant Cancer Cell Line Collection. The former show acquisition of multidrug resistance not correlated with P-glycoprotein (in contrast to resistance modeled through selective pressure). Further, similar gamma-H2AX foci are induced following XRT in DX and REL cells, despite cell survival differences, implicating a resistance mechanism downstream of genotoxic damage. Mitochondria isolated from REL tumors resist apoptosis induction when directly exposed to the death effectors tBid and BimBH3 using mitochondrial profiling in 7 of 7 tumor pairs, providing a functional biomarker that correlates with multidrug resistance. No differences in mito biomass (CS activity) or mtDNA (Affymetrix Mito2.0) were seen, but electron microscopy imaging show ER-mito contact number and gap-distance reduced up to 70% at REL in multiple pairs, confirmed by IB for organelle-specific proteins. Disrupting ER-mito contacts using shRNA against MFN2 or PACS2, or Cyclosporine-A exposure (a cyclophilin D inhibitor that reduces tethering) attenuated mitochondrial responses and phenocopied resistance. Calcium transfer measured by cytosolic and mito calcium reporters showed no changes, and shRNA of the mito calcium uniporter MCU showed only modest changes, suggesting altered calcium transfer does not play a principal role.

Conclusions: Our data implicate inter-organelle ER-mito contacts as physiologic regulators of apoptosis, which are disrupted in therapy resistant neuroblastomas, providing a novel mechanism for multidrug resistance. Studies to further assess calcium transfer, and to assess altered lipid transfer, as resistance mechanisms downstream of de-tethering are ongoing.

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Molecular Profiling by Plasma Cell-Free DNA Analysis in High-Risk Relapsed Neuroblastoma

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Background: Neuroblastoma (NB) is the most common extracranial solid tumor in children. 5-year survival rates for high-risk NB are < 50% despite intense multimodality treatment. There is often a higher mutational burden at progression or relapse in NB tumors as a result of clonal evolution. However, obtaining biopsy samples at relapse is often a challenge. Our study evaluates the feasibility of using cell-free DNA (cfDNA) to noninvasively characterize tumor profiles at relapse.

Methods: Tumor specimens (17/19 patients), plasma and matched control samples from 19 patients with high-risk stage 4 NB were collected during treatment. Samples were analyzed using targeted next generation sequencing panels covering 322 to 468 genes. Tumor samples were collected at diagnosis, disease progression, or relapse. Plasma samples were collected at a time of disease progression, at a median of 242.5 days (range of 3-1597 days) from tumor collection. Matched control samples were used to filter germline variants.

Results: We detected somatic mutations and copy number alterations in tumor tissues and cfDNA of 15/17 (2 tumors unavailable) and 11/19 patients, respectively. These included recurrent NB drivers such as MYCN amplification, ALK and ATRX mutations. Of 4 patients with MYCN-amplified neuroblastoma, cfDNA analysis revealed MYCN amplification in 3. In 7 patients, cfDNA analysis revealed somatic variants that were not detected in the original tumor specimens, including potentially targetable mutations in NRAS, MLL2, CIC and IDH2 as well as ARID1A and ARID1B mutations that are associated with poor prognosis. In a patient without available tumor for analysis, cfDNA analysis revealed two mutations, ERBB3 and CDKN2C.

Conclusions: This study suggests that it is feasible to noninvasively profile the dynamic genetic heterogeneity of NB by plasma cfDNA analysis. Such analysis can potentially supplement tumor profiling especially in the relapse setting to guide treatment plans and monitor response to treatment. Our findings call for incorporation of cfDNA analysis in clinical trials to further evaluate its utility for clinical management of NB patients.

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GD2 Bispecific Killer Cell Engager (BiKE) Induces Cytotoxicity of Neuroblastoma Cells

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Background: Anti-disialoganglioside (GD2) monoclonal antibodies have been successful in the treatment of children with high-risk neuroblastoma by inducing tumor lysis via antibody-dependent-cellular- cytotoxicity (ADCC). However, survival, despite improvement with anti-GD2, continues to be poor and novel therapeutic strategies are needed. We describe the development of recombinant bispecific antibodies that direct natural killer cells to kill GD2+ neuroblastoma cells in vitro.

Methods: Recombinant bispecific killer cell engager (BiKE) antibodies were constructed by linking the short chain variable fragments (scFv) of murine 14G2A (anti-GD2) with human NM3E2 (anti-CD16). Mammalian transient production using freestyle 293 cells was carried out. Binding specificity for GD2 and CD16 antigen was demonstrated with flow cytometry and enzyme linked immunosorbent assay (ELISA). In vitro cytotoxicity was demonstrated using DIMSCAN assay. The BiKE at varying concentrations was co-cultured with GD2+ cell lines in the presence of NK cells at effector: target (E:T) ratios of 1:1 with IL-2, and cell viability was assessed at 2, 4, and 6 hours.

Results: There was successful production of the purified BiKE with an estimated molecular weight of 57 kDa. The BiKE showed increased binding to GD2+ cell line CHLA255 with increasing concentrations on flow cytometry. As expected, there was no binding to GD2- cell line LAN6. On ELISA, the BiKE had a binding affinity (Kd) of 279.6 nM to GD2 and 996 nM to CD16. The BiKE demonstrated statistically significant cytotoxicity against multiple GD2+ neuroblastoma cell lines in vitro at a concentration of 10 µg/ml at 4 hours.

Conclusions: We have successfully produced a novel GD2 x CD16 BiKE which demonstrates binding specificity to both antigen targets. It induces ADCC in vitro against GD2+ neuroblastoma cell lines in the presence of NK cells. Although its binding and cytotoxicity is lower than dinutuximab, its smaller size may be more beneficial for tissue and CNS penetration. We are now constructing a new variant of this BiKE which has a high affinity mutation to enhance its killing. The production of these recombinant BiKE antibodies show promise for the development of a novel immunotherapy utilizing the innate immune system in treating neuroblastoma.

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Long-term Survival of Immunocompetent Murine Model of Neuroblastoma Post Chemotherapy and Immune Check Point Blockers

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Background: Children with high risk MYCN non-amplified Neuroblastoma (NBL-NA) continue to have poor survival rate. Considering the very immunogenic characteristic of NBL-NA tumors, therapies that alter the tumor microenvironment (TME) while enhancing T-cell response are an attractive option. In this project, we assessed the efficacy of immune checkpoint blockers (ICB) with and without chemotherapy in an immunocompetent murine model of neuroblastoma.

Methods: ICB against PD-1, CTLA-4, or their combination were used in subcutaneous and orthotopic NBL-NA model (NBT3L cell line derived from NB-Tag mice; Hadjidaniel et. al. 2017). ICBs were tested in minimal residual disease (MRD) setting and immune cell responses were evaluated from blood, spleen, and draining lymph nodes (dLN). Survival studies were conducted in established tumor model with/without prior chemotherapy.

Results: In MRD model where ICB treatment was started shortly after tumor cells inoculation, monotherapy with anti-PD-1, anti-CTLA-4, or their combination effectively prevented tumor formation and increased the survival compared to the isotype treated controls (86.7%, 88.9%, 80% versus 23.8%; p=0.0005). Immune composition of examined tissues in mice treated with anti-PD-1+CTLA-4 therapy revealed significantly increased CD3+ T-cells in dLNs and increase in PD-1+ and CD28+ subsets of CD4/CD8 T-cells in dLNs and spleen compared to all other groups. While anti-PD-1 therapy increased the number of circulating PD-1+CD4 cells, anti-CTLA-4 significantly increased the number of CD28+CD8 cells in blood. Anti-PD-1 treatment also increased the number of circulating neutrophils, while anti-CTLA-4 increased the number of NK cells in LNs. In established tumor models, combination therapy of anti-PD1+CTLA-4 significantly delayed tumor growth and increased survival (p=0.04), however all mice eventually succumbed to their disease. Treatment of established tumors with chemotherapy (5-day Cyclophosphamide+Topotecan) prior to ICB therapy significantly improved survival in mice treated with anti-PD1 or anti-PD1+CTLA-4 (70% v. 25% p=0.004, and 85% v. 25% p=0.0002 respectively).

Conclusion: Our studies reveals activation of distinct subsets of immune cells in ICB treated animals with significant delay in tumor growth observed in animals with established tumors treated with anti-PD1+anti-CTLA4. Long-term survival benefits in animals with established tumor were achieved with initiation of chemotherapy prior to ICB treatment, with anti-PD1, or anti-PD1+anti-CTLA4 providing best outcome.

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Treatment and Outcome of Adult-Onset Neuroblastoma

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Background: Adult-onset of neuroblastoma is rare, and little is known about its biology and clinical course. There is no established therapy for adult neuroblastoma. Anti-GD2 immunotherapy is now standard therapy in children with high-risk neuroblastoma; however, its use has not been reported in adults.

Methods: After obtaining permission from the Institutional Review Board of Memorial Sloan Kettering Cancer Center (MSKCC), medical records of adults with neuroblastoma treated between 1979 and 2015 were reviewed retrospectively. Progression-free (PFS) and overall survival (OS) were measured from the time of diagnosis. Prognostic factors were tested by log-rank test. Cox-regression was used for multivariate analysis. Response was assessed by International Neuroblastoma Response Criteria.

Results: Forty-four patients with adult-onset (18-71 years old) neuroblastoma were identified (17 receiving initial treatment at MSKCC). Five, 1, 5, and 33 patients had INSS stage 1, 2, 3, and 4 diseases respectively. Genetic abnormalities included somatic ATRX (58%) and ALK mutations (42%) but not MYCN-amplification. Among 33 adults with stage 4 neuroblastoma, 7 (21%) achieved complete response (CR) after induction chemotherapy +/- surgery. Seven patients with primary refractory neuroblastoma (all with osteomedullary but no soft tissue disease) received anti-GD2 antibodies, mouse or humanized 3F8, with/without GM-CSF. Adverse events of immunotherapy were similar to those in children; responses were noted in 5/7 (71.4%) patients. In patients with stage 4 disease at diagnosis, five-year PFS was only 9.7±5.3% and most patients who were alive with disease at 5 years died of neuroblastoma over the next 5 years, 10-year OS being only 19.0±8.2%. Patients who achieved CR after induction therapy (n=7) had superior PFS and OS (p=0.006, p=0.031 respectively). In the 11 patients with locoregional disease, 5-year PFS and OS were 70.7±14.3% and 81.8±11.6% respectively. However, 4 patients relapsed >5 years after diagnosis, resulting in 10-year PFS and OS of 35.4±16.1% and 61.4±15.3% respectively.

Conclusions: Adult-onset neuroblastoma appeared to have different biology from pediatric neuroblastoma. Adults had worse outcomes regardless of stage compared to pediatric or adolescent patients. Complete disease control appeared to improve long-term survival. Anti-GD2 immunotherapy was well tolerated and might be beneficial in adults with neuroblastoma.

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Novel T Cell Engaging Antibodies Against L1CAM For Neuroblastoma

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Background: The L1 cell adhesion molecule (L1CAM) is a glycoprotein consisting of 6 Ig-like domains and 5 fibronectin-like repeats in the ectodomain. L1CAM is overexpressed on neuroblastoma (NB) (Bosse et al., Cancer Cell 2017), and stimulates tumor proliferation, migration, and metastasis (Valiente et all, Cell 2014). Chimeric IgG1 antibody, chCE7, targeting the 6th Ig-like domain of L1CAM, had high affinity (KD = 0.63 nM) and when 131I-labeled, had superior sensitivity and specificity over 131I-MIBG in NB detection. However, chCE7 lacked ADCC activity. Bispecific antibodies (BsAbs) that engage CD3 on T cells can exploit polyclonal T cell-mediated tumor cytotoxicity bypassing the classic dependence on MHC.

Methods: Humanized E71 (huE71)-BsAb (specific for 2nd Ig-like domain) and huE72 (the humanized version of CE7)-BsAb were generated using the IgG(L)-scFv BsAb platform (Xu et al, Can Immunol Res 2016), and their functions were compared. Binding to L1CAM and CD3 was measured by SPR and by FACS. In vitro T cell activation and cytokine release were assessed by FACS and by ELISA, respectively. In vitro tumor cytotoxicity was measured by 4-hour 51Cr release at an E:T ratio of 10:1. In vivo tumor targeting was performed by using 89Zr-labeled huE71 and huE72 IgG1 in xenografted nude mice. Anti-tumor effect was assessed in Balb/c-Rag2-/-IL-2R-c-KO (DKO) mice xenografted with s.c. NB patient derived xenografts (PDX).

Results: By SPR, huE71-BsAb retained its parental affinity, while that of huE72-BsAb decreased by 8-fold (KD of 5.15nM and 5.28nM, respectively). Both BsAbs induced CD25 expression on T cells as well as Th1 cytokine (e.g. TNFα) release from PBMCs in the presence of IMR32 NB cells. Both BsAbs mediated T cell cytotoxicity on L1CAM(+) cell lines with pM EC50s. In nude mice with s.c. L1CAM(+) tumor, i.v. huE71 IgG1 showed higher tumor targeting efficiency than huE72 IgG1. In DKO mice with s.c. L1CAM(+) NB PDX and s.c. PBMCs, i.v. huE71-BsAb significantly suppressed tumor growth while huE72-BsAb had no anti-tumor effect.

Conclusions: Despite near identical in vitro binding characteristics, BsAbs targeting different epitopes on the same target have strikingly different anti-tumor properties in vivo. HuE71-BsAb is a potential T cell engaging immunotherapeutic for NB.

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Targeting Anaplastic Lymphoma Kinase (ALK) Gene Alteration by Using an Alkylating Pyrrole-Imidazole Polyamide in Neuroblastoma

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Background: Aberrant status of anaplastic lymphoma kinase (ALK) and MYCN are two major causes in the development of aggressive neuroblastoma. Activating mutations and the overexpression of ALK often associated with its gene amplification have been described in both familial and sporadic neuroblastoma disease. Since MYCN and ALK have been important therapeutic targets in developing novel drugs against aggressive neuroblastoma, we are currently developing CCC-002 and CCC-003, novel MYCN- and mutant ALK-targeting DNA-alkylating pyrrole-imidazole (PI) polyamides, respectively.

Methods: CCC-002 has been designed to bind to the minor groove of genomic DNA in the coding region of MYCN in a sequence-specific manner. In this study, we tested CCC-003 and CCC-002, which may downregulate the ALK gene through potential binding sites of CCC-002, for their anti-tumor effect in neuroblastoma cells with ALK amplification.

Results: The IC50 of CCC-002 in ALK-amplified NB-39-nu cells was 13 nM, demonstrating potency over conventional ALK inhibitors, such as crizotinib and alectinib, (21 and 16 nM, respectively). In addition to the down-regulation of MYCN, CCC-002 treatment suppressed the phosphorylated form and total expression of ALK in ALK-amplified cells. We also tested CCC-003 designed for a ALK F1174L mutant and identified suppression of ALK expression and growth inhibition showing lower IC50 than crizotinib and alectinib in ALK F1174L mutated Kelly and SH-SY5Y cell lines.

Conclusion: Alkylating PI polyamide conjugates can be a promising and an innovative therapeutic drug designed for ALK-amplified neuroblastoma.

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Diagnosis and Treatment of Childhood Neuroblastoma with CCCG-NB-2015 in Multicenter

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Background: The CCCG-NB-2015 program is formulated by the Pediatric Oncology Committee of China Anti-Cancer Association based on the CCCG-NB-2009 program.

Objective: This study aimed to summarize the recent clinical status of the application of CCCG-NB-2015 in the treatment of neuroblastoma in multicenter.

Methods: The clinical data of children diagnosed and treated with neuroblastoma by CCCG-NB-2015 were collected from January 2014 to August 2016 in our hospital.

Results: A total of 128 cases were identified, with 27 patients give up treatment, ultimately, 101 cases were included in this study, of which only 89 cases received regular treatment. The risk group: 32 cases (31.7%) in low 22 cases (21.8%) in median risk group, 47 cases (46.5%) in high-risk group. Histopathological type of prognosis: 63 cases (62.4%) in the FH group and 38 cases (38.6%) in the UH group. Treatment: In 32 children of low risk group, a total of 30 cases underwent radical surgery, 2 cases of partial resection, CR or VGPR rate was 96.9%, survival time 11 ~ 39 months (median 24.5 months), all survived. In 22 children of median risk group, the CR or VGPR rate was 86.1%, 19 patients survived, 3 patients died, and the survival time was 9-51 months (median 26 months). As for the 47 cases of high-risk group, CR or VGPR rate 85.1%, 14 cases accepted autologous peripheral blood stem cell transplantation (including two sequential transplants in 7 cases), 19 cases received radiotherapy. Finally, 36 cases survived, and 14 cases relapsed, 1 case progressed, 11 patients died, the survival time range from 8 to 46 months (median 23 months). There were significant differences (both $P < 0.001$) between the median-risk group and the high-risk group; regular and irregular treatment group. The higher the risk, the worse the prognosis ($P = 0.04$).

Conclusion: The results show that CCCG-NB-2015 is reasonable in the risk sub-group assignment, and risk level is closely related with patients' prognosis. Regular treatment can significantly improve the survival rate of children, indicating that the current treatment plan has great potential value, which still needs to be further clinical validated.

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Bispecific Antibody Does Not Induce T-Cell Death Mediated by Chimeric Antigen Receptor Against Disialoganglioside GD2

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Background: Immunotherapy using chimeric antigen receptors (CAR) and bispecific antibodies (BsAb) are exciting strategies to exploit T cells in cancer therapy. Despite encouraging results from clinical trials in hematologic malignancies, few solid tumors have shown significant response to such immunotherapy. Understanding the immunobiology of such clinical failures should help improve the design and implementation of more effective CAR and BsAb therapies. Disialoganglioside GD2 is expressed on solid tumors including neuroblastoma.

Method: We developed a second-generation CAR containing the scFv of humanized 3F8 anti-GD2 antibody, the 4-1BB costimulatory domain and CD3z signaling moiety. The humanized anti-GD2×CD3 BsAb using the IgG-scFv platform was described previously. CART cells and BsAb-redirected T cells were compared in their in vitro proliferation, viability, and cytotoxicity against GD2(+) neuroblastoma cells. The anti-tumor activity of the two modalities against GD2(+) melanoma xenograft was evaluated in mouse.

Results: In vitro, high-CAR density T cells were depleted upon exposure to GD2, anti-idiotype, or GD2(+) target cells while the BsAb-redirected T cells survived and proliferated. The anti-tumor activity of the surviving low-CAR density T cells was inferior compared to the BsAb-engaged T cells. In vivo, BsAb-engaged and CART cells penetrated established subcutaneously-injected tumor xenografts and provided potent anti-tumor effect; however, the higher number of tumor-infiltrating lymphocytes (TIL) in the BsAb group translated to more rapid and complete tumor suppression. Furthermore, the CD4:CD8 ratio of TILs was substantially lower in CAR treated tumors versus BsAb groups.

Conclusion: BsAb versus CAR mediated stronger in vitro and in vivo anti-tumor effect against GD2(+) targets.

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Targeting the Yes-Associated Protein in Neuroblastoma

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Background: Survival for relapsed high-risk neuroblastoma (rNB) is < 5%, underscoring the critical need for novel therapies. rNBs have increased RAS/RAF/MAPK mutations and increased Yes-associated protein (YAP) transcriptional activity. YAP is a transcriptional co-activator that binds with TEA-Domain (TEAD) transcription factors to regulate cellular proliferation, self-renewal, and survival. We found that shRNA inhibition of YAP decreases NB cell proliferation and sensitizes RAS-mutated NBs to MEK inhibitors, supporting YAP as a tractable therapeutic target. Verteporfin (VP), a photodynamic drug used for macular degeneration, is the only drug found to inhibit YAP expression or YAP:TEAD binding to kill tumor-derived cells. Peptide 17 is a 17mer YAP peptidomimetic that also disrupts YAP:TEAD interactions. We sought to determine whether these compounds are potent in NB via YAP direct effects.

Methods: YAP expressing (NLF, SK-N-AS) or YAP null (NGP, LAN5, SK-N-AS-shYAP) human-derived NBs were incubated with VP, with and without direct light exposure, or with Peptide 17. CellTiter-Glo and Immunoblots were used to assess for cell death and YAP-downstream protein expression, respectively.

Results: Without direct light exposure, VP inhibits YAP expression at nM dosing, yet no NB cell death was observed at equal or higher concentrations. EGFR and ERK1/2 were inhibited along with YAP, confirming YAP/RAS pathway co-regulation. When VP was exposed to direct incandescent light for 30 minutes, > 80% NB cell death occurred in all NBs tested, even those lacking YAP. Peptide 17 caused no cell death or YAP inhibition up to 75 uM.

Conclusions: Neuroblastomas are resistant to VP at doses sufficient to inhibit YAP expression. In macular degeneration, light-activated VP produces reactive oxygen species, which we hypothesize is the off-target mechanism killing NBs independent of YAP. Given the off-target effects and the need for light activation, VP is not an ideal preclinical or clinical YAP inhibitor. Accordingly, Peptide 17 has poor cell permeability and low TEAD affinity, leading to its lack of efficacy. Given the relevance of YAP in rNB and other cancers, we are chemically optimizing a YAP peptidomimetic with enhanced permeability, nuclear localization, and TEAD affinity to create a bonafide YAP inhibitor for preclinical and clinical application.

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Investigating Impacts of Cell-Cycle Dependent Interaction Between N-Myc and TFIIIC On Chromatin Architecture

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Amplification of the transcription factor N-Myc is a major driver event of neuroblastoma. Understanding the molecular basis of N-Myc on gene expression therefore provides in-depth insight on neuroblastoma tumorigenesis. Our group have reported that N-Myc interacts with TFIIIC in a cell-cycle dependent manner for regulating promoter escape and pause release of RNA polymerase II. Intriguingly, TFIIIC is an architectural protein that governs genome three-dimensional organization. Chromatin immunoprecipitation (ChIP)-seq experiment revealed joint-binding sites of TFIIIC and N-Myc downstream of transcription start site (TSS). It also demonstrated TFIIIC co-localization with architectural proteins cohesin and CTCF at 5' TSS, posing an open question about how N-Myc/TFIIIC complex mediates spatial configuration of chromatin (Buechel et al., 2017). This study aims to decipher the chromatin organization landscape of MYCN-driven neuroblastoma. We hypothesized TFIIIC can mediate N-MYC dependent transcriptional regulation by altering chromatin architecture. Chromatin conformation capture-based methods were employed to evaluate changes in chromatin organization under cell-cycle synchronized, TFIIIC depleted and N-Myc over-expressed conditions. Histone marks ChIP-seq that identified enhancers (H3K4me1 and H3K27ac) and promoters (H3K4me3) were performed for further analysis of looping between enhancer and promoter. Initial data from 4C experiment showed an increase in loop formation in G1-phase in which N-Myc and TFIIIC interaction takes place. We have also established HiChIP-protocol to directly visualize three-dimensional interactions of N-MYC and TFIIIC. This study will highlight the role of TFIIIC in assisting N-Myc transcriptional regulation via chromatin interaction, contributing to more profound understanding of the neuroblastoma pathogenesis.

Reference: Buechel, G., Carstensen, A., Mak, K. Y., Roeschert, I., Leen, E., Sumara, O., Hofstetter, J., Herold, S., Kalb, J., Baluapuri, A., et al. (2017). Association with Aurora-A Controls N-MYC-Dependent Promoter Escape and Pause Release of RNA Polymerase II during the Cell Cycle. Cell reports 21, 3483-3497.

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Dual PI3K/HDAC Inhibitor CUDC-907 Blocks Tumor Growth and Potently Sensitizes MYCN Amplified Neuroblastoma To BCL-2 Inhibition by Venetoclax (ABT-199)

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Amplification of MYCN proto-oncogene occurs commonly in high-risk neuroblastoma and marks a particularly aggressive and high-risk form of the disease. Recent advances in therapy for neuroblastoma have been mostly through toxic escalations in conventional cytotoxic chemotherapy and radiation. Most high-risk neuroblastoma patients will relapse with highly refractory disease. Therefore, new therapies for neuroblastoma are focused on more targeted approaches, many of which involve MYCN regulation. Small molecule inhibitors of phosphoinositide 3-kinase (PI3K) and histone deacetylase (HDAC), which regulates MYCN expression, have shown some pre-clinical efficacy in neuroblastoma and several of these are in clinical trials as mono-therapy in children with neuroblastoma. However, resistance to targeted mono-therapies is common in cancers. One means of bypassing resistance is to target two or more pathways simultaneously through combinations of novel drugs. We hypothesize that inhibitors of MYCN and associated pathways each block distinct aspects of MYCN biology, and when used in combination, will both enhance efficacy and block emergent resistance.

For the first time, we show dual PI3K/HDAC inhibitor CUDC-907 is able to reduce neuroblastoma cell growth and is more effective against MYCN amplified cell lines. CUDC-907 suppresses MYCN transcriptional expression by regulating acetylated histone H3 and degrades MYCN protein by regulating the phosphorylation of GSK3β. CUDC-907 is highly effective as a monotherapy, but like most monotherapy approaches, was not curative in our preclinical models. We hypothesized that resistance might be through PI3K/HDAC modulation of apoptotic pathways. Using protein array assays, we determined that several apoptotic pathways are altered, potentially priming neuroblastoma cells for blockade of apoptotic pathways. We screened targeted drug combinations with mechanistically distinct activities for efficacy against neuroblastoma and MYCN protein using cell-based assays. We identified synergy between CUDC-907 and the BCL-2 inhibitor venetoclax (ABT-199). Combination treatment with CUDC-907 and venetoclax were able to significantly decrease neuroblastoma cell growth by preventing cells from entering S-phase during mitosis and thus inducing cell cycle arrest. Combination treatment also induces cytochrome c release and activation of caspase-3 leading to apoptosis.

Our research provides strong evidence demonstrating the efficacy of CUDC-907 and venetoclax as novel targeted therapy for the treatment of neuroblastoma.

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Phase II Trial of GD2-KLH/GD3-KLH Vaccine for Stage 4 Neuroblastoma In ≥2nd Remission: Induced Anti-GD2 Titer Strongly Correlates with Survival

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Background: Anti-GD2 antibodies mouse 3F8 and hu3F8 have proven survival benefit in patients with high risk stage 4 neuroblastoma (HR-NB). Both antibodies induced de novo anti-GD2 antibody response. A Phase I vaccine trial utilizing GD2-keyhole limpet hemocyanin (KLH)/GD3-KLH in 15 patients found no dose-limiting toxicities (Kushner et al., CCR 2014).

Methods: In this Phase II trial, 7 doses of 60ug of GD2-KLH/GD3-KLH conjugate vaccine mixed with 150ug of adjuvant OPT821 were administered subcutaneously in outpatient setting over one year in 84 patients with HR-NB in ≥2nd remission; oral yeast beta-glucan at 40mg/kg/day x 2 weeks q month x 10 months was included to enhance antibody mediated cytotoxicity. Progression-free survival (PFS) and overall survival (OS) were estimated by Kaplan Meier analyses.

Results: All 84 patients had prior relapse, 57 treated in 2nd remission, 18 in 3rd remission, and the rest in 4th to 7th remission. All had prior exposure to either mouse 3F8 (63%), and/or hu3F8 (57%), and/or dinutuximab (46%). Median follow-up was 16 months; PFS was 51%±7% and OS were 90%±5% at 2 years with no ≥grade 3 toxicities. Serum anti-GD2 and anti-GD3 IgG1 antibodies were measured using ELISA at serial time points, integrated, and expressed as area-under-the-curve per month. Anti-GD2 titer was positive pre-vaccine in 14% of patients (median=39ng/ml), and positive post-vaccine in 78% (median=134 ng/mL/month). Developing anti-GD2 antibody titer did not result in any patient having pain or neuropathy. There was no correlation between pre-vaccine and post-vaccine titer. Anti-GD2 antibody titer >134ng/ml/month was prognostic for improved PFS and OS (p=0.033 and 0.025, respectively). In contrast, developing anti-GD3 response had no prognostic significance for survival. There was no impact on patient outcome based on age at diagnosis, time from diagnosis, MYCN amplification, number of prior relapses, pre-vaccine anti-GD2 antibody therapy, as well as pre-vaccine anti-GD2 serum titer.

Conclusion: These results confirmed the safety of GD2-KLH/GD3-KLH vaccine and the potential influence of anti-GD2 but not anti-GD3 seroconversion on PFS and OS. If the 90% OS is confirmed in a phase III randomized trial, GD2 vaccine could provide a viable option to improve the outlook for patients with relapsed HR-NB.

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The Synergistic Targeting of Pediatric Solid Tumors with BRD4 and AURKA Inhibitors

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Background: Children diagnosed with high-risk neuroblastoma face considerable clinical challenges. Despite aggressive multimodal therapy, nearly half of all patients have treatment-refractory or recurrent disease, ultimately dying of the cancers. Novel therapeutics have been developed to target biological pathways in neuroblastoma, including the BRD4 inhibitor, IBET-151, and the aurora kinase A (AURKA) inhibitor, MLN8237. Both drugs were tolerated in clinical trials but lack efficacy alone. Our in vitro studies show that, when used together, the drugs synergistically inhibit cell growth and viability of neuroblastoma cell lines.

Hypothesis: We hypothesized that IBET-151 and MLN8237 act synergistically against neuroblastoma by targeting common pathways, including MYC/MYCN, through different mechanisms.

Aims: We tested this hypothesis through two specific aims: 1) validation of the synergistic effects of the drug combination against neuroblastoma tumor xenografts in immunodeficient mice and 2) confirmation of the pharmacodynamic effects of dual drug treatment on common biological targets through RNA and protein analysis.

Results: Dual drug use significantly slowed xenograft growth in all models tested as compared to control ($p<0.03$). The combination of IBET-151 and MLN8237 significantly extended survival as compared to either drug alone against tumor xenografts derived from two MYCN-nonamplified neuroblastoma cell lines, SK-N-AS and SK-N-SH ($p<0.025$). The two drugs were superior to IBET-151 alone against tumor xenografts of the MYCN-amplified NB1643 cell line, and the two drugs were more effective in maintaining tumor suppression after drug washout than MLN8237 ($p<0.05$). MLN8237 use was associated with increased target gene expression, but concomitant use of IBET-151 repressed that increase. The use of both drugs was more effective than either drug alone at repressing protein expression downstream targets, including MYC, MYCN, MCL1, CDK4, and CDK6, as evaluated by western blot.

Conclusion: Combined BRD4 and AURKA inhibition is effective against neuroblastoma. Assessment of the drug combination with synergistic chemotherapy is ongoing. These data provide support for advancing the drug combination towards clinical trial alone or in combination with chemotherapy as a novel approach against neuroblastoma.

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LIN28B Promotes Neuroblastoma Metastatic Dissemination

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Background: LIN28B is an RNA binding protein that plays key roles in normal development; mechanistically, it blocks the processing of the let-7 family of tumor suppressors and binds mRNAs directly. When deregulated, LIN28B promotes tumorigenesis across diverse histotypes. We previously demonstrated that LIN28B induces neuroblastoma proliferation, in part by regulating the expression of RAN GTPase and Aurora kinase A. However, in addition to increased proliferation, high-risk neuroblastoma exhibits a striking proclivity for widespread metastases. Although LIN28B has been shown to influence metastatic dissemination in colon and esophageal cancer, adult and pediatric cancers differ significantly with respect to mutational burden and outcomes, among other factors. Thus, in the present study, we investigated how LIN28B influences neuroblastoma metastasis.

Methods: We used gain and loss of function approaches to genetically manipulate transcripts of interest in neuroblastoma cells, and then measured effects on self-renewal, proliferation, invasion, and downstream signaling. To examine the impact of LIN28B on dissemination, we generated GFP-luciferase expressing neuroblastoma cell line models in which LIN28B levels were manipulated, injected these lines into the tail veins of NSG mice, and tracked dissemination using an IVIS Spectrum system.

Results: Depletion of LIN28B significantly delayed the onset of tumor metastasis, reduced tumor burden, and extended mouse survival (103 days versus 50 days, $p<0.0001$) compared with control cells expressing scrambled shRNA. Moreover, although LIN28B did not impact anoikis resistance, it did increase both tumorsphere number and size, linking self-renewal to metastatic dissemination. Overexpression of let-7 only partly mimicked LIN28B depletion, suggesting that the promotion of metastasis by LIN28B is not fully let-7 dependent. Interestingly, Gene Set Enrichment analysis showed that LIN28B signaling was most strongly and positively correlated with BRCA1 signaling (ranked 1/231 gene sets tested), in both MYCN and non-MYCN amplified tumors.

Conclusions: LIN28B promotes self-renewal and metastasis of neuroblastoma. Current investigations are further delineating the effects of LIN28B/let-7 signaling on migration and invasion and studying whether LIN28B directly promotes enhanced BRCA1 signaling/DNA damage repair to increase metastatic fitness in neuroblastoma cells.

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Application of a Sustained Release Carrier Containing an Anticancer Drug Which is Generated by Electrospinning

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Aim: The purpose of this study is to evaluate and apply a drug delivery system(DDS) for controlled release of anticancer drugs. The material similar to this DDS, called ReBOSSIS is used in the healthcare setting and has already got the authorization from FDA as a material for osteoplasty. We investigated the efficacy of the newly-developed DDS on neuroblastoma.

Methods: We prepared the cotton wool-like poly(DL-lactide-co-glycolide) (PLGA) as a DDS using the electrospinning method, a fiber production method which uses electric force to draw charged threads. To assess the DDS, we used an animal model of neuroblastoma, Th-MYCN mice. In the model, neuroblastoma develops in homozygous mice at two weeks of age and the mice die at around seven or eight weeks of age. Carboplatin conjugated with a copolymer(poly(DL-lactide-co-glycolide); 30 times quantity (equaled 450 mg carboplatin/body) was placed in the abdominal cavity of the four-week-old homozygous mice. At the same time, we also treated the mice with DDS without carboplatin as a control.

Results: Th-MYCN homozygous mice died of tumor around eight weeks of age in our observation this time (n=11). The mice which received carboplatin (quantity of LD50) without the copolymer died of serious side effect in a few days (n=3). On the other hand, mice which received carboplatin with the copolymer (30 times quantity) survived until more than ten weeks of age without any side effects (n=11). We did not observe remaining tumor cells confirmed by histological assessment. But some mice which received carboplatin with copolymer (60 times quantity (equaled 900 mg carboplatin/body)) died of side effect (n=5). The DDS which did not contain carboplatin died within eight weeks of age (n=7).

Conclusions: Our DDS using electrospinning for carboplatin efficiently suppressed in situ tumor growth without any serious side effects of the anticancer drug. This DDS may be useful for cancer treatment including neuroblastoma.

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Non-Invasive Differential Diagnosis of Neuroblastoma with Other Paediatric Solid Tumours Using Methylation Profiling of Circulating Cell-Free DNA

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Introduction: Paediatric solid tumours are often difficult to biopsy because of the location or clinical condition of the patient. Furthermore, surgical biopsies are invasive causing anxiety and discomfort to the patients. Although al lot can be learned from imaging studies, obtaining diagnostic certainty is rarely possible. An affordable, fast and non-invasive test to establish the pathological diagnosis of a paediatric patient with a solid tumour will greatly improve the diagnostic process.

Methodology: As a proof-of-concept, whole genome bisulfite sequencing (WGBS) was performed on 10 ng of cell-free circulating serum DNA from serum of two patients with neuroblastoma (MYCN amplified and MYCN non-amplified) at 10-fold coverage. A workflow was developed to integrate the methylation calls from our cell-free WGBS data (after mapping with Bismark) with the public tissue data from Illumina Infinium 450K microarray output, available in the TARGET database.

Results: Unsupervised hierarchical clustering allowed to distinguish four paediatric tumours (osteosarcoma, Wilms tumour, neuroblastoma and clear cell sarcoma of the kidney, total n = 463) based on the epigenetic profile. In addition, a support vector machine (SVM) was able to classify the four tumour types to their corresponding group with an accuracy and recall of 98% using cross-validation. The two methylation profiles of our two neuroblastoma cfDNA samples correctly clustered together within the neuroblastoma group and the SVM classifier predicted both cases to be neuroblastoma. Interestingly, also MYCN status and copy number profile analysis could be performed on the cfDNA WGBS data and showed a good concordance with the arrayCGH profile.

Conclusion: The methylome of cfDNA yields potential for the tissue-of-origin assessment in the diagnostic work-up of paediatric solid tumours. In future experiments, tumour types in which the differential diagnosis is more challenging (e.g. brain tumours) will be included. Our workflow is currently limited by the extremely high cost of WGBS. Overcoming the cost of WGBS is necessary for the implementation in routine diagnostics. This will require the development of more cost-effective methylation-sensitive sequencing technologies, which is a currently ongoing development in several laboratories.

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Proteomic Characterization of Human Neuroblastoma

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Neuroblastoma is a heterogeneous embryonic malignancy originating from neural crest cells. The low frequency of genomic mutations has made genomic characterization of neuroblastoma less success as that in adult tumors. Only few genes have actually been shown to drive the disease at the molecular level. To identify new signatures either for individualized diagnosis or therapeutic intervention, we performed quantitative proteome profiling of 95 neuroblastoma tissue samples and 5 cell lines using chemical labeling-based quantitative mass spectrometry. Primary data analysis showed that approximately 7000 proteins were identified from each sample and around 4000 proteins were identified and quantified across all the samples. Statistical analysis highlighted the significantly expressed proteins inter the samples. Supervised analysis of the neuroblastoma (NB) ganglioneuroblastoma (GNB) and ganglioneuroma (GN) revealed different signaling pathways overrepresented in three sample groups. Particularly, the cancer relevant signaling pathway, i.e. Splicesome, DNA replication, Mismatch repair, were significantly enriched in NB group but not GNB and GN group. Unsupervised analysis could clearly separate the samples into four subgroups either using the proteins with top standard deviations (SD) or proteins with top significant p values. Interestingly tow of the subgroups were clearly correlated to the clinical parameters, such as 2-year overall survival rates, age at diagnosis and so on. Supervised cluster analysis of the samples with or without chemotherapy highlighted several chemotherapy related proteins, including the known drug resistant relevant proteins, i.e. TOP2A, also some unknown but potentially drug sensitivity correlated proteins, such as MCM2, CTCF and so on. In conclusion, the straightforward proteome analysis provided an alternative strategy for molecular characterization of neuroblastoma and the primary findings highlighted the potential of developing new diagnostic and therapeutic options for neuroblastoma patients.

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The Important Role of Tumor-Derived Galectin-1 As Tumor-Promoting Protein in A Novel Immunocompetent Orthotopic NB Mouse Model

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Question: We recently introduced the carbohydrate-binding protein Galectin-1 (Gal-1) as a novel tolerogeneic molecule in neuroblastoma (NB). NB-secreted Gal-1 suppressed the maturation of dendritic cells (DC) and inhibited T cell function. However, the mechanisms underlying Gal-1 suppressive capacities on effector cell compartments and the importance of tumor vs host Gal-1 in NB progression are unexplored. To address these issues, we first needed to move to an immunocmpetent orthotopic mouse model in C57/Bl6J background to be able to employ transgenic mice.

Methods: C57Bl/6J-derived orthotopic growing NB cells (named NB975A) were used for all culture as well in vivo experiments Gal-1 knock-down clones (GL) were generated by stable transfection of wt NB975A cells (NBA) with the antisense Gal-1 expression vector (NBA-GL), (kindly provided by Dr. Rabinovich). Blasticidine resistant (5 µg/ml) transfectants were cloned by limited dilution. Protein expression of Gal-1 was determined by Western blot. Orthotopic NB tumors were generated in C57Bl/6J wild type and Gal-1-knock-out (Lgals-/-) female mice by subcapsular tumor cell injection of NBA-GL or wild type NBA ($1x10^6$, 100 µl PBS) into the left kidney. Primary tumor growth was analyzed by using high frequency ultrasound measurement. Mice were sacrificed 23 days after tumor inoculation and tumor tissue was harvested for further analysis.

Results: NBA-GL showed 50% reduced protein expression of Gal-1 compared to control wild type NBA cells. In vitro viability and proliferation of the tumor cells was not affected by the transfection. Mice inoculated with NBA-GL cells had significantly smaller primary tumors at all when compared with control animals that received the NBA cells. Comparing Gal-1-deficient mice as hosts for NB cells where Gal-1 levels were preserved, we found that only tumor but not host Gal-1 was critical for promoting tumor growth.

Conclusion: Our data support the important role of tumor Gal-1 as tumor-promoting protein in a novel immunocompetent orthotopic NB mouse model.

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Image-Defined Risk Factors in Patients Enrolled in the Low-Risk Protocol (JN-L-10) From the Japan Children's Cancer Group Neuroblastoma Committee

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Objective: The Japan Children's Cancer Group (JCCG) Neuroblastoma Committee (JNBSG) conducted the JN-L-10 for low-risk patients to minimize treatment complications using Image-defined Risk Factors (IDRFs) as the main factor for determining treatment. In this study, we analyzed the IDRF results in JN-L-10 to clarify the usefulness of IDRFs in low-risk neuroblastoma patients.

Patients and Methods: Sixty patients were enrolled in JN-L-10, and the IDRF results were available in 58. IDRFs were evaluated at the diagnosis and after every three courses of chemotherapy. Three low-dose chemotherapy protocols were included in this study: LI-A (VCR/CPA), LI-B (VCR/CPA/THP-ADR), and LI-C (VCR/CPA/CBDCA). We analyzed the relationship between surgical complications and IDRFs as well as the changes in IDRFs after chemotherapy.

Results: All 26 patients who had IDRFs at the onset of disease underwent chemotherapy. All IDRFs disappeared in 3 of these 26 patients after 3, 5 and 6 courses of chemotherapy. Two of the 3 patients underwent surgery. IDRFs remained after chemotherapy in the other 23 patients. Three patients underwent surgery, and 20 patients did not undergo any surgery after chemotherapy. Twenty-eight of 32 patients with no IDRFs at the onset of disease underwent surgery. One patient did not undergo any treatment. Three patients underwent chemotherapy only. Overall, 34 patients underwent surgery rather than a biopsy. IDRFs were present in four patients at the time of surgery. In these four patients, surgery was performed after six courses of chemotherapy in two patients and after nine courses of chemotherapy in the other two patients. Major surgical complications were noted in 5 of 58 (8.6%) patients, including pleural effusion in one patient, renal atrophy in one patient, vascular injury in three patients and intraoperative hemorrhaging (class 3, 4) in two patients. Of these, only one patient had IDRFs at the time of surgery.

Conclusions: IDRFs were present in 44.8% of low-risk NB patients at the diagnosis. Of these, all IDRFs disappeared in only 3 (11.5%) patients after chemotherapy. Major surgical complications were observed in 8.6% of the patients.

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Expression and Modulation of Potassium Channels in Neuroblastoma

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Background: Potassium channels (denoted by KCNx e.g. KCNQ2) are a diverse family of ion channels which are ubiquitously expressed in normal and malignant cells. They have been implicated in a number of oncogenic processes including cell proliferation, apoptosis, and angiogenesis. Resultantly, modulation of potassium channel activity has been proposed as a therapeutic target in cancer. The biological role of potassium channels in neuroblastoma is currently unclear. We sought to examine the expression of selected potassium channels in neuroblastoma cell lines and assess the effect of specific channel modulation on cell viability, proliferation and differentiation.

Methods: Potassium channel expression was investigated by quantitative PCR and Western Blotting in two human neuroblastoma cell lines representative of high risk disease, SK-N-BE(2)-C (MYCN amplified) and SK-N-AS (11q deletion). SK-N-BE(2)-C and SK-N-AS cells were treated with different concentrations of a KCNQ2 channel inhibitor (XE991) and activator (ML213) and tested for cell viability (MTT assay), proliferation (Ki67 fluorescence assay) and differentiation (fluorescence microscopy). Fluorescence activated cell sorting (FACS) was used to determine the effect of KCNQ2 modulation on mitotic progression.

Results: The expression of KCNQ2, KCNB1 and KCNG1 was 101.51 ± 17.52 ($p<0.001$), 71.91 ± 46.85 ($p<0.01$) and 5.31 ± 2.41 ($p<0.05$) fold greater in SK-N-BE(2)-C relative to SK-N-AS. The KCNQ2 channel was selected for further investigation. A reduction in the number of viable cells after treatment with XE991 was observed in both SK-N-BE(2)-C (10.10 ± 1.49%, $p>0.05$) and SK-N-AS (11.97 ± 1.06%, $p>0.05$). Treatment of SK-N-BE(2)-C cells with 1 µM and 10 µM XE991 caused a 13.33 ± 3.94% ($p>0.05$) and 32.85 ± 5.87% ($p<0.05$) reduction in SK-N-BE(2)-C cell proliferation. XE991 was found to induce morphological changes, akin to neuronal differentiation, in SK-N-BE(2)C cells. Treating SK-N-BE(2)-C cells with XE991 had no effect on mitotic progression.

Conclusion: We have demonstrated differential expression of potassium channels between two heterogeneous neuroblastoma cell lines. Our findings suggest inhibition of KCNQ2 activity influences neuroblastoma cell viability, proliferation and differentiation. Variations in potassium channel expression may contribute to the biological heterogeneity observed in neuroblastoma cell lines and primary tumours. Potassium channels may be an interesting novel therapeutic target in neuroblastoma.

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Retrospective Analysis of Patients with Stage 3 Neuroblastoma without MYCN Amplification – The Pilot Study

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Neuroblastoma (NBL) is one of the most common cause of death in pediatric oncology patients. In stage 3 NBL without MYCN amplification, although prognosis is better than in stage 4 or in MYCN amplified tumors, still about 30% of patients die of disease.

From 2001, 33 patients with stage 3, MYCN non-amplified NBL were treated in single pediatric oncology and hematology center. Tumor tissue was stored either embedded in parafine (FFPE) other/and as deep-frozen tumors. In the pilot study, we evaluated the comparative genomic hybridization (CGH) results in 7 patients, whose tumors were available both as frozen tissue and FFPE.

The patient's DNA was extracted automatically from deep frozen tumors tissues and FFPE samples using MagCore® Automated Nucleic Acid Extractor, according to the manufacturer's protocol (RBC Bioscience, New Taipei City, Taiwan). It was used 350 ng of genome DNA for further analysis. The Agilent SurePrint G3 CGH ISCA v2 Microarray Kit 8x60K array platform was used for genome evaluation, according to the manufacturer's instructions with enzymatic digestion (Agilent Technologies, Santa Clara, CA). The resolution for aCGH evaluation was established on 150 kb. Frozen and FFPE aCGH profiles were matched for each patient to confirm and validate results.

In 4/7 (57%) patients the results of CGH obtained from frozen tumors and FFPE samples were identical (in 3 cases numerical aberrations only). Quality of DNA was better in frozen tumors. Due to the poor quality of DNA, in 1 patient analysis of FFPE was not possible and in the other one we found lower number but concordant aberration in FFPE. In 1 patient, we found segmental and numerical aberrations in the FFPE which were not found in deep frozen tumor, but it was the only patient with analyzed material taken in different time points. This case will be further analyzed.

The study will be continued with evaluation of both type of samples, with fresh tumor as reference, to correlate results with the clinical course of disease. DNA obtained from the both type of material can be used for analysis, although the quality of DNA from frozen tumor is better.

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Ototoxicity and Speech Impairment After Therapy of High Risk Neuroblastoma

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Ototoxicity is a common late side effect of cisplatin therapy, which is one of the main drugs used in neuroblastoma therapy. As most of the neuroblastoma patients are young children, during the process of speech development, it may be a severe problem in communication and learning. Moreover, in the ototoxicity typical for cisplatin is observed in frequencies typical for many sounds used in Polish language. We evaluated ototoxicity in 24 patients after the end of high risk neuroblastoma treatment in 2015-2017. There were 14 girls and 10 boys, age 1,5 – 8 years (mean 3,8 years). All children had hearing evaluation done by tympanometry and distortion-product otoacoustic emission test (DPOAE). At the same time children had speech evaluation performed by the psychologist and the logopedists with the employment of objective tests adjusted to the age of the patients. The parents of patients were also asked about their subjective feelings of hear impairment and speech development in their children.

We found hear impairment in 10 among 24 evaluated patients, grade 1-3 according to Brock scale. In 25% of evaluated children the results of verbal tests evaluated by psychologists were below average. The results were not correlated with the degree of hearing impairment. In articulation tests done by logopedists in 37,7% of patients the results were below average and only in 18% - above average. Only patients in whom standardized tests could be used are evaluated.

The observed disturbances in both tests in the speech development were not clearly correlated with ototoxicity. Moreover, parents did not observe neither problems with hearing nor with speech in children with objective hear impairment.

Ototoxicity was observed in 42% of patients at the end of therapy and probably the incidence will grow with time. At the early stages, it is not necessarily correlated with speech impairment, although increased frequency of lateral lisping at sibilitanst (typical in Polish, high frequency sounds) was observed. As usually ototoxicity is not noticed by parents, especially in lower stages, these patients must be carefully followed. Early support of psychologist and logopedist may influence development and learning abilities, especially in younger children.

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Galectin-1 Modulates CD8+ T-Cell Response Towards Tolerance and Can Be Targeted by DNA Vaccine to Suppress Tumor Growth in Neuroblastoma

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Background: We recently identified Galectin-1 (Gal-1), a β-galactoside-binding lectin, as a novel immune regulator in neuroblastoma (NB) capable of impairing T cell and dendritic cell function and blunting host immune responses. Here, we evaluated the impact of Gal-1 expression by primary NB tumors on the function of CD8+ T cells. Secondly, we generated minigene DNA vaccines encoding Gal-1 with the ultimate goal of breaking immune tolerance and inducing immune response against NB.

Methods: A/J mice were challenged with lethal doses of NXS-2 NB cells (2 x 10^6 s.c.). Depletion of CD8+ T cells was performed by i. p. injections with anti-CD8 antibodies. Vaccination was performed in a prophylactic setting by oral gavage of attenuated SL7207 (AroA-, 10^8 per mouse) carrying minigene or pU control plasmids. The minigenes encode for Gal-1-derived peptides with superior MHC class I binding affinities.

Results: We were able to abrogate the reduced primary tumor growth by serial injections of anti-CD8 mAb. Next, we carried out epitope screening with online syfpeithi database and computer docking experiments. “FDQADLTI” (FDQ), “GDFKIKCV” (GDF) and “AHGDANTI” (AHG) were predicted with superior H2-Kk and “KFPNRLNM” (KFP), “DGDFKIKCV” (DGD) and “LGKDSNNL” (LGK) with superior H2-Dd binding affinities. Minigenes encoding for FDQ-GDF-AHG (G1-KK), KFP-DGD-LGK (G1-DD) or a triplet of highest affinity G1-epitopes FDQ-GDF-KFP (G1-H) were generated by overlapping PCR and cloned into a ubiquitin containing plasmid (pU). Mice receiving the pUG1-KK or pUG-1H presented up to 80% reduction in s.c. tumor volume and weight in contrast to control and were associated with increased cytotoxicity of isolated splenocytes. Vaccination with pUG1-DD plasmid showed less suppressive capabilities on primary tumor progression.

Conclusion: Gal-1 expression by NB negatively regulates CD8+ T cells. Vaccination with Gal-1-encoding DNA plasmids may be effective against NB.

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Targeting A Key Enzyme in Serine/Glycine Metabolism for Neuroblastoma Therapy

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Neuroblastoma is the most common extracranial childhood tumor of the sympathetic nervous system. Some genetic alterations have been shown to be prognostic of high-risk clinical courses, such as the amplification of V-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog (MYCN), which was found in approximately 20% of neuroblastomas. The patients with advanced neuroblastoma are still hard to cure, and 5-year survival could only be improved to 50% under the current therapeutic regimen. Here, through a gene expression-based integrative analysis, we uncovered several genes including phosphoglycerate dehydrogenase (PHGDH) associated with the aggressive biology by MYCN amplification in high-risk neuroblastoma. PHGDH is the first enzyme in serine biosynthesis and also essential in downstream glycine synthesis. Recently, a large body of work has demonstrated that reprogramming metabolism in cancer is a promising therapeutic strategy. In the study, silencing PHGDH reduces the MYCN-amplified neuroblastoma cell survival and tumor formation. Additionally, using a myriad of perturbation profiles across multiple cell lines from Library of Integrated Network-based Cellular Signatures (LINCS), we inferred recurrent 'perturbation−affected gene' relationships. We proposed an expression-based therapeutic discovery strategy by searching for drugs that can achieve the 'reversal' effect given a disease profile. Interesting, homoharringtonine (HHT) is screened out as one of the potential new PHGDH inhibitors and reduces PHGDH mRNA but not protein expression level. Isothermal titration calorimetry (ITC) displayed HHT inhibited PHGDH enzyme activity via competing with its substrate NAD+. We confirmed HHT cytotoxicity at a nanomolar concentration in MYCN-amplified neuroblastoma cell lines. To test the therapeutic efficacy in vivo, we used the xenograft mouse model via subcutaneous injection of the MYCN-amplified neuroblastoma cell line SK-N-BE (2)C. Consistently, we found that HHT significantly reduced tumor volume and increased survival in this neuroblastoma mouse model but has no significant changes in weights of total body and the organs including liver, spleen, and kidney as well as no observable toxicity to these organs. Metabolite profiling reveals that HHT causes a reduction in serine/glycine biosynthesis but a promotion in tricarboxylic acid (TCA) cycle. These findings suggest that reprogramming serine/glycine metabolism by a new PHGDH inhibitor HHT is a powerful therapeutic strategy for high-risk neuroblastoma.

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Predictors of Differential Response to Induction Chemotherapy in High-Risk Neuroblastoma: A Report from the Children's Oncology Group (COG)

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Background: Induction chemotherapy is important in the management of patients with high-risk neuroblastoma. Predictors of response to Induction therapy itself are largely lacking. We sought to describe clinical and biological features associated with differential response to Induction.

Patients and Methods: Patients from the following COG high-risk trials with at least one disease evaluation during Induction were included: A3973; ANBL02P1; ANBL0532; and ANBL12P1. Response at end-Induction was evaluated by the 1993 International Neuroblastoma Response Criteria. The primary endpoint was partial response (PR) or better. Secondary analyses evaluated complete response (CR) and progressive disease (PD). A series of univariate analyses (Fisher's exact or chi-squared tests) were performed to compare response as a function of clinical or biologic predictor variables. For each predictor variable, the Holm-Bonferroni method was used to correct for multiple testing, using an overall α=0.05. A multivariate logistic regression model using significant predictors from univariate analyses was constructed to model PR or better.

Results: The analytic cohort included 1,242 patients (79.8% with PR or better; 20.8% with CR; 9.1% with PD). Baseline factors significantly associated with a PR or better included age <18 months (87.4% with PR or better vs. 78.7% if older; p=0.0103), age <5 years (82.0% vs. 70.6% if older; p<0.0001), INSS <Stage 4 (89.0% vs. 78.4% if Stage 4; p=0.0016), MYCN amplification (85.5% vs. 77.1% if non-amplified; p=0.0006), 1p loss of heterozygosity (LOH; 85.6% vs. 76.0% if no LOH; p=0.0085), no 11q LOH (84.8% vs. 70.9% if 11q LOH; p=0.0004), and high mitosis-karyorrhexis index (MKI); 84.5% vs. 77.5% if low-intermediate MKI; p=0.0098). Factors significantly associated with CR included INSS <Stage 4 (p<0.0001), MYCN amplification (p<0.0001), no 11q LOH (p=0.0196), high MKI (p=0.0066), and MIBG non-avid tumor (p<0.0001). The only baseline factor significantly associated with PD was 11q LOH (p=0.0491). On multivariate analysis (n=407), the absence of 11q LOH was the only factor that remained significantly associated with PR or better (odds ratio: 1.962 compared to 11q LOH; 95% confidence interval 1.104-3.487; p=0.0216).

Conclusions: Clinical and biological factors are associated with differential response to Induction chemotherapy. These findings may further improve our ability to predict treatment response.

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Sepin-1, an Inhibitor of Separase, as a Novel Agent for Neuroblastoma Therapy

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Background: Separase, a chromosomal cohesion-resolving enzyme during cell division, is an oncogene overexpressed in multiple tumors, including neuroblastoma. Over 80% of neuroblastoma tumors overexpress Separase transcripts. In mouse models, Separase overexpression has been shown to induce aneuploidy, genomic instability, mammary and osteogenic tumorigenesis, and intratumoral heterogeneity. Using a high throughput screen, we identified a small molecule Separase inhibitor (Sepin-1) that non-competitively inhibits Separase. Sepin-1 inhibits the growth of leukemia and breast tumor cell lines, as well as Separase-overexpressing human triple negative breast cancer xenografts in mice in a Separase-dependent manner. This is the first description of Sepin-1 in neuroblastoma.

Methods: We tested the effect of Sepin-1 on neuroblastoma cell growth in tissue culture and mouse xenografts. To test Sepin-1 in mouse xenograft model, we transduced SKNSH cells with construct expressing GFP and fly luciferase using lentivirus. The derived cells were implanted in adrenal glands of NCr nude mice. The mice were treated with 15mg/kg daily Monday to Friday for 3 weeks via oral gavage. We also investigated the synergistic anti-tumor effect of Sepin-1 and 13-cis retinoic acid (13-cRA), vincristine or topotecan using SKNSH cells. Cells were cultured at 0.7 X105 cells/mL overnight and treated in the presence or absence of Sepin-1 with the second drug at varying concentrations for 72 hrs.

Results: The IC50 of Sepin-1 in inhibiting the growth of 9 NB cell lines ranging from 4.8 to 62.5 μM. In neuroblastoma mouse xenograft models, compared to the PBS control, Sepin-1 significantly inhibited the tumor growth as determined by both fluorescence intensity and tumor mass. MTT cell viability assay showed Sepin-1 plus 13-cRA or Vincristine has synergistic effect at IC75 drug concentrations on inhibiting SKNSH cell growth with combination indices of 0,5 and 0.32, respectively. However, no synergy was observed when cells were treated with Sepin-1 and topotecan.

Conclusion: These results suggest that inhibition of Separase represents a new line of therapy to treat neuroblastoma and other tumors addicted to Separase overexpression. Blocking overexpressed Separase activity can potentially be used both to both kill neuoroblastoma cells, as well as to sensitize resistant cells to chemotherapy.

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AVEN Is an Indirectly Druggable Mediator of Neuroblastoma Cell Tumorigenicity and Chemotherapy Resistance

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AVEN inhibits apoptosis by binding APAF1. Here we explored AVEN expression in neuroblastoma in relation to clinical characteristics, exploited its control mechanisms as potential therapeutic targets and functionally analyzed the phenotypic consequences of modifying AVEN expression in neuroblastoma cells. High-level AVEN expression from mRNA-Seq data in 649 primary neuroblastomas positively correlated with established clinical and molecular markers for unfavorable tumor biology and negatively correlated with patient survival. Correlations were confirmed in microarray expression data from an independent 122-tumor cohort and in technical replicates from 498 neuroblastomas. Multivariate analyses with the 649-tumor cohort revealed that the prognostic value of AVEN expression provides additional predictive power over the established prognostic markers, stage, age, MYCN and 1p. AVEN immunohistochemistry in 17 selected tumor samples demonstrated that the differential pattern of AVEN expression is translated to the protein level in neuroblastomas having the most divergent tumor biologies. Correlating AVEN copy number alterations assessed by WGS, WES and/or aCGH with AVEN expression in corresponding mRNA-Seq data from 256 neuroblastomas showed hardly any correlation, suggesting epigenetic regulation. In-depth time-resolved mRNA-Seq combined with microRNA profiling of a neuroblastoma differentiation model using the clinically approved histone deacetylase inhibitor, panobinostat, showed that AVEN was strongly down-regulated by miR-630, which was the most dramatically induced microRNA in the model. Stably enforcing AVEN expression in BE (2)-C cells markedly promoted growth in subcutaneous xenografts of these cells in female NMRI-Foxn1nu/nu mice, while CRISP/Cas9-mediated AVEN knockout in BE (2)-C cells substantially mitigated subcutaneous xenograft growth and sensitized the tumors to systemic doxorubicin treatment. FACS-based cell cycle analyses of BE (2)-C and IMR5/75 cells and matched CRISP/Cas9 knockout or enforced expression clones revealed that AVEN knockout caused G0/G1 arrest and apoptosis (sub-G1 fraction increase), while enforcing AVEN expression increased the proportion of cells in S-phase cells and reduced the apoptotic fraction. Colony forming assays with all BE (2)-C and IMR5/75 clones mirrored findings in xenografts. SILAC experiments revealed that AVEN knockout caused the differential expression of 33 proteins in BE (2)-C cells, mostly involved in RNA/DNA-binding, transcriptional regulation, cell cycle control and differentiation. AVEN is a critical and indirectly druggable mediator of neuroblastoma cell tumorigenicity and chemotherapy resistance.

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ENCIT-01: A Phase 1 Study of Autologous T-Cells Lentivirally Transduced to Express CD171-Specific Chimeric Antigen Receptors for Recurrent/Refractory High-Risk Neuroblastoma

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Background: Anti-ganglioside (GD2) antibody immunotherapy improves outcome for HR-NB but 35% of patients (pts) will still recur. L1-CAM (CD171) is expressed by neuroblastoma, offering an alternative immunotherapy target for HR-NB.

Methods: Pts with recurrent/refractory HR-NB were enrolled to examine the safety and feasibility of administering autologous, peripheral blood-derived T cells genetically modified using a SIN lentiviral vector to express either scFv-IgG4hinge-CD28tm-4-1BB-zeta (second generation, Arm A) or CD171-specific scFv-IgG4hinge-CD28tm/cyto-4-1BB-zeta (Arm B) CD171 (L1CAM)-specific chimeric antigen receptor (CAR) and the selection/tracking/suicide construct EGFRt. All patients received lymphodepleting chemotherapy after which cryopreserved CD4 and CD8 CARs were administered at a 1:1 ratio at the prescribed dose level. Dose range is 1x10^6/kg-1x10^8/kg. The MTD is determined using a “k-in-a-row” up-and-down design on each of 2 study arms, enhanced with additional safety rules.

Results: From 11/2014-12/2017, 24 pts have enrolled with CAR manufacturing successful in 22 pts. Sixteen pts (median age 7.5 yrs; range 3-18 yrs) have been treated at dose level (DL) 1 (n=3-A and n=1-B), DL2 (n=2-A and n=5-B), DL3 (n=1-A and n=1-B), DL4 (n=3-A) and DL5 (n=1-A). Dose limiting hyponatremia attributable to CAR T was seen in 2 patients at DL 3A and DL 5B. Self-limited skin rash occurred in 5 patients (DL 2B, 3B, 4A and 5A), skin biopsy performed in 2/5 pts demonstrated EGFRt+ CAR infiltration. CAR T persistence documented by EGFRt- flow improved with both increasing dose levels and CAR generation (3rd>2nd). Two patients exhibited tumor pseudoprogression with documented T cell tumor infiltration in 1 patient who consented to tumor biopsy (DL 3B). There were no objective responses, 2 pts with early progressive disease are alive without subsequent treatment 15 (DL 5-2G) and 30 months (DL 1-3G) post CAR infusion, respectively.

Conclusions: It is feasible to manufacture CAR T cells from heavily pre-treated pts with HR-NB. The initially planned T cell infusion doses were insufficient to generate persistence in HR NB patients; however, increased T cell dose and 3G CAR constructs may improve persistence. Observed toxicities may represent on-target-off-tumor cytotoxicity. Expansion of the current dose cohorts is underway to further evaluate safety and efficacy.

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BGA002 (Anti-MYCN Antigene PNA) Exerts Potent and Specific Anti-Tumor Activity In MYCN-Amplified/Expressing Neuroblastoma

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MYCN amplification is present in almost 30% of Neuroblastoma (NB) and is associated with tumor aggressiveness and poor prognosis. Moreover, in some MYCN-unamplified NB, increase of N-Myc protein by altered degradation is also associated with poor prognosis. MYCN is highly expressed in fetus and tumoral mass, while it shows a very restricted pattern of expression in healthy tissues after birth; consequently, a MYCN-directed targeted therapy for MYCN-amplified/expressing NB is not expected to result in adverse effects. We previously showed in MYCN-expressing Rhabdomyosarcoma that the antigene oligonucleotide strategy to inhibit MYCN transcription, results in improved efficacy compared to inhibition of MYCN translation by antisense oligonucleotide strategy. We present the in-vitro and in-vivo preclinical anti-tumor activity of BGA002 (an antigene PNA oligonucleotide, specific for the inhibition of the MYCN transcription, that granted orphan drug designation by FDA and EMA for NB treatment).

BGA002 caused a dose-dependent MYCN transcription inhibition, in both MYCN-amplified/overexpressing and MYCN-unamplified/expressing NB cell-lines. BGA002 was also effective in MYCN-amplified/overexpressing and p53-mutated (therapy-related) NB cell-lines. BGA002 showed a dose-dependent N-Myc protein reduction. Potent, MYCN-specific and dose-dependent cell-growth inhibition was observed in MYCN-expressing NB cell lines after treatment with BGA002, that correlated with the level of MYCN mRNA expression. The highest activity was observed in MYCN-amplified/overexpressing NB cell-lines, followed by apoptosis. BGA002 caused gene expression modifications (up-and down-regulation) in previously related and unrelated MYC-target genes.

In-vivo anti-tumor effect after systemic administration of BGA002 performed in MYCN-amplified NB Xenograft mouse-model showed potent dose-dependent anti-tumor activity. The in-vivo pharmacodynamic response was demonstrated by reduction of N-Myc protein amount in immunohistochemical analysis from extracted tumours. In-vivo pharmacokinetic profile was characterized in rodents and rabbits. The in-vivo toxicological and safety pharmacology regulatory profile showed that BGA002 was well tolerated.
In summary, the in-vitro and in-vivo preclinical studies of BGA002 showed potent and specific anti-tumor activity for MYCN-amplified/expressing NB. Further analysis is under evaluation to characterize the molecular pathways underlying the specific activity in this highly aggressive subtype of NB. Considering the consistent efficacy and well tolerated safety profile, BGA002 is progressing to a clinical phase-I evaluation in patients affected by NB with MYCN-expression.

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The Novel Neuroblastoma Model OHC-NB1 Reflects Intratumor Heterogeneity and Offers A Variety of Culture Systems

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Accurate disease modeling creates a bottleneck against effective translation of novel neuroblastoma therapies and therapy combinations into the clinic. The intratumoral genetic heterogeneity of neuroblastoma has recently been described and calls for preclinical models that reflect this characteristic. Flexibility in a testing platform is also desirable to support assessment of different endpoints and tumor cell characteristics, including automated in vitro testing, modeling cell-cell and cell-matrix interactions and assessing responses in a 3D microenvironment. We present the novel neuroblastoma model OHC-NB1, which was propagated from a bone marrow metastasis from a patient with INRG stage M, MYCN-amplified neuroblastoma at first diagnosis. To model different aspects of disease, several preclinical models were developed from the single bone marrow aspirate: a tissue plastic-adherent cell line, a 3-dimensional (3D) spheroid culture, tumor organoids growing in matrigel and a patient-derived xenograft (PDX) serially subcutaneously passaged in mice. Growth characteristics, immunophenotypes, expression of key proteins and the molecular genetic background (using whole-exome sequencing) were examined to characterize this neuroblastoma model set. Immunophenotyping revealed surface expression of the GD2 and NCAM1 neuroblastoma markers by cells in plastic-adherent monolayer and spheroid cultures. Xenograft tumors expressed the neuroblastoma markers, synaptophysin and NB84. Immunohistochemical target screening detected HDAC5, HDAC9 and EGFR overexpression. The OHC-NB1 model fulfills typical aspects of MYCN-amplified neuroblastoma, with MYCN amplification in double minutes, 1p deletion, 17q gain, diploid karyotype and no evidence of either TERT rearrangement or ATRX mutation, but with TERT overexpression. ALK was not mutated. Whole-exome sequencing revealed intratumoral heterogeneity in the bone marrow metastasis and genetic heterogeneity in the culture models. Cells maintained in all culture types harbored the same 1p deletion, 17q gain and MYCN amplification as the primary metastasis. Smaller copy number variations accumulated as xenografts were passaged (passage 4 compared with passage 1). Sample-specific single-nucleotide variants not present in the bone marrow metastasis were detected in all culture models, especially in the monolayer culture. The set of different OHC-NB1 culture systems and the genetically heterogeneous MYCN-amplified neuroblastoma background provide a flexible preclinical testing system that recapitulates important characteristics of metastatic neuroblastoma.

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PD-L1 Expression in Donor Car-T Cells Predicts the Benefit from Co-Treatment with Nivolumab

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Despite the remarkable potency of CD19-targeting CAR-T cells against hematological malignancies, similar results have not yet been achieved for CAR-T cell therapy of solid tumors. Programmed death-ligand 1 (PD-L1) is thought to help create the immunosuppressive microenvironment in solid tumors and to impair CAR-T cell efficacy. We evaluated the PD-1/PD-L1 pathway in CD171-specific CAR-T cells co-cultured with neuroblastoma cells and assessed the influence of PD-1 inhibition on immunotherapy efficacy. The neuroblastoma-specific CD171-targeting CAR-T cells were cultured separately or co-cultured with SK-N-BE(2) neuroblastoma cells, and FACS-based expression analysis assessed PD-1 and PD-L1 in CAR-T cells and PD-L1 in neuroblastoma cells before and after co-culture. We generated the SK-N-BE(2)PD-L1+ neuroblastoma cell model, which stably expresses PD-L1 from a lentiviral vector, to assess the impact of high PD-L1 expression by tumor cells in our in vitro co-culture system. FACS analyses, luciferase reporter assays and cytokine release assays were used to assess CAR-T cell activation, exhaustion and neuroblastoma cell killing after co-culture with SK-N-BE(2) or SK-N-BE(2)PD-L1+ cells. Co-culture with CD171-CAR-T cells upregulated PD-L1 expression in SK-N-BE(2) cells from 6.6% to up to 47.8%. Nivolumab increased CD171-CAR-T cell-directed killing of both SK-N-BE(2)PD-L1+ and SK-N-BE(2) cells in co-culture experiments, even though this benefit was not reflected in altered CAR-T cell activation, exhaustion or cytokine release. Interestingly, Nivolumab-enhanced CD171-CAR-T cell killing did not correlate with PD-L1 expression in neuroblastoma cells. Expression of both PD-1 (5. 9% to 39.2%) and PD-L1 (3.3% to 29.7%) was significantly increased on CD171-CAR-T cells after co-culture with neuroblastoma cells. CD171-CAR-T cells were manufactured from T cells isolated from donor blood, and PD1/PD-L1 expression varied on CD171-CAR-T cells from different donors. Nivolumab enhancement of CD171-CAR-T cell killing was positively correlated with PD-1/PD-L1 expression on the CD171-CAR-T cells. Thus, PD-1 inhibition by Nivolumab depended on PD-1/PD-L1 expression levels in T cells not tumor cells. The upregulation of PD-1/PD-L1 caused only by interaction between neuroblastoma and CD171-CAR-T cells emphasizes the importance of the PD-1/PD-L1 pathway in suppressing CAR-T cell efficacy in patients. Screening donor T cells for PD-1/PD-L1 expression could help determine when to combine CD171-CAR-T cell therapy with checkpoint inhibitors to improve treatment efficacy.

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In Vitro and In Vivo Validation of Combination Strategies with Venetoclax For Relapsed Neuroblastoma

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Background: The majority of all neuroblastoma patients overexpress the anti-apoptotic protein B cell lymphoma/leukaemia 2 (BCL-2). We previously showed that the BCL-2 inhibitor Venetoclax causes programmed cell death in BCL-2-dependent neuroblastoma, which contributed to the initiation of a phase I trial to study the safety and pharmacokinetics of Venetoclax in children with relapsed or refractory neuroblastoma. In the current study, we aim at the identification and validation of targeted combination strategies to prevent or overcome neuroblastoma resistance to Venetoclax.

Results: To identify effective combination treatment options for relapsed neuroblastoma patients we subdivided our model systems in subgroups with specific genomic backgrounds: ALK mutated, RAS-MAPK pathway mutated and p53 wild-type. Cells were screened using concentration series of libraries of targeted compounds in combination with concentration series of Venetoclax. Surprisingly, BCL-2-dependent neuroblastoma cell lines harboring an additional ALK mutation did not yield ALK inhibitors as most effective or synergistic drugs in combination with Venetoclax. Instead, PARP inhibitor Talazoparib, PI3K/HDAC inhibitor CUDC-907 and CHK1 inhibitor Prexasertib were the most promising hits for combination therapy with Venetoclax in the ALK-mutated subgroup. The same hits were found for the p53 wild-type subgroup. In RAS-MAPK-mutated neuroblastoma cells, the MEK inhibitor Trametinib showed synergistic responses with Venetoclax. This was related to MEK-mediated phosphorylation of BIM. This synergistic combination could be confirmed in vivo.

Strategies to overcome Venetoclax resistance have also been studied. In previous preclinical studies, we demonstrated that Venetoclax resistance occurs due to upregulation of the anti-apoptotic protein MCL-1. In vitro combination studies confirmed a strong synergistic interaction between Venetoclax and the novel MCL-1 inhibitor S63845. High-throughput screening of non-resistant versus Venetoclax-resistant BCL-2-dependent cell lines with wild-type p53 uncovered the MDM2 inhibitor Idasanutlin as one of the strongest re-sensitizers to overcome resistance. In vivo combination of Venetoclax with Idasanutlin resulted in a remarkably improved anticancer effect compared to single agent therapy, with very good partial and complete responses in BCL-2-dependent neuroblastoma xenografts.

Conclusion: We identified various targeted combination strategies that might improve the clinical use of Venetoclax for the treatment of children with BCL-2-dependent neuroblastoma.

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Association of Image-Defined Risk Factors with Clinical Features, Histopathology, and Outcomes in Neuroblastoma

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Background: Clinical and histopathologic features guide treatment for neuroblastoma but obtaining tumor tissue may cause complications and is subject to potential sampling error due to tumor heterogeneity. The association of specific imaging characteristics of neuroblastoma with clinical features, histopathology, and outcomes is unclear.

Methods: We performed a retrospective cohort study of patients with neuroblastoma or ganglioneuroblastoma enrolled on the Children's Oncology Group biology study (ANBL00B1) between 2000-2015 at UCSF Benioff Children's Hospital in San Francisco. Data collected included clinical features (age, stage, primary tumor site, surgical complications); histopathology (MYCN status, ploidy, mitosis-karyorrhexis index [MKI]); detailed radiographic review of diagnostic CT scans for image-defined risk factors (IDRFs); and event-free survival (EFS). IDRFs were evaluated in five separate binary variable categories: extension within multiple body compartments, vascular encasement, airway compression, infiltration of adjacent organs/structures, and intraspinal extension.

Results: Our analytic cohort included 36 patients. Thirty-two patients (89%) had tumors with IDRFs. Of the clinical and histopathologic features, only the proportion of patients who had surgical complications differed statistically when compared between those with and without IDRFs (62% vs. 0%, p=0.02). Using a stepwise regression model, the presence of IDRFs was significantly correlated with the presence of surgical complications (p=0.03), abdominal/retroperitoneal primary site (p=0.04), and hypodiploid/diploid tumors (p=0.02). Additional multivariable stepwise regression analyses were performed to investigate the correlation of histopathology with the five separate categories of IDRFs. MYCN amplification (p=0.01) and high MKI (p=0.01) were significantly correlated with tumor infiltration to adjacent organs/structures. Hypodiploid/diploid tumors were significantly correlated with tumor extension within multiple body compartments (p=0.05). The 5-year EFS was 100% in patients who had neuroblastic tumors without IDRFs compared to 71.2% (95% CI, 49.3-85.5%; p=0.25) for those patients with tumors with IDRFs.

Conclusions: There is significant correlation between the presence of IDRFs with primary tumor site, DNA ploidy, and surgical complications. MYCN status, MKI, and ploidy were correlated with tumor infiltration to adjacent organs/structures and tumor extension within multiple body compartments. These findings suggest that specific imaging characteristics may be associated with important clinical and histopathologic features in neuroblastoma. Analysis in a larger cohort of patients is ongoing.

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Induction Chemotherapy with An Anti-GD2 Monoclonal Antibody (Dinutuximab) and Cytokines in Children with Newly Diagnosed High-Risk Neuroblastoma: A Case Series

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Background: Recent studies conducted in patients with relapsed/refractory neuroblastoma demonstrated that treatment regimens with chemotherapy plus an anti-GD2 monoclonal antibody (mAb) and cytokines were well-tolerated and had a positive clinical response. A current phase II trial (NB2012) for patients with newly diagnosed high-risk neuroblastoma (HRNB) evaluating an anti-GD2 mAb, hu14.18K322A, combined with Induction chemotherapy and cytokines demonstrated a significantly improved early response rate. We report the results of three patients with newly diagnosed HRNB treated with combined Induction chemotherapy, dinutuximab, GM-CSF and IL-2.

Methods: Children with newly-diagnosed HRNB received 6 cycles of Induction chemotherapy combined with dinutuximab (17.5mg/m2/dose IV, days 2-5), GM-CSF (250mcg/m2/day subcutaneous (sc), days 7-nadir) and IL-2 (1 million units/m2/dose sc, every other day for 6 doses). Chemotherapy regimens included cyclophosphamide/topotecan (cycles 1,2), cisplatin/etoposide (cycles 3,5) and cyclophosphamide, doxorubicin and vincristine (cycles 4,6). Surgical resection of the primary tumor was performed when feasible. Following Induction therapy patients received Consolidation (busulfan and melphalan with peripheral blood stem cell rescue and radiation therapy) and Post-Consolidation (dinutuximab, GM-CSF, IL-2 and cis-retinoic acid). Response was evaluated using the revised International Neuroblastoma Response Criteria (INRC).

Results: Three patients with newly-diagnosed HRNB (2 females, median age 4 years; range 3-5 years) completed Induction and Consolidation therapy. All three patients had stage 4, MYCN non-amplified HRNB. Curie scores at diagnosis were 1, 14 and 21. The mean duration of admission for chemotherapy was 7.9 days. During Induction, dinutuximab was infused over 10 hours with no respiratory complications or hypotension. Common toxicities included grade 4 myelosuppression (3/3 patients), grade 3 anorexia (3/3 patients) grade 3-4 fever with neutropenia (3/3 patients), grade 3 mucositis (2/3 patients), grade 2 pain (3/3 patients), grade 2 urinary retention (2/3 patients) and grade 4 sepsis (1/3 patients). All three patients achieved greater than 85% primary tumor resection. At the end of Induction, Curie scores were 0 and 3/3 patients had a complete response. All 3 patients are receiving Post-Consolidation therapy.

Conclusions: Administration of Induction chemotherapy with dinutuximab, GM-CSF and IL-2 in patients with newly-diagnosed HRNB is feasible and demonstrated clinical response. Further studies using this approach are warranted.

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Modulators of Multidrug Resistance Protein 1 Show Promising In Vitro and In Vivo Activity in Neuroblastoma

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Multidrug resistance protein 1 (MRP1) is frequently overexpressed in tumors where it effluxes chemotherapeutic agents, protecting tumor cells from chemotherapy. This is strongly illustrated in neuroblastoma, where MRP1 expression is highly prognostic of clinical outcome and contributes to chemoresistance in vivo. Furthermore, MRP1 also effluxes low levels of glutathione (GSH), a major antioxidant linked with resistance to chemotherapy and radiotherapy. Based on our previously identified MRP1 inhibitor Reversan, we have developed small molecule MRP1 inhibitors, which simultaneously block drug transport and enhance GSH efflux We are investigating the utility of dual-function MRP1 modulators for neuroblastoma treatment, with a view to identifying a candidate molecule suitable for clinical use.

Methods: In vitro activity and selectivity were determined in cells overexpressing drug transporters (MRP1, P-glycoprotein, ABCG2, MRP2 and MRP3), treated with established drug substrates in combination with MRP1 modulators, using cytotoxicity assays. GSH levels were determined by glutathione recycling assay. In vivo activity was assessed in the transgenic Th-MYCN and human xenograft mouse models of neuroblastoma, in combination with the MRP1 substrate drug etoposide. Specificity for MRP1 was tested using MRP1-deficient Th-MYCN mice.

Results: Our modulators demonstrated excellent selectivity for MRP1 over P-glycoprotein, ABCG2, MRP2 and MRP3, and sensitized neuroblastoma and other cancer cell lines at least three-fold to the substrate drugs etoposide, vincristine and arsenic trioxide in vitro ($P<0.001$). The modulators showed promising on-target activity in the Th-MYCN and Kelly xenograft models treated with etoposide, doubling median survival over etoposide alone (11 days to 21–23 days; $P<0.001$) in Th-MYCN mice, and extending median survival over etoposide alone (15 to 21 days) in the xenograft mice. No impact was observed on etoposide pharmacokinetics. Our MRP1 modulator depleted intracellular GSH in an MRP1-dependent manner and worked synergistically with GSH synthesis inhibitor L-buthionine sulfoximine (BSO), to deplete GSH, abolish clonogenic capacity, and sensitize cells to chemotherapy ($CI<0.01$, $P<0.05$).

Conclusion: Our modulators show strong selectivity for MRP1 and may increase the therapeutic window for standard-of-care drugs in MRP1 overexpressing cancers. Current studies examine the ability of MRP1 modulators to deplete intracellular GSH and whether this would increase survival in our animal model.

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Novel Method for Minimal Residual Disease Detection by Next Generation Flow Cytometry, Discriminating Neuroblastoma from CD56+ Mesenchymal Bone Marrow Cells

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Background: Detection of minimal residual disease (MRD) in neuroblastoma (NBL) by multiparameter flow cytometry (FC) is limited by low sensitivity with false negative and false positive results. Recent advances in deep MRD detection by next generation FC (NGF) in multiple myeloma, prompted us to apply similar methodology for NBL. Recent description of CD56+ human bone marrow (BM) derived mesenchymal stromal cells (CD56+BM-MSCs), led us to investigate their possible interference in NBL-MRD detection by MFC.

Aim: This study was to formulate a NGF-MRD method for NBL and propose a one-tube multicolour assay, based on antigens, discriminating CD56+BM-MSCs from NBL cells.

Patients and Methods As negative biologic controls in NBL-MRD detection were assessed 9 BM childhood-ALL samples during treatment. As positive control served 6 NBL patient BMs at diagnosis and 2 NBL cell cultures. The NBL associated antigens assessed were: CD56, CD45, GD2, CD81, CD24, CD200, CD9, NG2, CD117, CD99; BM-MSCs associated were: CD56, CD73, CD90, CD146, CD13, CD10. Nuclear stain Syto16 was used to avoid debris and apoptotic cells. Ammonium chloride bulky pre-lysis of BM volumes 0.8-1.5ml was also conducted. Initial 5-colour combinations were used with: Syto16/CD45/CD56 in every tube, leaving 2 positions to assess another 2 antigens per tube. Samples were analysed by Navios Beckman-Coulter apparatus, acquiring 2x106 events per tube. Discriminant analysis of NBL and CD56+BM-MSC was conducted.

Results: Two discrete CD45-negative subpopulations of CD56+BM-MSCs were detected in negative ALL controls with different CD56 intensity, CD56weak-MSCs and CD56bright-MSCs. Their BM frequencies and % antigen expressions, median value (range) were: a)CD56weak-MSCs: log10=-3.7(-4.4 to -3.1), CD81=86(54-100), CD24=3(0-21), CD200=83(36-97), CD73=74(20-96), CD90=58(17-83), CD146=40(3-74), NG2=8(0-61), CD13=69(50-98), CD9=13(5-50), CD117=2(0-29), CD99=67(0-94), CD10=80(57-93), GD2=27(4-32) b)CD56bright-MSCs: log10=-4.2(-4.5 to -3.9), CD81=99(90-100), CD24=18(0-58), CD200=94(80-100), CD73=91(32-98), CD90=30(0-58), CD146=18(5-100), NG2=39(15-86), CD13=88(76-100), CD9=88(65-94), CD117=1(0-13), CD99=69(2-100), CD10=78(18-100), GD2=34(3-55).

Conclusion: Deep NGF MRD detection with bulky prelysis, acquiring 2x106 events, revealed CD56+BM-MSCs with NBL antigen expressions, overlapping in given negative ALL controls. Discriminant analysis showed that CD24, GD2 and CD13 antigens were the ones actually dissecting between NBL and CD56+BM-MSCs cells. A 7-colour combination SYTO16/GD2-PE/CD24-ECD/CD13-PerCP5.5/CD45-PC7/CD81-APC/CD56-APC700 is proposed for further evaluation for fast, accurate and precise measurement of NBL MRD in BM.

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KIR-ligand Incompatible Allogeneic Cord Blood Transplantation Reduces Relapse and Improves Survival in Children with Primary High-Risk Stage 4 Neuroblastoma

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Introduction: Killer cell immunoglobulin-like receptor (KIR) / HLA genotypes predictive of missing KIR-ligands are reported to have a better outcome after autologous hematopoietic stem cell transplantation in patients with high risk neuroblastoma (Venstrom JM et al. Clin Cancer Res. 2009). We conducted a prospective study of KIR-ligand incompatible allogeneic cord blood transplantation (CBT) in 20 consecutive primary patients with high-risk neuroblastoma (NB).

Methods: The eligibility criteria were newly diagnosed International Neuroblastoma Staging System (INSS) stage 4 and International Neuroblastoma Risk Group (INRG) high risk NB patients aged <18 years with one of the following factors: chemo-refractory (MIBG positive disease after 4 courses of chemotherapy), ≥10 years old at diagnosis, or MYCN amplification. We scheduled KIR-ligand incompatible CBT with a reduced-intensity conditioning regimen 3 months after high-dose chemotherapy followed by autologous stem cell transplantation. Natural killer (NK) cells expressing single inhibitory KIR were monitored by flow cytometry before and after CBT up to 1 year.

Results: Twenty consecutive patients who met the eligibility criteria underwent CBT (17 chemo-refractory patients, 9 patients with MYCN amplification, and one >10 years old patient). The median age at diagnosis was 2.4 years (range: 0.7–10.5 years). The median follow-up period from CBT was 43 months (range: 24–104 months). Three-year event free survival and overall survival was 85.0% and 90.0%, respectively. No patients relapsed in bone marrow but only one patient developed isolated central nervous system relapse 307 days after CBT, and he achieved the 2nd complete remission without further bone marrow relapse after salvage craniospinal irradiation. Three-year cumulative incidence of relapse was 5.3%. Three patients developed grade II–IV acute graft-versus-host disease (GVHD) while no patients developed chronic GVHD. No patients died of relapse or progression of neuroblastoma in this cohort. NK cells expressing single inhibitory KIR that were considered as alloreactive NK cells significantly expanded after CBT and reached to donor CB level ($p = 0.0009$) in vivo.

Conclusions: These results suggest that KIR-ligand incompatible allogeneic CBT reduces bone marrow relapse and improves survival in children with high-risk NB.

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Single-Cell Transcriptomic Analysis Reveals the Early Separation of Neuroblastoma Fate in Th-MYCN Mice

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Introduction: The aim of this study is to understand the enigmatic phenomena “spontaneous regression” in neuroblastoma. To this end, we performed single-cell transcriptomic analysis to reveal the early fate determination of neuroblastoma observed in Th-MYCN mice.

Methods: We investigated the early-stage tumor tissue in Th-MYCN mice individually and performed droplet-type single-cell RNA sequencing by chromium single cell 3’ assay (10x Genomics). To validate the analysis, we carried out single-molecule fluorescence in situ hybridization (smFISH).

Results: Firstly, survival analysis showed that 80% of Th-MYCN hemizygous mice died of tumor by 10 to 20 weeks of age while 20% of them never developed neuroblastoma. Secondly, when we histologically investigated tumor origin of the mice, all of them had clusters of neuroblastoma cells until 3 weeks of age. Therefore, although neuroblastoma cells appeared in all cases, the fate to either develop tumor further or disappear was likely determined during early ages. We assumed the latter fate as spontaneous regression-like phenomena in Th-MYCN mice. Thirdly, to understand the observed fate determination, we obtained single-cell transcriptome of early neuroblastoma cells from tissues of seven 3-week-old and one 6-week-old (tumor-developing) Th-MYCN mice. The analysis revealed distinct cell types in the tissues, including MYCN+ neuroblastoma cells, ganglion cells, glial cells and so on. Within MYCN+ neuroblastoma cells, there were distinct sub-populations and the proportion of cells was different individually, suggesting that different fates were captured at single cell level. The sub-populations were characterized by the differential expressions of target genes regulated by certain transcription factors. Lastly, SmFISH analysis validated the results of single-cell analysis.

Conclusion: Single-cell transcriptomic analysis captured the separation of neuroblastoma fate observed in early ages of Th-MYCN mice. Early-stage subpopulation of MYCN+ neuroblastoma cells can be discriminated by the differential expressions of several genes, which possibly explains the mechanisms of fate determination, i.e. spontaneous regression, in neuroblastoma.

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Results of Myeloablative Therapy and Peripheral Blood Stem Cell Rescue in Patients with High-Risk Neuroblastoma

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Introduction: Despite the development of new treatment options, the prognosis of high-risk neuroblastoma patients still remains poor. Intensification of consolidation therapy with autologous stem cell rescue (PBSCR) after myeloablative doses of chemotherapy could contribute to the improved survival of children with high-risk neuroblastoma.

Aim: To investigate the 5-year overall survival of patients with high-risk neuroblastoma treated with myeloablative therapy and PBSCR in the National Cancer Institute of Ukraine.

Methods: One hundred forty-three patients with neuroblastoma were enrolled into the study: 74 standard-risk (SR) patients and 69 high-risk (HR) patients. The risk group of patients was determined on the basis of the most common prognostic factors include the child's age, stage, genetic markers (MYCN amplification, DNA ploidy) and some additional molecular-genetic markers: 1p36, 17p deletions, MDM2 gene expression. All enrolled patients received treatment according to NB-2004 and HR-NBL-1/ESIOP protocols. At the late stage of treatment, 54 high-risk patients received high-dose chemotherapy (HDHT) with PBSCR, of these, 13 patients were cured with tandem HDHT.

Results: Our previous study has been shown that higher MDM2 expression level was associated with unfavorable clinical neuroblastoma features, indicating a link between MDM2 overexpression and the HR phenotype as well as significant decrease in event-free survival of neuroblastoma patients ($p < 0.001$). The 5-year OS was 67 % for SR patients and 30.4% for HR patients. Depending on the child's age: the OS was 58.8 % for patients less than 1 year of age, while the OS was 19.2 % for patients aged 1 year or over. We also analyzed the survival rate of HR patients based on MYCN gene status. The OS was 49.8 % for N-myc negative patients and 24.3% for MYCN positive patients. Currently, OS is 69.2 % for HR patients who were treated with tandem HDHT with PBSCR.

Conclusion: The obtained results show that the OS of HR patients aged 1 year or over positive for MYCN was worst greatly than in patients with normal MYCN status. The results of HR neuroblastoma patient's treatment who received tandem HDHT with autologous steam cell support are encouraging.

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A Double-Conditioning Regimen with Thiotepa and Melphalan is Effective for MYCN-Amplified High-Risk Neuroblastoma

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Background/Objectives: Appropriate high-dose chemotherapy (HDC) for high-risk neuroblastoma remains unestablished. We reported previously a double-conditioning regimen with two cycles of thiotepa (total 800 mg/m2) and melphalan (total 280 mg/m2) for high-risk neuroblastoma. In this report, we analyzed retrospectively the outcomes of the double-conditioning regimen for patients with INSS stage4 neuroblastoma in three Japanese institutions.

Patients/Methods: The medical records of consecutive 40 patients with INSS stage4 neuroblastoma who received the double-conditioning regimen followed by autologous peripheral blood stem cell rescue (PBSCT) between 2002 and 2012 were reviewed retrospectively.

Results: The median patient age at diagnosis was 35 months (range, 8–75). MYCN-amplified tumors were observed in 20 patients. All patients underwent surgical resection of the primary tumor and radiotherapy to the residual lesion. The regimen of induction chemotherapy and timing of surgery varied depending on the institutions. None of the patients received anti-GD2 immunotherapy. The median length of follow-up for censored cases was 9.1 years (range, 3.9–14.8). The event-free survival (EFS) rate and the overall survival (OS) rate at 5 years from PBSCT was 47.5% ± 7.7% and 57.0% ±7.7%, respectively. Three patients died from regimen-related toxicity (infection (2) and microangiopathy (1)). The five-year EFS of patients with MYCN-amplified tumors were 65.0% ± 10.7%, which was significantly superior to those with MYCN-non-amplified tumors (24.0% ± 9.8%; P=0.001). The five-year EFS of patients with CR+VGPR at HDC (n=12, 75.0 ±12.5%) was significantly better than that with PR+NR (n=28, 32.1 ±8.8%; P=0.004). Notably, the five-year EFS of patients with MYCN-amplified tumors and CR+VGPR at HDC (n=9) was 88.9% ± 10.5%. In a multivariate analysis, increased probability of EFS was demonstrated in patients with MYCN-amplified tumor (HR=2.85; P=0.026) and good remission status before HDC (HR=3.57; P=0.049).

Conclusions: The double-conditioning regimen of thiotepa and melphalan could improve survival in MYCN-amplified high-risk neuroblastoma. On the contrary, we might need to treat MYCN-non-amplified patients with an alternative approach other than intensified chemotherapies.

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Targeting Prohibitin to Inhibit MAP Kinase Pathway Activation in Neuroblastoma

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Whole-genome sequencing efforts in the last few years have revealed activating mutations in components of the MAP kinase pathway as common features of relapsed neuroblastomas. As a means of inhibiting the MAP kinase pathway in neuroblastoma cells, we chose to target the plasma membrane scaffold factor prohibitin (PHB), a protein required for RAF1 activation by Ras family members. PHB is located in the 17q21-17qter region of chromosome 17, the most commonly gained genomic region in neuroblastomas. Gain of this region is a cytogenetic hallmark of poor prognosis. Combining whole-genome and RNA sequencing data from primary neuroblastomas identified PHB as frequently present in 17q gains and showed that high PHB expression is associated with adverse patient outcome. Western blot analysis of 8 neuroblastoma cell lines detected PHB phosphorylated at threonine 258, a modification associated with plasma membrane localization. PHB knockdown using shRNAs reduced viability of 4 neuroblastoma cell lines and viability was partially restored by ectopic expression of recombinant PHB, suggesting that PHB is required for neuroblastoma cell survival. PHB knockdown was sufficient to reduce phosphorylation of ERK1/2 at activating residues. Neuroblastoma cells were sensitive to rocaglamide, a drug previously demonstrated to disrupt the interaction between PHB and RAF1, at nanomolar concentrations. Rocaglamide treatment recapitulated the effects of shRNA knockdown on cell viability and ERK1/2 phosphorylation. High-throughput RNA sequencing revealed that PHB knockdown increased expression of NTRK1, known to be a marker of favorable outcome, as well as other genes involved in neurotrophic signaling. Our results present PHB as a molecular target for inhibiting MAP kinase signaling in neuroblastoma cells in a kinase-domain-independent manner, suggesting it is a therapeutic option for patients with high-risk disease exhibiting aberrant MAPK pathway activation.

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Identification of Novel Compounds Which Compromise the Cytoskeleton of Neuroblastoma

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The actin cytoskeleton mediates a range of cancer cell functions including cell migration, intracellular transport, cell structure and proliferation; functions which are altered in all cancers. Compounds which compromise the organisation and function of the actin cytoskeleton in neuroblastoma are therefore candidates for development as anti-neuroblastoma agents. We have screened an 115,000-compound library to identify candidates which impact the actin cytoskeleton of SK-N-SH neuroblastoma cells. SK-N-SH cells were chosen because they have a very well organised actin cytoskeleton which is readily quantified in terms of its organisation. Eight hundred and eighty-one compounds resulted in an altered actin cytoskeleton and they could be grouped into a set of 29 phenotypes. Examination of the phenotypes allowed us to identify novel compounds which have characteristics of anti-tropomyosin, anti-ROCK, Latrunculin and Jasplakinolide activities. In addition, one set of compounds inhibits the ability of the cells to move because of increased attachment to the substratum and another set of compounds promotes the morphological differentiation of SK-N-SH cells. A subset of compounds which impact substratum attachment also compromise neuroblastoma viability with low micromolar IC50s. Representative members of each group and subgroup are being tested for synergy with currently used neuroblastoma therapeutics. These new families of anti-actin cytoskeleton compounds provide new potential approaches to compromise the growth and metastasis of neuroblastoma.

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Zero Childhood Cancer: A Comprehensive Precision Medicine Platform for Children with High-Risk Cancer

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Molecular genomics analyses aim to identify the subset of patients harbouring actionable mutations as a pathway to better targeted treatment selection. Low mutation rates and the paucity of clinical data linking targeted treatments with mutations in paediatric cancer suggests genomic analysis alone has limitations for translation into clinical benefit.

Zero Childhood Cancer aims to assess the feasibility of a comprehensive precision medicine platform to identify targeted therapeutic agents for high-risk (HR) paediatric malignancies (expected survival <30%). The program combines molecular genomic analysis (WGS of tumour and germline DNA, deep sequencing of a panel of cancer associated genes, and whole transcriptome (RNASeq), with in vitro high-throughput drug screening, and patient-derived xenograft (PDX) drug efficacy testing followed by recommendations by a National Multidisciplinary Tumour Board (MTB).

The Pilot Feasibility Study enrolled 59 patients with a range of tumour types (47% central nervous system tumours, 20% sarcoma, 12% leukaemia, 9% neuroblastoma, 12% other) over 28 months (49% at diagnosis; 51% at relapse). In 54 curated cases, the complete molecular platform identified reportable somatic SNVs, fusions, and CNVs in 56%, 24% and 40% of patients, respectively. 5 patients had a reportable germline cancer predisposition variant and in 3 patients, the genomic findings changed the primary diagnosis. Fresh tissue collection permitted in vitro high-throughput drug screening (112 compound library single agent) in 31% of cases to date. Patient-derived xenograft model engraftment was successful in 55% of cases. Final in vitro drug screens, and in vivo drug efficacy studies testing actionable mutations or in vitro drug hits are ongoing. Overall, 57% of patients received a personalised medicine recommendation (therapy, change in diagnosis, germline mutation referral) and 36% of patients with a therapy recommendation have currently received that therapy.

A national, multicentre prospective study (PRISM) testing the implementation of the platform opened in September 2017 for Australian children with HR cancer. 27 patients have been registered from 6 paediatric oncology hospitals, with 20 fully enrolled on the study to date and 14 patients discussed in an MTB meeting (December 2017). Zero Childhood Cancer demonstrates the potential of the program to enhance clinical outcomes for HR childhood cancers.

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Therapeutic Effect of Novel Chelators DpC on Neuroblastoma

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Background: High risk neuroblastoma still has poor prognosis despite current therapeutic approaches. Chelator such as di-2-pyridylketone-4,4-dimethyl-3-thiosemicarbazone (Dp44mT) has been tried as novel anti-cancer agent but side-effects of methemoglobin formation and hypoxia hindered its clinical application. Synthesized di-2-pyridylketone-4-cyclohexyl-4-methyl-3-thiosemicarbazone (DpC) has less toxicity profile than Dp44mT. We investigated the therapeutic effectiveness and safety of DPC & DP44MT on neuroblastoma by in vitro and in vivo orthotopic models.

Methods: The cytotoxic effect of DpC/Dp44mT/L1 on a panel of neuroblastoma (SK-N-LP/SK-N-AS/Be(2)C/SH-SY5Y) and normal cell lines [cardiac (H9C2), hepatocyte (MIHA), kidney (HK2) and mesenchymal stem cell (MSC)] were assessed by flow cytometry and XTT test. Orthotopic SK-N-LP/Luciferase nude mice were used as in vivo model. Tissue expression of Caspase 3, TNFα and Hematoxylin-eosin (H-E) staining were used to assess the cytoxicity of DPC. Reactive oxygen species (ROS) and neuroglobin & cytoglobin expression post-DPC/DP44MT treatment were monitored in vitro & in vivo.

Results: DpC has more potent cytotoxic effect than Dp44mT or deferiprone (L1) for both neuroblastoma and normal cells in vitro. For in vivo study, after 2-weeks treatment, tumor significantly decreased in DpC group compared with control ($P<0.05$) but all mice (4/4) died within 2 days after DP44MT treatment. In DPC treatment group, tissues of heart, liver and lung did not show any significant changes. We found significantly higher Caspase 3 and TNFα expression in tumor tissue ($P<0.05$). The neuroglobin and cytoglobin expression increased while DCF (indicator of ROS) decreased in mouse tumor tissue ($P<0.05$).

Conclusion: The new generation of thiosemicarbazone, DpC has better cytotoxic profile than Dp44mT for neuroblastoma in vivo despite relatively higher in vitro toxicity in normal cells. Compensatory mechanisms such as increase expression of neuroglobin and cytoglobin may be involved in the protective mechanism for normal tissues in vivo.

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The PA2G4-MycN Protein-Protein Interface Is a Treatment Target in Neuroblastoma

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High level MycN protein expression is a key driver of neuroblastoma tumorigenesis. MycN is a member of the Myc oncoprotein family which are overexpressed or activated in the majority of human cancer. Myc proteins affect diverse cellular functions in disease through protein-protein interaction sites which are potential therapeutic targets in cancer cells. The development of Myc inhibitors has proven challenging, as Myc is a largely unstructured protein with no deep pockets for drug design. We have previously reported that the proliferation associated gene 2G4 (PA2G4) was a poor prognostic factor in primary human neuroblastoma tissues, which bound MycN in neuroblastoma cells and increased its protein stability. Knockdown of PA2G4 in neuroblastoma cells in vitro or in xenografts reduced the malignant phenotype.

Here we used molecular modelling, surface plasmon resonance (SPR), differential scanning fluorimetry and mutant studies, to map the MycN-PA2G4 interaction site to a thirteen amino acid sequence in MycN Box IIIb, and a surface crevice in PA2G4. Competitive chemical inhibition of the MycN-PA2G4 protein-protein interface with a small molecule known to bind PA2G4, WS6, reduced MycN and PA2G4 levels, and neuroblastoma tumorigenesis. SPR gave clear dose-response binding to immobilised PA2G4 with a KD of 24.8 ± 0.61 µM (n=5). SPR competition experiments showed repression of MycN oligopeptide dose-response binding to PA2G4 when 10 µM WS6 was pre-incubated with peptide. Co-IP showed that transiently transfected MycN point mutants, at the PA2G4-MycN binding site, disrupted PA2G4 binding. Overexpression of PA2G4 partially blocked the cytopathic effects of WS6. WS6 (IC50 0.65□M) had comparable single agent potency with the USP7 inhibitor, P22077, the FACT inhibitor, CBL0137, and the allosteric AurA inhibitor, CD532, but not the AurA kinase inhibitor, MLN8232 (IC50 0.043□M). Examination of the Cancer Genome Atlas (TCGA) revealed that MycN-driven human neuroendocrine prostate cancer (NEPC) and Myc-driven uterine carcinosarcoma both exhibited a high incidence of PA2G4 amplification, indicating PA2G4 may be also be a driver in these malignancies. Together, these data strongly support PA2G4 as an oncogenic cofactor in Myc- and MycN-driven cancer and for the first time a novel chemotype for the MycN oncogenic signal.

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CFC1 is a Cancer Stemness-Regulating Factor in Neuroblastoma

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Background: Despite the use of aggressive therapy, survival rates among high-risk neuroblastoma (NB) patients remain poor. Cancer stem cells (CSCs) are considered to be critically involved in the recurrence and metastasis of NB and are isolated as NB spheres.

Methods: The gene expression profiling of adherent (control) and sphere-forming primary NB cells was conducted using a gene expression microarray. CFC1, which functions in the development of embryos and decides the left-right axis, was strongly expressed in sphere-forming cells only and was related to the unfavorable prognosis of NB patients. The knockdown and overexpression of CFC1 were performed using a lentiviral system in NB cell lines. Sphere formation, cell proliferation, colony formation in soft agar, and xenograft tumor formation were analyzed. Gene expression microarray of CFC1 overexpressing cells was performed and the relation between CFC1 and Activin A was studied.

Results: The overexpression of CFC1 increased sphere formation, cell growth, and colony formation. These phenotypes, particularly sphere formation, and xenograft tumor formation were significantly suppressed by the knockdown of CFC1. The genes belonging Activin A signaling pathway were differentially expressed in CFC1-overexpressing cells. CFC1inhibited Activin A-induced NB cell differentiation and Smad2 phosphorylation in NB cell lines, and Activin receptor inhibitor SB431542 induced NB cell sphere formation similar to CFC1 overexpression, indicating its involvement in tumorigenesis related to EGF-CFC co-receptor family molecule pathways. Collectively, these results indicate that CFC1 is a candidate molecule for the development of CSC-targeted therapy for NB.

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The Roles of Endogenous Ligand for Aryl Hydrocarbon Receptor in Neural Development and Tumorigenesis of Neuroblastoma

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Neuroblastoma (NB) is a highly malignant pediatric cancer derived from the sympathoadrenal lineage of the neural crest during development. Our previous studies suggested that the expression level of aryl hydrocarbon receptor (AHR), a receptor for dioxin-like compounds, correlated well with histological stages of NB tumors. Positive expression of AHR also predicted a favorable prognosis of NB patients. In addition, overexpression or activation of AHR promotes neural differentiation of NB cells. These evidences suggested that AHR is an important regulator for the tumorigenesis of NB. However, the roles of AHR in the neural development and the mechanism by which AHR affects NB tumorigenesis remained elusive. In the current study, we aim to investigate the roles of AHR in neural development. By an ultrasensitive bioassay for dioxin-like compounds, a novel endogenous AHR ligand X was identified. We further confirmed that ligand X effectively promotes AHR translocation into nucleus and activates CYP1A1 expression. In addition, the expression levels of neural differentiation markers including NSE and GAP43, were upregulated by ligand X treatment in SK-N-DZ NB cells. In zebrafish model, ligand X treatment significantly promotes the expression of neural development associated proteins, and the induction effects were abolished by zAHR2 morpholino (MO) treatment. Moreover, ligand X treatment improves the mobility of zebrafish larvae in zAHR2 knock-downed zebrafish. These results suggest that ligand X is an endogenous ligand of AHR and plays critical roles during neuronal differentiation and oligodendrocyte development. Our results may contribute to the development of future treatment for NB patients.

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Synergy of Anti-Microtubule Plus Anti-Tropomyosin Agents in Neuroblastoma

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We have developed compounds which destabilise the actin cytoskeleton of neuroblastoma. The compounds target one of the major components of the actin cytoskeleton, the tropomyosin isoform Tpm3.1. We have previously shown that tropomyosins control the functional capabilities of actin filaments in an isoform dependent manner by determining which motors and binding proteins can interact with the actin filament. The isoform Tpm3.1 is enriched in neuroblastoma and also in a range of other paediatric and adult cancers. Anti-Tpm3.1 compounds show activity against neuroblastoma cells in culture where they cause the disassembly of the actin cytoskeleton and induce intrinsic apoptosis. We tested the ability of anti-Tpm3.1 compounds to synergise with drugs currently used to treat solid tumours in children. Only two classes of drugs consistently displayed synergy with anti-Tpm3.1 compounds, vinca alkaloids and taxanes. The extent of synergy varies between neuroblastoma lines and shows up to 20-fold synergy in the most sensitive lines. Synergy is also observed in vivo. Mice inoculated with CHLA20 cells were treated with each agent alone or in combination after tumours reached 400mm3. Only the combination resulted in reduction of the size of the tumour and in some cases, disappearance. The mechanism of synergy does not involve collapse of both the actin cytoskeleton and the microtubules. Rather, synergy results in failure of mitosis, similar to the mechanism of anti-microtubule agents. However, unlike anti-microtubule agents alone, the drug combination causes a defect in assembling the metaphase plate with accumulation of cells in prometaphase. The drug combination also causes a substantial increase in multipolar spindles which likely accounts for the ability of cells to assemble a symmetrical metaphase plate. We conclude that anti-tropomyosin compounds enhance the anti-mitotic activity of anti-microtubule drugs and may allow for dose reduction of these agents without compromising efficacy.

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A Preclinical Study of Epigenetic Drug-Based Differentiation Therapy for Neuroblastoma

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We previously showed that the CpG island methylator phenotype (CIMP) of neuroblastoma (NBL) was strongly associated with poor prognosis, and also suggested that CIMP may be a target for DNA demethylation therapy. At the same time, differentiation therapy with 13-cis-retinoic acid has been established as a standard maintenance therapy for high-risk NBLs in the USA. In this study, we conducted a preclinical study of an "epigenetic drug-based differentiation therapy" using a combination of a DNA demethylating agent (decitabine: DAC) and a differentiation agent (tamibarotene: TBT), a new synthetic retinoid developed in Japan. Treatment with DAC suppressed the growth of 12 NBL cell lines by increasing the number of cells in the S-phase. Genome-wide DNA methylation analysis revealed that DAC treatment induced global DNA hypomethylation, and that the genes related to cell death and neurological processes were enriched among the genes whose promoter CpG islands were hypomethylated. TBT induced neural extension and upregulation of differentiation markers, such as HOXD4, NGFR, and NTRK1, in five NBL cell lines. Pretreatment of the five cell lines with DAC enhanced the upregulation levels of differentiation markers, indicating that DAC enhanced TBT-induced differentiation. Finally, the tumor suppression effect of DAC and TBT in vivo was investigated using a mouse xenograft model of NB-1 cell line. Monotherapy with DAC or TBT could induce significant tumor regression without severe side-effects although their synergistic effect was not apparent. From these data, an epigenetic drug-based differentiation therapy is a promising therapeutic strategy for refractory NBLs. Now, an investigator-initiated phase I trial of TBT as a single agent has been completed, and a phase I/II trial of a combination of DAC and TBT is planned.

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P-Glycoprotein Limits the Effectiveness of Conventional Chemotherapies in High-Risk Neuroblastoma

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Despite improvements in therapy, high-risk neuroblastoma patients have a survival rate of less than 50% due in part to intrinsic or acquired resistance to chemotherapy. ABCB1/P-glycoprotein (P-gp), an efflux membrane transporter of the ATP binding cassette (ABC) family is a contributor to multidrug resistance (MDR) in several human cancers and can efflux a range of conventional and targeted agents, including doxorubicin, vincristine and the ALK inhibitor crizotinib [1]. However, in neuroblastoma, some controversy exists regarding the importance of P-gp in MDR [2,3]. While some studies have shown a correlation between high P-gp expression and chemotherapy failure [3], others have dismissed P-gp as a predictor of treatment outcome [2]. We investigated whether P-gp contributes to the resistance of neuroblastoma cells to standard-of-care conventional chemotherapies and to newer targeted agents, including alisertib, dasatinib and the ALK inhibitors ceritinib, crizotinib and alectinib.

Using RT-PCR and western blot for P-gp on a panel of neuroblastoma patient derived xenografts and cell lines, we demonstrated that high P-gp expression is common in high-risk neuroblastoma. Furthermore, analysis of P-gp mRNA levels in large patient cohorts indicates that high P-gp expression is associated with poorer outcome in high-risk disease. The selective P-gp inhibitor tariquidar sensitized high P-gp expressing neuroblastoma cells to the P-gp substrates vincristine and doxorubicin in vitro, indicating that P-gp levels were sufficient to confer chemoresistance. In contrast, no chemosensitization was observed when tariquidar was combined with the non-P-gp substrate cisplatin, or with irinotecan (SN-38), temozolomide, cyclophosphamide (mafosfamide), topotecan, etoposide, alisertib, or dasatinib. Surprisingly given recent reports (ALKi P-gp [1]), tariquidar did not sensitize ALK mutant or amplified cells to ALK inhibitors.

P-gp expression is known to correlate with resistance to a number of chemotherapies; however, the use of P-gp inhibitors has shown limited clinical success [4,5]. Our findings suggest that tumour P-gp levels might help indicate where particular agents may be less effective and where non-P-gp substrate chemotherapies might be substituted.

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Initial Results from the Zero Childhood Cancer Pilot Feasibility Study for High-Risk Neuroblastoma Patients

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The Zero Childhood Cancer program aims to assess the feasibility of a comprehensive precision medicine platform to identify targeted therapeutic agents for high-risk paediatric malignancies. The platform combines molecular profiling, including WGS of tumour and germline DNA, deep sequencing of a panel of cancer associated genes, and whole transcriptome (RNASeq) with sensitivity testing based on ex vivo high-throughput drug screening, and patient-derived xenograft (PDX) drug efficacy testing.

Five relapsed high-risk neuroblastoma patients were enrolled on the Pilot Feasibility Study. For each patient, the complete molecular profiling platform was applied, and for three of five patients, a potentially actionable molecular aberration was identified (NF1 mutation/loss, ALK amplification, 11q loss including ATM). PDX models were successfully generated for all five patients, with time to model establishment 1–6 months. Ex vivo drug sensitivity screening (>120 approved oncology drugs) was conducted for each patient, either directly on patient material or on PDX material. For one patient where molecular profiling did not identify a targetable aberration, ex vivo screening identified high sensitivity to the Bcl2 inhibitor venetoclax (IC_{50} 9 nM). Efficacy testing was conducted in the PDX models where a target could be identified. ALK amplification and ex vivo venetoclax sensitivity were validated by in vivo sensitivity of the PDX models to the ALK inhibitor ceritinib and venetoclax respectively. The PDX model with NF1 mutation/loss responded only minimally to the MEK inhibitor trametinib, or to trametinib in combination with isotretinoin. The PDX model with ATM loss is currently being assessed for sensitivity to the PARP inhibitor olaparib in combination with temozolomide and irinotecan.

Based on our Pilot Feasibility Study, we conclude that PDX models can be established for high-risk neuroblastoma patients at relapse with an excellent success rate, and that ex vivo drug sensitivity screening can identify therapeutic options in the absence of actionable aberrations identified by molecular profiling.

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Targeting ABCE1-Mediated Translation Selectively Blocks the Malignant Phenotypes of MYCN-Driven Neuroblastoma

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Amplification of MYCN confers poor prognosis in neuroblastoma. When dysregulated, transcription factors of the MYC family up-regulate protein synthesis to drive cancer progression. Since inhibiting protein synthesis is detrimental to the progression of c-MYC driven cancers, targeting this process may also offer therapeutic benefit for MYCN-driven neuroblastoma. ABCE1 is a MYC target gene and encodes an ATP-binding cassette protein that plays a key role in mRNA translation by powering the ATP-dependent dissociation of ribosomes into their small and large subunits. This process allows translation re-initiation and continued protein synthesis to provide essential building blocks for cancer growth and metastasis. Furthermore, high tumor ABCE1 expression is correlated with reduced survival of neuroblastoma patients. ABCE1 is thus a putative therapeutic target in MYCN-driven neuroblastoma. To investigate this possibility further, we conducted experiments to test whether targeting ABCE1-mediated translation can block neuroblastoma progression.

ABCE1 suppression by short interfering RNAs (siRNAs) severely impaired the proliferation of three MYCN-amplified neuroblastoma cell lines, and a patient derived xenograft (PDX) cultured ex vivo cell line ($P<0.001$). ABCE1 knockdown also inhibited the migration of these lines ($P<0.0001$). In contrast, ABCE1 knockdown did not affect these malignant characteristics in neuroblastoma or fibroblast cell lines lacking MYCN amplification. Polysome profiling showed that ABCE1 knockdown in MYCN-amplified SK-N-BE(2) neuroblastoma cells reduced the proportion of translating ribosomes ($P<0.001$) and slowed the rate of translation ($P<0.0001$). Similar decreases in translation were observed in all other MYCN-amplified cell lines tested, but not in neuroblastoma cell lines lacking MYCN amplification. Induction of MYCN overexpression in the SH-EP Tet21N neuroblastoma cell line substantially increased translation; however, ABCE1 knockdown completely abolished this increase, returning translation to basal levels. These data indicate that ABCE1 is required for the elevated translation driven by MYCN dysregulation. Notably, in mice xenografted with MYCN-amplified neuroblastoma cells, ABCE1 suppression delayed tumor growth at both subcutaneous and orthotopic (bone marrow) sites ($P<0.001$), prolonging the survival of tumor-bearing mice. Our study shows that targeting ABCE1-mediated translation is a promising approach to selectively impair the progression of MYCN-amplified neuroblastoma.

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Investigating Deregulation of Mitosis as A Mechanism of Tumourigenesis in MYCN-Driven Neuroblastoma

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MYCN amplification occurs in 20% of neuroblastoma patients and correlates with poor prognosis. The oncogenic function of MYCN has been demonstrated in a transgenic mouse model overexpressing human MYCN in the sympathetic ganglia and develops neuroblastoma at 6 – 7 weeks of age. Single cell technology has the potential to study the cellular heterogeneity in premalignant tissue to elucidate key tumourigenic components.

Single cell PCR of 150 cells derived from 2-week old TH-MYCN sympathetic ganglia identified a mitotic gene signature (MGS) that was over-expressed in a subpopulation of premalignant cells. This MGS was also found to be overexpressed in single cells derived from TH-MYCN tumours from 6-week old mice. Furthermore, prophylactic treatment using selective antimitotic agents significantly delayed the onset of tumour formation and prolonged survival in TH-MYCN mice. These data suggest that mitotic deregulation is a key feature of early tumour initiation that drives subsequent tumourigenesis.

In a large neuroblastoma patient cohort, we found that over-expression of the MGS correlated with MYCN amplification and poor prognosis among 649 neuroblastoma patients. MGS genes that were involved in different stages of mitosis (BUB1B, BUB1, KIFC1, ASPM) were subjected to siRNA mediated gene silencing in human neuroblastoma cell lines. This revealed an underlying requirement of these genes in maintaining cell viability and clonogenicity. To explore the therapeutic potential of targeting MGS members in the context of MYCN driven apoptosis, several targeted antimitotic agents were used in combination with the BCL2 inhibitor, ABT263. Synergy was observed in all four human neuroblastoma cells lines tested. MYCN-amplified cell lines were more sensitive to the combination treatment, which supported the hypothesis that targeting mitotic deregulation can reactivate apoptotic pathways in MYCN-amplified neuroblastoma.

In summary, we have identified mitotic deregulation as a feature of early MYCN-driven tumour initiation, and a potential therapeutic vulnerability in MYCN-amplified neuroblastoma.

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Network Modelling of microRNA-mRNA Interactions in Neuroblastoma Tumorigenesis Identifies miR-204 as A Direct Inhibitor of MYCN

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Neuroblastoma is a pediatric cancer of the sympathetic nervous system. MYCN amplification is a key indicator of poor prognosis for the disease, however, mechanisms by which MYCN promotes neuroblastoma tumorigenesis are not fully understood. In this study, we analyzed global miRNA and mRNA expression profiles of tissues at different stages of tumorigenesis from TH-MYCN transgenic mice, a model of MYCN-driven neuroblastoma. Based on a Bayesian learning network model in which we compared pre-tumor ganglia from TH-MYCN+/+ mice to age-matched wild-type controls, we devised a predicted miRNA-mRNA interaction network. Among the miRNA-mRNA interactions operating during human neuroblastoma tumorigenesis, we identified that miR-204 is a tumor suppressor miRNA that inhibits a subnetwork of oncogenes strongly associated with MYCN-amplified neuroblastoma and poor patient outcome. We found that MYCN was bound to the miR-204 promoter and repressed miR-204 transcription and similarly, miR-204 directly bound MYCN mRNA and repressed MYCN expression. In support of a tumor suppressor role, miR-204 overexpression significantly inhibited neuroblastoma cell proliferation in vitro and tumorigenesis in vivo. Together these findings identify novel tumorigenic miRNA gene networks and miR-204 as a tumor suppressor that regulates MYCN expression in neuroblastoma tumorigenesis.

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Modulation of Immune Responses and Radio Resistance by Neuroblastoma-Derived and Host-Derived TrkB-target Galectin-1 In Vitro and In Vivo

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The multifunctional protein Galectin-1 (Gal-1) has been described to promote tumor growth by inducing angiogenesis and to contribute to both, the tumor immune escape and resistance to radiotherapy. We had previously identified Gal-1 as a downstream target of the neurotrophin receptor TrkB in preclinical models of aggressive neuroblastoma (NB). However, the clinical and biological relevance of Gal-1 in this tumor entity remains unclear. X-Ray irradiation of different murine and human neuroblastoma cell lines revealed an upregulation of Gal-1 expression as deduced from Western Blot and semi-quantitative RT-PCR analysis. Knock-down of Gal-1 by shRNA sensitized murine NHO2A cells towards ionizing radiation (IR). However, colony formation assays and cell cycle analysis revealed no differences in long term survival or induction of apoptosis, respectively, for irradiated cells with or without Gal-1 expression. In vivo, targeted Gal-1 gene disruption (Gal-1-/-) in tumors of TH-MYCN mice correlated with impaired tumor angiogenesis, and impaired T cell infiltration, while tumor incidence was unaltered. However, Gal-1 deficient mice displayed splenomegalies consistent with the finding that migratory activity of CD4+ T cells requires Gal-1. These results are in line with a paracrine rather than an autocrine role of Gal-1 in modulating responses to radiotherapy. Interfering with Gal-1 functions in vivo will inform about the role of the TrkB/Gal-1 axis in response to radiation and contribute to a better understanding of the complex tumor-host interaction during chemo- and radiotherapy of neuroblastoma.

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Multi-Region Sequencing Dissects Intratumor Heterogeneity in Neuroblastoma

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Intratumor heterogeneity (ITH) has been described as a major cause for disease progression and treatment failure for several tumor entities. While proof-of-concept studies have demonstrated its presence in neuroblastoma, the extent of ITH has not yet been explored. To investigate ITH and to understand tumor evolution in neuroblastoma, we performed a multi-region whole-exome sequencing (WES) on 56 spatially separated tumor samples derived from 9 primary neuroblastomas (2 low-risk, 1 intermediate-risk, 6 high-risk) and 1 relapsed neuroblastoma treated in the NB2004 trial. Samples included 3 metastatic sites collected at the time of second-look resection of 3 high-risk primary tumors. We also assessed the impact of chemotherapy on clonal expansion by WES of 19 separate tumor regions from 1 intermediate-risk and 1 high-risk tumor with matched samples at diagnosis and after 4 cycles of chemotherapy according to the NB2004 trial protocol. To increase detection sensitivity for less abundant genetic aberrations, we macrodissected areas with high tumor cell purity (>70%) from cryosections. WES data were generated for tumor regions and matched germline control samples at a mean exome coverage of 300x. We identified a wide range of somatic single-nucleotide variations ranging from 20 to a few hundred per tumor region. We classified each mutation as ubiquitous (present in all tumor regions), partial (>1 region, but not ubiquitous) or specific (only 1 tumor region). A high rate of spatial heterogeneity was detected in these 10 patients, with 30 to 90% of mutations being classified as specific. Importantly, spatial genetic heterogeneity was also apparent for mutations of known cancer-related genes in high-risk tumors, whereas such mutations were absent from all samples derived from patients in complete remission. Our multi-region deep sequencing approach also defined the clonal composition of 1 high-risk and 1 intermediate-risk tumor before and after chemotherapy, dissecting out potentially relevant driver mutations and copy-number aberrations. Our data provide the first evidence for extensive spatial ITH in primary neuroblastomas and clonal selection under chemotherapy, both of which could have important implications for the clinical interpretation of molecular diagnostic results and the use of experimental drugs in second-line treatment approaches.

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Combined Inhibition of Histone Deacetylases and The Histone Chaperone FACT Leads to Complete Neuroblastoma Regression in Th-MYCN Transgenic Mice

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CBL0137, the lead curaxin compound, is a safe, non-DNA damaging small-molecule inhibitor currently in phase 1 clinical trials for advanced adult cancer. It acts as a chromatin destabilizer by interfering with the binding of the histone chaperone protein FACT to DNA. In neuroblastoma, high risk and aggressive tumors are frequently characterized by elevated MYCN expression, which we have previously shown to be maintained by FACT in a feed-forward manner (Carter et al, Science Translational Medicine 7(312): 312ra176). Using the highly penetrant Th-MYCN transgenic mouse model, where neuroblastomas develop spontaneously by 7 weeks of age, we have found that CBL0137, alone or combined with conventional chemotherapy, suppressed MYCN expression and significantly extended survival rates. To explore targeted therapeutic agents that potentiate CBL0137 efficacy, we tested a range of compounds in vitro and found that the FDA-approved histone deacetylase (HDAC) inhibitor panobinostat worked synergistically with CBL0137 to reduce cell viability and tumor clonogenicity of human MYCN amplified neuroblastoma cell lines. More strikingly, combination treatment of CBL0137 and panobinostat eradicated tumors in tumor-bearing Th-MYCN mice, leading to complete regression in the long-term. Mechanistic studies have shown that panobinostat markedly enhances chromatin destabilization induced by CBL0137, resulting in increased loss of histone subunit H1 from chromatin. Moreover, CBL0137 suppresses repair of DNA damage induced by panobinostat, thereby activating apoptosis. We have also demonstrated the in vivo synergy between CBL0137 and panobinostat in preclinical models of two other pediatric tumor types with dismal outcome, namely, patient-derived xenografts of diffuse intrinsic pontine glioma (DIPG) and mixed lineage leukemia (MLL)-rearranged leukemia. Our studies have identified a highly effective drug combination for neuroblastoma and other poor-prognosis pediatric malignancies and have provided insights into the mechanisms of interaction of CBL0137 and panobinostat, which will greatly facilitate clinical development of effective and non-toxic therapies for childhood cancer.

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Genetic Alterations of ALK In High-Risk Neuroblastoma Patients; A SIOPEN Study

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Background: In neuroblastoma (NB), the ALK receptor tyrosine kinase can be constitutively activated either through genomic amplification or activating point mutations. We studied ALK genetic alterations in high-risk NB patients to determine their frequency and prognostic impact.

Methods: Diagnostic NB samples from 1039 patients enrolled in the SIOPEN-HR-NBL1 trial were studied to determine the ALK amplification status (copy number analysis; n=337), the ALK mutational profile (Sanger and/or NGS including deep sequencing covering hotspots in exons 23-25, n=203) or both (n=499).

Results: High level genomic ALK amplifications were detected in 4.4% of cases (37/836); all but 2 showed MYCN amplification. As for MYCN amplification, ALK amplification was more frequently observed in children aged<18 months at diagnosis (p=0.01). No correlation with the primary tumor site was observed. ALK mutations were detected at a clonal level (>10% mutated allele fraction, MAF) in 9.8% of cases (69/702) (F1174 n= 25, R1275 n=32, both F1174 and R1275 n=1, F1245 n=6, others n=5). Additionally, 3.7% of patients (22/586 by NGS) harbored ALK mutations at a subclonal level (MAF 0.5-10%) (F1174 n=11, R1275 n=6, both F1174 and R1275 or F1174 and F1245 n=3, other n=2). Although not statistically significant, ALK mutations were observed slightly more frequently in non-adrenal compared to adrenal primary tumors (p=0.08). Whereas no statistically significant difference in survival was observed between patients with and without ALK mutations, patients with ALK amplification had a significantly poorer event free (EFS) and overall survival compared to those without ALK amplification (logrank, p<0.0001). A multivariate analysis was performed to determine which parameters independently predicted EFSin this high-risk population. Among 450 patients with complete datasets, a Cox proportional hazards procedure retained stage 4 disease (as opposed to non-stage 4) and ALK amplification as factors with a higher hazard of relapse/progression (hazard 2.3 and 2.2, respectively), whereas ALK mutation, MYCN amplification and age>18 months were not retained.

Conclusion: Taking into account amplifications, clonal and subclonal mutations, genetic alterations of ALK were observed in 17% of tumor samples in high-risk NB patients, of importance when considering ALK targeted therapies. Among the different genetic alterations, only ALK amplification predicted poorer survival.

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Combined Loss of 1p36 Gene KIF1Bbeta and NF1 Causes Neuroblastoma, Pheochromocytoma and Composite Tumors in Mice

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Hemizygous loss of the distal part of human chromosome 1 (1p36) is strongly correlated to poor prognosis in neuroblastoma. We recently identified pathogenic mutations in the 1p36 gene KIF1Bβ that are impaired in developmental apoptosis and sympathoblast differentiation in developing neuroblasts. We deleted KIF1Bβ in the mouse sympatho-adrenal lineage and observed that KIF1Bβ is required for neuron maturation and function in the sympathetic nervous system. We discovered that KIF1Bβ is required for nerve growth factor (NGF)-dependent neuronal differentiation through anterograde transport of the NGF receptor TRKA. Moreover, pathogenic KIF1Bβ mutations identified in neuroblastoma impair TRKA transport. Clustering non-MYCN-amplified tumors according to expression of genes down-regulated in KIF1Bβ-deficient sympathetic ganglia was prognostically relevant in regard to risk classification, disease stage, and survival. Our analysis of neuroblastoma tumors indicates that specific loss of KIF1Bβ contributes to less differentiated and more aggressive disease independent of MYCN amplification and the loss of genes neighboring KIF1B on 1p36.

Importantly, loss of KIF1Bβ in combination with loss of NF1 develop malignant neuroblastoma (NB) and pheochromcytoma (PCC), demonstrating that KIF1Bβ is a bona fide 1p36 tumor suppressor gene. Interestingly, two tumors were diagnosed as composite tumors, consisting of both PCC and NB components, provoking the question if NB and PCC might have a common progenitor. Tumors are now analysed by single cell transcriptomics to validate the cells of origin and mechanisms of tumor formation initiation. We hope to identify gene expression clusters that are prominent in specific embryonic sympatho-adrenal developmental stages.

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Clinical Significance of MYCN Amplification in Patients with High-Risk Neuroblastoma

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Purpose: We investigated the clinical significance of MYCN amplification within high-risk neuroblastoma (NB).

Materials and Methods: Medical records of 135 patients who were diagnosed with high-risk NB from 2004 to 2016 were reviewed.

Results: Fifty-one (37.8%) patients had MYCN amplified tumors and the remaining 84 (62.2%) had MYCN non-amplified tumors. MYCN amplification was associated with abdominal primary site, less differentiated pathology, higher level of LDH and NSE, lower VMA level, and larger primary tumor volume at diagnosis. MYCN amplification was associated with a better early response (faster reduction of primary tumor volume and NSE level after three cycles of chemotherapy). The proportion of patients in CR or VGPR after induction treatment was relatively higher in MYCN amplified tumors than in non-amplified tumors; however, all progressions during induction treatment occurred only in MYCN amplified tumors (p=0.006). There was no difference in the frequency of treatment-related mortality or second malignancy according to the MYCN amplification. The time to relapse/progression was shorter (1.5 years vs. 1.9 years, p=0.037) and OS after relapse/progression was worse in MYCN amplified tumors (3-year OS: 7.7 ± 7.4% vs. 20.5 ± 8.8%, p=0.046). As results, there was no difference in EFS and OS between MYCN amplified and non-amplified tumors.

Conclusion: MYCN amplification was associated with more aggressive clinical features at diagnosis, a better early response, but a higher progression rate during induction treatment and a worse survival after relapse/progression. As results, there is no difference in survival rates according to MYCN amplification in patients with high-risk NB.

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Characterization of Neuroblastoma Vulnerability to CDK13 Knockdown

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Background: High-risk neuroblastoma patients with amplified MYCN frequently suffer from relapses after chemotherapy and acquire resistances to the applied drugs. In cell culture, a subset of MYCN-driven cells is able to overcome cell cycle arrest induced by doxorubicin. At the time of treatment, these cells are newly born and reside in M or early G_1 phase. New treatment options are urgently needed that target these resister cells. This study characterizes the effect of CDK13 knockdown, a top hit of a MYCN synthetic lethal screen in neuroblastoma cells.

Methods: The expression level of CDK13 in the course of the cell cycle was examined in synchronized IMR5/75 cells. CDK13 expression was knocked down by siRNA in 8 neuroblastoma cell lines and cell viability was analyzed. For further characterization, we generated a stable IMR5/75 cell line with CRISPR interference-based inducible knockdown. Cell viability and colony formation capacity were evaluated by Giemsa staining, cell death was detected by propidium iodide (PI) staining and cell cycle was analyzed by flow cytometry.

Results: The expression of CDK13 peaked in mitosis and $G_o/_1$ phase. We identified 4 out of 8 cell lines as strong and another 2 as intermediate responders to knockdown of CDK13. Knockdown with CRISPRi reduced the protein level by 50-70%, which led to a reduced viability of the cells (40-60%). The ability to form clones was almost completely abolished upon knockdown. 90% of the cells stained PI-positive, indicating high cell death. The cell cycle distribution was shifted towards G_1 phase (12% increase).

Conclusion: CDK13 inhibition may represent a promising new treatment option in MYCN-driven neuroblastoma. In combination therapies, it might allow to target resister cells which escape chemotherapy.

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Validation of the Paediatric Solid Tumour Clinical Next Generation Sequencing (NGS) Panel for the Detection of TERT Rearrangements in Neuroblastoma

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Background: Whole genome sequencing studies have recently identified a group of neuroblastoma patients with genomic rearrangements proximal to the telomerase reverse transcriptase (TERT) gene, associated with increased TERT expression and poor outcome. Furthermore, TERT rearrangements are mutually exclusive to other poor outcome groups defined by MYCN amplification and ATRX mutations. It is essential that methodologies allowing routine detection of TERT rearrangements are integrated into upcoming clinical trials in order to better characterise these distinct groups within clinical trial datasets.

Methods: We have been offering NGS panel sequencing with clinical reporting to children with solid tumours since March 2016. In 2017 the panel was updated to include the intronic region 50 kb upstream to the TERT promoter in order to capture TERT rearrangements. We have sequenced the TERT rearranged CLGBA cell line with the updated panel and analysed results using bioinformatics tools. For further validation we are currently sequencing retrospective samples with TERT status determined by Break-Apart FISH and samples from patients treated on the SIOPEN-High Risk 1 study. Results from the SIOPEN samples will be further cross-validated with results from a SIOPEN biology group retrospective NGS panel.

Results: We have successfully NGS panel sequenced and clinically reported 140 samples from children with solid tumours in the UK, of which 20 had a diagnosis of neuroblastoma. MYCN amplification was detected in 20% of cases, ALK mutations in 15% and ATRX mutations in 5%. Sequencing of the CLBGA cell line confirmed the presence of a 5:21 chimera with a break in the UTR of TERT. We are currently validating the ability to detect TERT rearrangements in patient samples.

Conclusions and Discussion: Incorporating prospective molecular data collection into upcoming clinical trial datasets will enable a greater understanding of genotype-phenotype correlation, leading to more accurate risk stratification and better, more tailored therapies in the future. We demonstrate that clinical NGS panel sequencing is feasible, can detect complex rearrangements in addition to other genetic alterations and can be tailored to the needs of the study population.

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Genetic Predisposition Responsible for Variable Treatment Outcome in Patients with Neuroblastoma

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Purpose: We performed this study to discover genetic polymorphism responsible for variable treatment outcome among patients with neuroblastoma (NB).

Methods: Firstly, we investigated whether the degree of neutropenia, absolute neutrophil count (ANC) at nadir, after first cycle of chemotherapy is associated with the treatment outcome in 264 NB patients who received same induction chemotherapy. And then, we performed genome-wide association study (GWAS) to discover genetic polymorphism responsible for the variable degree of neutropenia and variable treatment outcome among patients.

Results: PFS was higher in patients with severe neutropenia (ANC < 20/μL) than in those with moderate (ANC 20-100/μL) or mild (ANC > 100/μL) neutropenia (P = 0.005). Interestingly, treatment-related mortality (TRM) was more frequent in patients with severe neutropenia than in those with moderate or mild neutropenia (P = 0.015). Among the most significant 33 common SNPs associated with the degree of neutropenia in GWAS, AA genotype at rs2818421 of DPYSL4 was associated with a higher PFS (P = 0.042) and AA genotype at rs11786984 of CSMD1 was associated with a higher frequency of non-progression event including TRM and second malignancy (P = 0.008). When the patients were stratified into low and high genetic-risk groups according to the prediction score incorporating the most significant 33 common SNPs, there was significant difference in survival rates between genetic risk groups (10-year PFS: 89.6 ± 3.0% vs. 67.9 ± 4.8%, P < 0.001; 10-year EFS: 82.1 ± 4.2% vs. 52.8 ± 5.5%, P < 0.001). In the multivariate analysis, high genetic risk was an independent poor prognostic factor for survival (HR for PFS = 3.03, P = 0.002; HR for EFS 3.07, P < 0.001). When the analysis was confined to only clinical high-risk patients, findings described above became more prominent.

Conclusion: The degree of neutropenia after chemotherapy can be used as a surrogate marker of patient's genetic characteristics predicting the treatment outcome. Our study suggests that treatment outcome is affected by genetic factor of patients and, therefore, future treatment needs to be tailored based on not only clinical or somatic factors but also genetic characteristics of the patients.

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The Core Regulatory Circuit Component TBX2 Is Implicated in Cell Cycle Control and Proliferation in Neuroblastoma

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Introduction: Neuroblastoma (NB) is a mutational silent childhood tumor but exhibits frequent recurrent DNA copy number alterations including 17q gain in most high risk MYCN amplified and non-amplified cases. The finding of gains for this syntenic human 17q region in MYCN driven NB mouse tumors further suggests a crucial role for dosage sensitive genes during tumor initiation and/or progression. We hypothesized that one or more dosage sensitive super-enhancer marked lineage transcription factors are present on 17q.

Material and methods: We performed a combined super-enhancers (SE) analysis in 26 NB cell lines, followed by selection of super-enhancer driven genes for impact on patient prognosis and dosage sensitivity, based on extensive copy number and gene expression analysis in a large primary tumor cohort. The selected top candidate TBX2 was further investigated by genomic and functional analysis.

Results: We identified TBX2 as the strongest 17q dosage sensitive SE marked transcription factor. In further support, in 1 NB patient we detected a 1 Mb focal amplification encompassing the TBX2 locus. 4C sequencing demonstrated physical interaction of the TBX2 locus with its nearby SE. TBX2 ChIP-sequencing and further integration of available CRC gene ChIP binding profiles strongly suggest TBX2 being part of the CRC consisting of, amongst others, GATA3, HAND2, PHOX2B and MYCN. Transcriptome analysis following TBX2 knockdown revealed enrichment for gene sets involved in cell cycle such as G2/M checkpoint and FOXM1/E2F targets. Phenotypically, TBX2 knockdown caused reduced proliferation and induction of G1 cell cycle arrest. Interestingly, combined TBX2 and MYCN knockdown in NB cells yielded synergistic effect on viability and cell cycle. Given that CRC genes driven by SEs are highly dependent upon strong sustained high expression levels, we combined the CDK7 inhibitor THZ1 with the bromo-domain inhibitor JQ1 and observed synergistic effects on TBX2 downregulation and cell viability and apoptosis.

Conclusion: We propose the SE driven 17q dosage sensitive TBX2 transcription factor as an important component of the NB CRC and provide evidence suggesting that targeting one or more CRC components represents a therapeutic vulnerability for epigenetic drugging of high risk NB.

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A Molecularly Driven Pharmacological Approach for Neuroblastoma: Targeting TERT and CKS1b

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Neuroblastoma is the most common extracrainal solid tumour in childhood. The high risk, metastatic form of neuroblastoma has very poor prognosis, and the survival rate after 5 years is of only about 50% in the face of aggressive treatments. The protooncogene and transcription factor MYCN is a direct cause of the disease when activated by amplification in a fraction (30%) of high-risk neuroblastomas. Using a genome-wide shRNA screen, we have recently unveiled a network of druggable MYCN synthetic lethal genes. One of the synthetic lethal interactors identified in the screen is CKS1b, inhibited by the small molecule fluoxetine, also known as Prozac. Fluoxetine is an antipsychotic drug and due to its relatively low toxicity profile is also used in the context of childhood behavioural control. Another important genomic rearrangement present in about a third of high risk neuroblastomas occurs in the chromosomal 5p15.33 region, proximal to the telomerase reverse transcriptase gene (TERT). Notably, an oligonucleotide inhibitor of TERT activity developed by the biotech company Janssen, Imetelstat, has been recently used in multiple clinical trials for human solid and haematological malignancies.

In vitro, both Fluoxetine and Imetelstat reduced neuroblastoma cell proliferation and caused apotosis of MYCN-amplified neuroblastoma cell lines. The effect was much more pronounced when the drugs were used in combination. In vivo, the combination of Fluoxetine and Imetelstat significantly reduced the growth of small but not large tumour masses. Interestingly, Fluoxetine alone showed antitumour activity if treatments were started shortly after subcutaneous tumour cells inoculations.

In conclusion, Fluoxetine and Imetelstat could represent potential candidates for second line therapy in relapsing neuroblastoma.

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SOX11 Is a Transcriptional Circuit Dosage Sensitive Transcription Factor Controlling SWI/SNF Components

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Introduction: Core regulatory circuits (CRC) compose a complex network of transcription factors (TF) implicated in the NB phenotype. CRC component MYCN is commonly amplified in high risk cases whereas LIN28B was shown to be affected by rare copy number gains and amplifications. We report on SOX11 as a novel copy number affected TF in NB.

Methodology: DNA copy number analysis was performed in 384 NB. In vitro functional assays were performed following SOX11 knockdown in NB cells. ChIP-sequencing and RNAseq were performed to identify direct SOX11 targets. IP-MS was performed to identify SOX11 interacting proteins.

Results: A 400kb region of overlapping gains/amplification was identified on chromosome 2p containing SOX11 as only protein-coding gene. This TF has lineage dependency characteristics including high expression in adrenergic NB tumors and cell lines and developing sympathetic nervous system. SOX11 also shows dynamic upregulation in TH-MYCN driven mouse hyperplasia and NB as well as in NB tumors derived from MYCN transformed mouse neural crest cells. Knockdown of SOX11 reduced colony formation, increased DNA damage and G1-S cell cycle arrest. Further, MYCN and several other CRC components bind to a SOX11 upstream enhancer as determined by 4C-sequencing. ChIP-seq revealed significant overlap of SOX11 binding sites with binding sites for MYCN and other adrenergic CRC components. SOX11 regulates multiple SWI/SNF components including ARID1A, ARID1B, SMARCE1 and SMARCC1, suggesting a critical role in chromatin remodeling, in keeping with its involvement in Coffin-Siris syndrome caused by mutations in SWI/SNF complex components including SMARCE1 and SOX11. Given the recent discovery that ARID1A targets SWI/SNF complexes to enhancers, SOX11 may facilitate CRC controlled gene activation. SOX11 also regulates other epigenetic regulatory components including EZH2, genes involved in DNA repair, DNA replication and cell cycle. Analysis of the SOX11 interactome provided evidence for MYCN as well as cell cycle control proteins WHDH1 and WEE1 as physical interactors.

Conclusion: Our data suggest that SOX11 is a CRC interconnected dosage sensitive lineage dependency TF with a putative role in regulating essential epigenetic components of the major SWI/SNF and PRC2 epigenetic regulatory protein complexes.

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The Clinical Significance of the Expression of B7H3 and PD-L1 in Neuroblastoma

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Background: We investigated the clinical significance of the expression level of B7H3 and PD-L1 in neuroblastoma.

Methods: Clinical data and tumor paraffin tissues of NB were collected from Sun Yat-Sen University Cancer Center between January 2004 to December 2015. The expression of B7H3 and PD-L1 were detected by immunohistochemical technology. The relationship between the B7H3 and PD-L1 expression and the clinical prognosis of NB patients was analyzed.

Results: A total of 100 patients with NB, of which, the low risk, intermediate risk and high-risk NB were 15%,18% and 67% respectively. 79% of the patients were H7H3 positive, 58% were low expression and 45% were high expression. The B7H3 high expression was positively related with risk stratifications, stage, MYCN status. 4-year EFS of patients with low and high expression of B7H3 was 41.8% and 27.1%, respectively, P=0.051; 4-year OS was 58.6% and 63.8%, respectively, P=0.739. For the 33 non-high-risk patients, 4-year EFS of patients with low and high expression of B7H3 was 94.7% and 44.2%, respectively, P=0.003; 4-year OS was 100.0% and 90.9%, respectively, P=0.157. For the 67 high-risk patients, 4-year EFS of patients with low and high expression of B7H3 was 22.1% and 13.9%, respectively, P=0.805; 4-year OS was 57.7% and 36.3%, respectively, P=0.151. The positive rate of PD-L1 in NB tumor tissue was 57.0%, 98% of patients were low expression. The expression of PD-L1 in NB was not significantly correlated with tumor sizes, age, gender, stage, MYCN status, risk stratifications and survival. 45 cases had B7H3 and PD-L1 double positive and 10 patients had PD-L1 and B7H3 double negative. 4-year EFS of the patients with PD-L1 and B7H3 double positive and double negative was 16.4% and 90.0%, respectively, P<0.001. 4-year OS was 48.7% and 100.0% for the patients with PD-L1 and B7H3 double positive and double negative, respectively, P=0.001. Multivariate analysis showed that B7H3 positive, MYCN amplification were independent adverse prognostic factors for OS. B7H3 positive and advanced stage were independent prognostic factors for EFS (P <0.05).

Conclusions: This study showed B7H3 may be used as a new prognostic indicator in NB in the future.

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Outcome of Treatment Using antiGD2 in Maintenance Phase for Children with High Risk Neuroblastoma at the National University Hospital Singapore

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Introduction: The outcome of treatment for children with high risk neuroblastoma with standard treatment has been dismal. The addition of immunotherapy targeting the GD2 antigen present on the neuroblastoma cells has improved event free survival rate to 66% and overall survival at 86% (Yu A et al, NEJM, 2010). We recently treated patients with Dinutixumab beta and report their outcome.

Methods: Children with INRG high risk neuroblastoma received induction chemotherapy following the MSKCC or Siopen HR neuroblastoma protocol, surgery, myeloablative chemotherapy using BUMEL regimen, followed by autologous peripheral blood stem cell rescue and radiation. Maintenance began within 100 days from transplant provided the absolute neutrophil counts are at least 1.0 and platelet counts were stable without transfusion. Maintenance consisted of five cycles of Dinutuximab beta at 10 mg /m2/day for 10 days given without IL2, followed by oral cis retinoic acid at 160 mg/m2/day for 14 days.

Results: Ten children with high risk neuroblastoma received immunotherapy. Their ages range from 3 to 10 years (median 3 years). Nine children presented with metastatic disease and one L2 with Nmyc amplification. Two patients with Nmyc amplification. One patient relapsed at the metastatic site two years posttransplant and was in second remission. Nine patients were in complete remission and one partial remission posttransplant. Six patients completed five cycles. Reasons for not completing were persistent disease (1) and new onset lesions (3). All patients developed CRS grade 2 with fever, chills and pain during the first two cycles. One patient with high grade fevers and maculopapular rash had Influenzae B infection. Six patients are alive and in complete remission 21-34 months posttransplant. Three patients relapsed during immunotherapy, two deceased and one alive with disease at 9 months follow up. One patient relapsed in the bone marrow 2 months posttreatment but went into remission with chemotherapy.

Conclusion: Dinutuximab beta given over 10 days without IL2 is well tolerated. However, relapse rate remains significant in our group of patients. Further review into biology of lesions is needed to determine which patients will benefit from additional treatment aside from immunotherapy.

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Pan-Genomic Analysis of Neuroblastoma by Array Comparative Genomic Hybridization and Correlation with Pathology

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Background: The aim of the prospective biological study IC2007-09/PHRCNB07 (ClinicalTrials.org, NCT02864563) is to obtain genomic profiles (GP) using aCGH and histopathologic characterization of tumor samples obtained at diagnosis of all patients with neuroblastic tumors treated in the centers of the SFCE (Société Française des Cancers de l'Enfant).

Methods: For patients enrolled in the study, central histology review of diagnostic samples (obtained by surgical resection, surgical biopsy or fine-needle biopsy) was performed according to the International Neuroblastoma Pathology Committee (INPC). Neuroblastic tumor samples with >60% tumor cells were analyzed by aCGH in three reference platforms. Genomic profiles (GP) were classified into three groups: numerical-chromosome-alterations (NCA) only, segmental-chromosome-alterations (SCA) without or with additional numerical alterations, and MYCN amplification (MNA).

Results: Among 512 enrolled patients (INRG stages L1+L2/M/MS 28%, 71%, 1% respectively, age: >18 months 63%), GP was analyzed for 365 cases: 103 NCA, 172 SCA, 61 MNA and 29 non-contributive profiles. SCA were more frequent (74%, p<0.0001) in patients >18 months and in INRG stage M (78%, p<0.0001). Histopathological review was obtained for 358 cases (INPC favorable n=105, unfavorable n=103, non-evaluable n=150), with GP data available in 281 cases. Among ganglioneuroblastoma (4 GNB intermixed, 6 GNB nodular), GP revealed: 5 silent, 2 NCA, 3 SCA, 0 MNA profiles. In neuroblastoma, different GP were observed according to degree of differentiation: differentiating NB (3 silent, 0 NCA, 6 SCA, and 0 MNA), poorly differentiated (9 silent, 70 NCA, 99 SCA, 30 MNA) and undifferentiated NB (0 silent, 0 NCA, 1 SCA, 5 MNA). A strong correlation between INPC histoprognosis classification and GP was documented (INPC favorable: 7 silent, 43 NCA, 26 SCA, 1 MNA; unfavorable: 6 silent, 13 NCA, 49 SCA, 16 MNA; p<0.0001). In addition, among 150 samples for whom no INPC histoprognosis could be established due to sample size or technical issues, 120 GP could be analyzed: 11 silent, 27 NCA, 52 SCA, and 30 MNA.

Conclusion: Fine-needle biopsies allowed histoprognosis characterization according to INPC in 58%. In absence of a reliable or possible histoprognosis classification, GP was informative with a success rate of 91% (109/120).

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Identification of Novel Fusion Transcripts Occurring at High Frequency in High-Risk Neuroblastoma

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The paucity of recurrent mutations has hampered efforts to understand the pathogenesis of several pediatric cancers. High-risk neuroblastoma is characterized by MYCN amplification and large structural chromosomal rearrangements but few recurrent amino acid changing mutations. Through bioinformatic analysis of sequenced neuroblastoma tumors, we have identified primarily read-through fusion transcripts of adjacent genes. The high frequency of these intrachromosomal fusion events implies that they represent an overlooked mechanism that could provide high-risk neuroblastoma with oncogenic properties. We validated several candidate fusion transcripts as tumor specific. To investigate altered functional properties of fusion gene products we focused on the ZNF451-BAG2 chimeric transcript. This fusion generated a truncated BAG2 protein, ΔBAG2, which inhibits retinoic acid-induced neuronal differentiation in neuroblastoma. Thus, ΔBAG2 acts as a dominant negative factor obstructing key aspects of neuronal maturation, potentially leading to a less differentiated and more aggressive tumor.

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Heterogeneity of Neuroblastoma Cells in Bone Marrow of Children with High-Risk Disease: An NCRI CCL CSG Neuroblastoma Group Study

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Purpose: Neuroblastoma (NB) cells in the bone marrow are a hallmark of high-risk disease in children over the age of 1 year, identifying children for more intensive treatment and a frequent site of relapse. The elimination of these cells remains one of the greatest challenges for cure of some children.

Methods: NB cells were isolated from bone marrow aspirates (BM) from children with stage M high-risk NB using immune-magnetic bead selection for the cell surface disialoganglioside GD2. Self-renewal was assessed by seeding single cells in low and substrate adherent plates, and in soft agar. Migration was analysed using a 3D gelatin-based assay. Cells were characterised using total RNAseq, immunocytology and microscopy. Paired primary tumour, neuroblastoma cells from BM and NB self-renewing clones were compared (n=7).

Results : Median infiltration of BM with GD2 positive NB cells at diagnosis was 7% (range 0 – 60%; n=100). Expression of the neuroblastoma markers tyrosine hydroxylase, nestin, NCAM and PHOX2B and the drug resistance protein MRP-1 was heterogeneous. NB cells did not express the haematopoietic markers CD45 or CD57. High infiltration of BM with NB cells was predictive of a worse event free survival (p=0.017), as was cellular expression of PHOX2B protein (p=0.023). Migratory cells were identified in all NB cultures; the migration index (MI) was highly heterogeneous (median MI 106; range 8-428 n=71). Median spheroid forming efficiency from a single cell was 5% (range 0.5-27%; n=114) and colony formation efficiency in soft agar 1.3% (range 0.07-9% n=70). Substrate adherent single cell clones were established from 74% of propagated primary cells; in these cells median clone forming efficiency was 12% (range 0.5-40, n=45). Hierarchical clustering of RNAseq data revealed that the primary tumours and neuroblastoma cells isolated from the BM clustered independently from the NB single cell self-renewing clones.

Conclusion: NB cells from the BM of children with stage M high-risk disease are heterogeneous. A subpopulation of cells produce single cell clones which have a different RNA profile to parental NB cells derived from BM and the paired primary tumour. Therapeutic strategies targeting this biological diversity are needed to improve outcomes.

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Very Good Partial Response to Ceritinib Treatment in a Patient with Relapsed ALK-Mutated Neuroblastoma, Hepatopathy and Prolonged QTc Interval

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Treatment of relapsed neuroblastoma has remained a challenge to pediatric oncologists and may be hampered by long-term sequelae of first-line therapy. Small molecule ALK inhibitors represent a promising therapeutic option in patients with ALK-mutated neuroblastoma; however, currently available inhibitors bear the risk of hepatotoxicity and QT interval prolongation. Here, we report on a girl who was diagnosed at the age of 22 months with stage 4 abdominal neuroblastoma and metastases in the bone marrow, lung and liver. Genomic profiling revealed wild-type ALK at diagnosis. In addition, a prolonged QTc interval was noticed with a maximum of 480 ms, suspicious of long-QT syndrome. First-line therapy was performed according to the NB2004 high-risk trial protocol. After myeloablative chemotherapy and autologous stem cell rescue, the girl developed temporary renal failure, respiratory failure, invasive pulmonary aspergillosis and secondary sclerosing cholangitis with consecutive ascites, partial liver failure and cholestasis (grade 4 increase of bilirubin and GGT, and grade 3 increase of AP and transaminases). Liver function and laboratory findings improved over the following months but did not normalize. Twenty-one months after stem cell rescue, local progression of a pulmonary lesion was observed. Neuroblastoma was confirmed by biopsy, and a de novo ALKR1275Q mutation was detected in the tumor cells. The patient was then treated with temozolomide accompanied by local radiotherapy, however, metastatic progression with bone marrow infiltration and increasing urinary catecholamines occurred after six months. We therefore switched to oral ceritinib mono-therapy. At this time, liver impairment had improved to grade 1 to 2. The therapy was tolerated very well: We recorded a maximum of grade 2 liver toxicity, no change of QTc interval, and no other relevant adverse events. Furthermore, we observed a good response to ceritinib treatment; the pulmonary tumor lesion decreased in size, urinary catecholamines normalized and bone marrow metastases disappeared completely. The patient was on treatment for 10 months at last follow-up and was in excellent clinical condition throughout the entire period. In conclusion, our data suggest that ceritinib treatment may represent a valuable treatment option in patients with ALK-mutated neuroblastoma, even in the presence of hepatopathy and prolonged QTc interval.

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Genomic Profiling in Low and Intermediate Risk Neuroblastoma to Refine Treatment Stratification and Improve Patient Outcome; LINES: A SIOPEN Trial

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Background: Neuroblastomas (NB) with segmental chromosome alterations genomic profiling are associated with poorer outcome. For low risk (LR) patients, the SIOPEN LINES trial (Low and Intermediate Neuroblastoma European Study, ClinicalTrials.org:NCT01728155) aims to maintain or improve the excellent outcome, whilst diminishing overall treatment burden whenever possible, by applying treatment stratification according to genomic profiling (GP) and clinical parameters. In intermediate risk (IR) patients, GP is studied retrospectively.

Methods: MYCN non-amplified NB samples with >60% tumor cells are analyzed using pangenomic techniques, with central review, data registration into the SIOPEN-R-NET database, and release of clinically relevant conclusions to clinicians within six weeks following diagnosis. Tumors were classified into two groups: numerical-chromosome-alterations (NCA) only, versus segmental-chromosome-alterations (SCA) >3Mb known to occur recurrently in NB, without or with numerical alterations.

Among 441 screened patients, GP was performed in 349 cases (250 LR, 99 IR) using pangenomic techniques (aCGH, SNP-arrays, other). In 73 cases, only absence of MYCN amplification was taken into account, either because no other copy number changes were seen (n=11), or because of non-established prognostic relevance alterations identification (n=13), including small interstitial deletions of chromosomes 8p or 3p, deletions of 5q, 11p, 17p, 19q and 22q, 2 cases with focal amplification of 12q14 encompassing CDK4/MDM2, amplification of 1p34.2, or a gain of 18p. For 49 other cases, no GP was obtained due to insufficient material, technical issues or not-done in real time (IR patients).

A clinically decision-making-GP was determined in 276 cases (209 LR: 159 NCA, 50 SCA; 67 IR: 24 NCA, 43 SCA). The frequency of SCA varied between different patient groups : LR L2<18months 19.8% (126 pts; 101 NCA, 25 SCA); Ms<12 months 30% (83 pts, 58 NCA, 25 SCA); IR L2>18months 70% (40 pts, 12 NCA, 28 SCA), M<12months 52% (25 pts, 12 NCA, 13 SCA).The most frequent alterations were 17q gain (62 cases), 11q deletion (33 cases), 2p gain (28 cases) and 1p deletion (26 cases).

Conclusion: Real-time genomic profiling for patient group allocation and treatment stratification in the SIOPEN's LINES Study is feasible, with a success rate of 79% of analyzed cases.

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The Effectiveness of IDRF In Predicting Surgical Complications in Abdominal Neuroblastoma Patients Treated by Laparoscopic Surgery: A Single-Institution Experience

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Background: Recently, laparoscopic surgery for abdominal neuroblastomas (NBs) has been reported, even in the advanced cases. Although image defined risk factor (IDRF) are widely used to predict surgical complications in patients with localized NBs, the efficacy of IDRF as a predictor of complications in patients undergoing laparoscopic surgery is still unclear. On the other hand, we previously reported that contact with the renal vessels (an IDFR-positive component) was not associated with surgical complications in laparotomy. The aim of this study was to describe our experience with laparoscopic surgery for NB and to examine the association between IDRF, especially contact with the renal vessels, and surgical complications in NB patients treated with laparoscopic surgery.

Methods: We performed laparoscopic surgery to treat15 patients with abdominal NBs who were treated at Kyushu university from April 2010 to December 2017. We examined the clinical features and surgical outcomes of these NBs cases retrospectively.

Results: Six of the 15 patients were male (mean age, 35.7±48.9 months). The histological diagnoses were neuroblastoma (n=10), ganglioneuroblastoma (n=2) and ganglioneuroma (n=3). The mean tumor size was 4.7±2.7 cm (1.8-11.0 cm). Only one patient exhibited MYCN amplification. The INSS stages were as follows: stage 1 (n=10), stage 2A (n=1), stage 3 (n=1), and stage 4 (n=3). Ten cases were IDRF-negative and 5 cases were IDRF-positive. Total extirpation was performed for all 10 IDRF-negative cases. For the 5 IDRF-positive cases, total extirpation was performed for 3 cases and tumor biopsy was performed for 2 cases. Among the 5 IDRF-positive cases, the two biopsy cases showed the encasement of renal vessels and other vessels. The 3 totally extirpated cases only showed the contact with the renal vessels and did not show other any IDRF-positive components. No cases exhibited surgical complications, including renal atrophy and conversion to laparotomy. There were no episodes of recurrence.

Conclusions: Laparoscopic surgery was found to be a safe and effective modality which showed a good surgical outcome in IDRF-negative cases. Our findings suggest that total extirpation by laparoscopic surgery could be safely performed in IDRF-positive cases when the only IDRF-positive component was the contact with the renal vessels.

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CRISPR-Cas9 Genomic Editing and High Throughput Compound Screening Identifies Druggable Networks for the Treatment of ATRX Mutated Neuroblastoma

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Background: In neuroblastoma, mutations/deletions in ATRX (Alpha Thalassemia/Mental Retardation Syndrome X-Linked) define a distinct patient subgroup with a clear phenotype: older age at diagnosis, conventional therapy resistance and poor survival. There are no novel therapies for this group despite urgent need.

Methods: We performed high-throughput compound screening, comparing overall sensitivity in 8 neuroblastoma cell lines versus 112 other cancer subtypes. In the SK-N-SH cell line, we then generated stable ATRX deleted clones, utilising a CRISPR-Cas9 construct targeting the helicase domain (subsequent to CRISPR-Cas9 TP53 deletion) then subjected parent cell-lines and 3 stable ATRX deleted clones to compound screening. Linear mixed effects models were used to test for differences in compound sensitivity according to subtype and ATRX status. Significant results were further validated.

Results: We identified preferential sensitivity in the neuroblastoma cell lines for three different Poly(ADP-Ribose) Polymerase (PARP) inhibitors (Olaparib, Rucaparib, and Talazoparib). Additionally, loss of ATRX further sensitised cells to inhibition of key DNA damage repair (DDR) proteins including all three PARP inhibitors and the Ataxia Telangiectasia Mutated (ATM) inhibitor KU60019.

We specifically focused on PARP inhibition due to the magnitude of effect, and greater potential for rapid clinical translation. ATRX deleted clones are unable to co-localise RAD51 with BRCA1 in response to DNA damage, resulting in a homologous recombination repair (HRR) deficiency, and thus a reliance on PARP for DDR.

We also identified an increase in genomic instability and double stranded DNA breaks as a consequence of ATRX deletion and demonstrated sensitisation to Irinotecan (but not Temozolomide) with ATRX loss. Furthermore, Irinotecan sensitivity is significantly further enhanced with the addition of Olaparib. Finally, we have validated these results in the ATRX deleted neuroblastoma cell line CHLA-90, which has the same HRR deficiency and clear Irinotecan sensitisation with the addition of Olaparib. We are currently validating our results in-vivo in xenograft and PDX models.

Conclusions: We show increased sensitivity to pharmacological ATM and PARP inhibition in-vitro in ATRX mutated neuroblastoma. Furthermore, we show that ATRX loss results in HRR deficiency. This provides a rationale for combination of PARP inhibitors with conventional chemotherapy for patients with ATRX mutated neuroblastoma

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Prognostic Power of Neuroblastoma mRNAs In Bone Marrow from High-Risk Children After Induction Treatment and During MRD: A SIOPEN Study

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Purpose: To validate the prognostic power of paired-like homeobox 2b (PHOX2B) and tyrosine hydroxylase (TH) mRNA levels in bone marrow aspirates (BM) from children with high-risk stage M neuroblastoma at the end of induction and during treatment for minimal residual disease (MRD) in HR-NBL-1/SIOPEN (www.SIOPEN-R-NET.org).

Methods: Bone marrow aspirates (BM; n=770) were analysed by RTqPCR for PHOX2B and TH mRNAs as previously described.[1] The log10 mRNA levels were transformed to generate a high or low PCR score, where a high PCR score is above the published cut-points for TH and PHOX2B mRNA levels in BM at the end of induction and low is below the cut-points.[1]

Results: High levels of TH and PHOX2B mRNA in BM (n=144) at the end of induction treatment independently predict a poor EFS; 5-year EFS for children with a high PCR score was 13% compared to 32% for children with a low score (chi^2=10, HR= 1.8, p=0.002). This observation was validated in a second cohort (n=223); 5-year EFS in children with a high PCR score was 26% compared to 48% for children with a low score (chi^2=11, HR= 1.8, p=0.001). Using the same cut-point, in BM taken pre-treatment for minimal disease (n=235) children with a high PCR score had a 5 year EFS of 17% compared to 41% for those with a low score (chi^2=7.4, HR= 2.0, p=0.006) and in BM post treatment for MRD (n=168) children with a high PCR score had a 5 year EFS of 6% compared to 54% (chi^2=27.1, HR= 3.9, p<0.0001). Ninety-four percent (17/18) of children with high levels of TH and PHOX2B mRNAs in BM post treatment for MRD rapidly progressed.

Conclusion: High levels of TH and PHOX2B mRNA in BM at the end of induction treatment independently identifies children with an increased risk of an event. Using the same cut-points, we report for the first time that high levels of these neuroblastoma mRNAs in BM in the MRD setting strongly predicts for a worse EFS. This test could be used to select children after induction treatment for alternative clinical management.

[1]Viprey et al,2014, Journal of Clinical Oncology,32;1074-83

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Detailed Characterization of The Neuroblastoma Landscape by Integrating Multiple Matched 'Omics' Datasets

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While recurring mutations in protein-coding genes are relatively rare in neuroblastomas, massive genomic alterations are common. Even though recurrent gains and losses in certain chromosome regions have been observed in high-risk neuroblastomas, the oncogenic nature of such alterations is, thus far, only understood for cases involving MYCN amplification or TERT rearrangements. To explore how other recurrent regional changes, contribute to oncogenicity in neuroblastoma, we generated large-scale matched pan-OMICS datasets from 60 primary neuroblastomas representing the entire clinical risk spectrum. Whole-genome sequencing was performed with an average base coverage of 60x on tumors and matched control DNA from patient blood. Sequencing depth was very high, supporting identification of subclonal genomic events. Somatic genomic events at single base-pair resolution were called using EBCall and large-scale genomic copy-number variations were determined using Control-FREEC. We detected previously described alterations in the 60 neuroblastomas, such as MYCN amplification, ALK mutation, and TERT rearrangements. Each total (coding and non-coding) tumor transcriptome was profiled by sequencing the complete RNA compartment following rRNA depletion. On average, 100 million paired-end reads were generated per tumor. Observed effects on the expression levels or sequence composition of mRNAs or non-coding RNAs were linked to genomic events. Whole-genome bisulfite sequencing at 60x base coverage was used to assess DNA methylation in 24 of the 60 tumor samples. Hyper- and hypo-methylated regions were detected in specific tumor groups of tumors and correlated with RNA expression changes. Overlapping genome annotations derived from public databases and a statistical meta-analysis approach were used to integrate all 'omics' datasets for each tumor, and the integrated data were correlated with clinical tumor annotation. We detected genomic/transcriptomic events previously associated with neuroblastoma risk-group stratification as well as novel events. Importantly, intermediate-risk tumors, which were under-represented in previous datasets, could be distinguished from low-risk tumors using genomic and transcriptomic events. Our comprehensive integrative analysis of ultra-deep pan-OMICs data characterized 60 primary neuroblastomas in unprecedented detail. The high sequencing depth and inclusion of the previously under-represented intermediate-risk tumors allowed detection of known and novel genomic and transcriptomic events pertinent to neuroblastoma development and/or progression.

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Response and Resistance of ALK-mutated Neuroblastoma Cells to Ceritinib

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Despite intensive multimodal therapy, more than 50% of patients with high-risk neuroblastoma relapse, which nearly always results in a fatal outcome. Therefore, development of new treatment options for high-risk NB patients is an urgent clinical need. Activating mutations of the Anaplastic Lymphoma Kinase (ALK) are present in approx. 15% of high-risk neuroblastoma. Others and we previously demonstrated that mutated ALK is a driving oncogene in neuroblastoma and suggested ALK inhibition (ALKi) as a potential targeted therapeutic approach in those high-risk cases. Most recently, a pediatric phase I/II trial with the ALK inhibitor Ceritinib revealed a safe tolerable Ceritinib dose in children. While some patients with relapsed neuroblastoma even went into complete or strong partial remission under Ceritinib monotherapy, most of them eventually relapsed during Ceritinib treatment due to ALKi resistance. We subjected tumor biopsy material of two high-risk NB patients collected before Ceritinib therapy and after the time of relapse to targeted sequencing. We identified acquired genomic alterations inactivating NF1 in the relapse samples, suggesting that secondary genomic events activate RAS signaling as a mechanism to evade ALKi. For further analyses, we used patient-derived xenograft (PDX) models as a platform to model and recapitulate the acquired resistance. We were able to establish PDX from both patient neuroblastomas before and after relapse. First, we analyzed the transcriptional response, in particular the activation of cellular salvage pathways, to Ceritinib treatment in PDX cultures as well as in ALK-mutated Ceritinib-responsive neuroblastoma cell lines. We especially focused on the identification and functional evaluation of convergent targets, such as MAPK pathway activation. Our results provide a rationale for clinical trials that investigate those targets and Ceritinib in combinatorial treatments to tackle ALKi resistance mechanisms.

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Dual ALK/BRD4 Inhibitors Are A Novel Therapeutic Approach for the Treatment of High-Risk Neuroblastoma

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Introduction: In high-risk neuroblastoma, mutations within the kinase domain of anaplastic lymphoma kinase (ALK), co-segregate with amplification of the MYCN gene. Transcription of MYCN is directly upregulated by ALK, and also by BRD4, a bromodomain protein member of the BET family of transcriptional co-regulators. We generated dual ALK/BRD4 inhibitors, which target both oncogenic mutations of ALK and transcription of BRD4, as an effective treatment for these patients. The oncogenic activity of ALK is targeted via inhibition of the kinase domain, and transcription of MYCN via BRD4 inhibition. We aimed to test the hypothesis that a dual ALK/BRD4 inhibitor would have superior efficacy as compared to single inhibitors of ALK and BRD4, avoiding the need for combinatorial treatment.

Methods: We chose a known dual PLK-1/BRD4 inhibitor with modest potency at ALK, as our starting point. We designed and prepared analogues with the aims of increasing ALK activity, decreasing PLK-1 activity and maintaining activity against BRD4. Compounds underwent in vitro assessment for activity against ALK and ALKmut, BRD4 and PLK, prior to testing in a panel of model systems of neuroblastoma. We utilized our in-house immunoassay for the quantification of activity against phosphorylated ALK, immunoblotting of downstream ALK signaling pathways and qRT-PCR to assess the effect upon MYCN and MYCN transcriptional targets.

Results: Initial data provide insights on how ALK potency can be enhanced, whilst decreasing PLK-1 potency and maintaining potent engagement with BRD4. Our leading candidate compounds demonstrated good activity against ALK/BRD4 in in vitro assays and were further evaluated against clinical compounds in cellular assays, including lines with induced resistance to clinically available compounds. Our work highlights the challenges of designing and developing dual inhibitors, particularly in balancing dual inhibition with the physicochemical properties of these compounds, while maintaining selectivity against other bromodomains and kinases.

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Characterization and impact of the ODZ receptor family on neuroblastoma tumor growth

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Neuritogenesis is the process during embryonic development when neural crest cells migrate and differentiate into multiple cell types. Neuroblastoma originates from neural crest progenitor cells of the sympathoadrenal lineage. We performed whole genome sequencing of 40 neuroblastoma tumor samples. We found that three (7.5%) of the patient samples contained a somatic mutation in the receptor family called the teneurins (ODZ1-4). The ODZ proteins are not fully characterized, however they are highly expressed during the development and differentiation of the central nervous system in the drosophila fly. Evidence suggests a function for dysregulated expression of the ODZs in human tumors, but investigations of a deeper cellular and molecular understanding in neuroblastoma are missing. The aim of this study is to investigate the ODZ receptors to determine the role and function of these proteins in neuroblastoma tumor development, progression and metastasis. When investigated in publicly available and validated neuroblastoma cohorts, low expression levels of ODZ1, ODZ2 and ODZ3 were associated with poor overall survival, while low ODZ4 expression correlated with high overall survival. Our data demonstrated that the ODZ receptors were differently expressed in neuroblastoma cell lines. Preliminary results also showed that siRNA knockdown of ODZ4 in a neuroblastoma cell line led to a 50% decrease in cell viability compared to control, together with morphological changes such as more neurite extensions, suggesting that an inhibition of ODZ4 may result in differentiation. Knockdown of the four different ODZ transcripts also showed that the ODZ receptors might have compensatory mechanisms for each other. Activity of the ODZ receptors may be of importance for neuroblastoma development and manipulation could offer a possible therapy for neuroblastoma.

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Resection and Surgical Exposure Techniques Used in Long Term Survivors of Abdominal Stage 4 High-Risk Neuroblastoma

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Background: Data on the surgical exposure techniques and the correlation of surgical and postoperative extent of resection for children with abdominal high-risk neuroblastoma (A-HR-NB) are limited. We retrospectively analyzed a homogeneously treated subgroup of patients surviving 5 years or longer without event (EFS) in order to minimize the potential impact of biologic factors.

Methods: All children aged>18 months with (1) newly diagnosed disease, (2) INSS stage 4 + A-HR-NB + event-free survival, (3) treatment according to the GPOH NB97 trial, (4) available surgical reports of first abdominal resection, (5) available information on initial and postoperative topographic tumor distribution were included.

Six topographic regions were defined by location relative to inferior vena cava, aorta and renal vein. Resection was classified as complete when no macroscopic tumor rests were documented intraoperatively (S-MR) or at postoperative staging (P-MR). The surgical exposure was scored as extended (SE-E) if more than unilateral colon mobilization was performed and if renal artery exposure (RAE) was documented for tumors in contact with renal vessels, and not extended (SE-NE) otherwise.

Results: 51/74 (68.9%) children were included. Initially, ≥3 topographical regions were affected by the tumor in 70.6% and MYCN was amplified in 25.5%. SE-NE was done in 66.7%, SE-E was done in 33.3% of patients. In 13.7% patients, ≥3 topographical regions were still affected by the tumor postoperatively. SE-E did not correlate with higher rate of S-MR (p=1.000) and P-MR (p=.377). For tumors located in both upper abdominal regions bilateral colonic mobilization was documented in 3/29 (10.3%) cases. For tumors in contact with renal vessels RAE was documented in 16/28 cases (57.1%). S-MR was documented in 80.4% but only 65.9% of them were confirmed as P-MR (P=.013). S-MR (P=.011) but not SE-E (P=.293) correlated with a lower number of affected tumor regions at postoperative staging.

Conclusion: Among long term survivors with A-HR-NB, more than half of patients have tumors initially affecting ≥3 topographic regions and a quarter present MYCN amplification. Macroscopic surgical resection rather than surgical exposure alone correlates with tumor extent at postoperative staging. Current exposure and resection techniques need to be improved in order to enhance respectability.

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MIPOGG Clinical Decisional Algorithm for Identifying Patients with Genetic Susceptibility to Neuroblastic Tumors

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Background: Cancer predisposition syndromes (CPSs) are increasingly implicated in tumor development in childhood. CPS recognition is essential as early identification can impact treatment strategies, inform surveillance protocols and direct family counselling. Extensive research into the molecular and genetic determinants of neuroblastic tumors (NBL) has led to the identification of pathogenic variants in several cancer susceptibility genes. The McGill Interactive Pediatric OncoGenetic Guidelines (MIPOGG) prediction tool was developed to identify children at increased likelihood of having an underlying CPS. A clinical decisional algorithm for NBL is incorporated within this tool.

Methods: A systematic literature review identified all known CPSs associated with NBL and characterised the predominant phenotypic features. We formulated a decisional algorithm consisting of Yes/No questions incorporating 4 tumor-specific and 7 tumor-independent questions, with a recommendation for genetic evaluation in those at increased risk of a CPS, as assessed by the tool. Through retrospective chart review, we assessed the algorithm's performance in 242 consecutive patients with NBL at the Hospital for Sick Children, Toronto. Diagnostic data, personal and family history details and genetic information were reviewed.

Results: 178/242 patients had sufficient data to permit evaluation using the MIPOGG tool. 47/178 patients had clinical genetic evaluation/testing, of whom 6 had a confirmed CPS (PHOX2b, Beckwith-Wiedemann (2), Fanconi Anemia, RB1, 2p partial trisomy). Four patients had a known CPS prior to NBL development. 15/47 had congenital anomalies (5/6 with CPS, 10/41 without confirmed CPS). Significant family history was recognised in 3/6 (50%) patients with CPS and 10/41 (24%) without confirmed CPS. 8/178 patients had multifocal or bilateral disease, one of whom had a PHOX2B alteration. Using this algorithm, 46/178 patients were recommended for genetic evaluation, and all 6 patients with confirmed CPS were appropriately identified, meeting ≥1 MIPOGG tumor-specific criteria.

Conclusion: The MIPOGG decisional algorithm is a simple and effective screening tool to identify children with NBL at increased risk of having an underlying CPS, appropriately identifying all children with a confirmed CPS in our cohort, with several cancer susceptibility genes beyond ALK and PHOX2B implicated. Further validation of the MIPOGG tool in other cohorts and tumor types is ongoing.

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Targeting Replication Stress Response by Combined PARP and CHK1 Inhibition: A New Strategy to Treat MYCN Amplified Neuroblastoma

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Neuroblastomas with MYCN amplification (MNA) are classified as high-risk aggressive tumors, posing hard therapeutic challenges to paediatric oncologists. As MYCN is an undruggable target, great efforts are being devoted to uncover MYCN-associated vulnerabilities in druggable essential pathways recruited by this oncogene. We and others have shown that MYCN induces replication stress (RS), a condition potentially leading to DNA damage and cell death or genomic instability. However, it is becoming increasingly clear that MYCN orchestrates a complex gene expression program recruiting DNA repair and signaling proteins required to restrain the deleterious effects of oncogene-induced RS, allowing cancer cell survival and progression. In principle, pharmacological inhibition of multiple targets along these pathways might represent a novel approach for the therapy of this tumor subset.

High expression of RS-associated proteins, such as PARP1, PARP2 and CHK1, is a previously unrecognized prognostic factor for human neuroblastoma. In vitro, PARP1/2 and CHK1 are abundant in MNA and MYCN-overexpressing cells. Interestingly, PARP inhibitors with high “PARP trapping” potency, such as olaparib or talazoparib, yield RS-dependent DNA damage and cell death, in MNA cells. Notwithstanding the transient activation of a CHK1-dependent RS checkpoint, PARP-inhibited MYCN overexpressing cells fail to sustain a prolonged S-phase arrest, progress through mitosis with damaged DNA, eventually undergoing mitotic catastrophe. CHK1-targeted inhibition of the RS checkpoint exacerbated this phenotype, suggesting that PARP inhibitors further enhance the reliance of MYCN-overexpressing cells on a CHK1-dependent checkpoint, prompting us to test the efficacy of combined CHK1 and PARP inhibition in neuroblastoma preclinical models.

As expected, CHK1 inhibitors (PF-477736 and MK-8776) reduced cell viability with a certain degree of specificity for MNA cells. Their combination with PARP inhibitors (olaparib and talazoparib) strongly reduced their IC50s, in a panel of neuroblastoma cell lines. Indeed, olaparib and MK-8776 showed a potent synergistic effect, in the lower concentration range. Most importantly, in MNA neuroblastoma xenograft, combination with olaparib strongly enhances the activity of MK-8776 in reducing tumor growth at very low non-toxic doses, sharply increasing the therapeutic index of the CHK1 inhibitor. These data support the introduction of a combined PARP and CHK1 inhibition for the therapy of MNA neuroblastoma.

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Identification of Polymorphic Variants Associated with Outcome and Response to Treatment in Neuroblastoma

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Background: Treatment for neuroblastoma (NB) is still suboptimal. Genetic polymorphisms in drug transporters and metabolizing enzymes could play a role in the inter-individual variability in terms of efficacy and toxicity of chemotherapeutic agents. The aim of this study was to identify genetic variants influencing response to treatment and outcome in NB.

Patients and methods: A pharmacogenetic study of 96 single-nucleotide polymorphisms (SNPs) was performed in 60 key genes relevant to drugs used to treat NB. Samples from 104 NB patients were analyzed by MassArray (Agena Bioscience). The association of genotypes with event-free (EFS) and overall survival (OS) was evaluated using a Cox Elastic-Net model, including MYCN, age and stage as covariables. The influence of genotypes on response to induction therapy (RIT) was assessed in a cohort of 41stage M high-risk (HR) NB patients treated with COJEC chemotherapeutic regimen.

Results: The strongest association with poorer OS was observed with rs1544410 GA variant (HR=1.39) while rs7186128 GG (HR=0.83) and rs1801133 TC (HR=0.65) were associated with better outcome. We also identified associations with lower EFS for rs45511401 GT (HR=1.79), rs1544410 GA (HR=1.75) and rs6539570 GG (HR=1.61) variants. On the other side rs4880 TC (HR=0.72), rs3814058 TT (HR=0.62) and rs2032582 GA (HR=0.48) variants were associated with better EFS. Remarkably, rs1544410 GA is associated with both OS and EFS. Regarding RIT, AG variant in rs726501 (OR= 2.87), GG variant in rs3740066 (OR= 1.79) or rs2010963 (OR=1.23) and TT variant in rs1143684 TT (OR=1.143) were associated with metastatic complete response. However, GG variant in rs8133052 (OR=0.53), TC variant in rs4149056 (OR=0.64), TT variant in rs10276036 (OR=0.67) and GA variant in rs1544410 (OR=0.68) were associated to incomplete metastatic response to COJEC induction regimen.

Conclusions: We identified polymorphisms in VDR, MTHFR, ABCC1, NR1I2 and ABCB1 genes associated with EFS and OS as well as in MAP3K1, VEGFA, CBR3, SLCO1B1, VDR, ABCB1 and ABCC2 genes influencing on HR patients' RIT. These associations must be replicated in a large independent cohort in international multicenter studies. Our results highlight the potential utility of pharmacogenetic analysis for identifying new prognostic markers and signatures as well as poor responders.

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The Neuroblastoma-Specific lncRNA NESPR Activates PHOX2B Expression and Is Essential for Neuroblastoma Cell Survival

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Long non-coding RNAs (lncRNAs) form a novel class of RNA molecules that are often characterized by an exquisite tissue-specificity, making them extremely attractive as targets for therapeutic intervention.

By reprocessing RNA-sequencing data from 900 cancer cell lines, we identified several neuroblastoma-specific lncRNAs including NESPR (NEuroblastoma Specific Phox2B Regulatory rna). NESPR is located in the super-enhancer region upstream of the familial neuroblastoma gene PHOX2B. Unlike many lncRNAs, NESPR is abundantly expressed, efficiently spliced and highly conserved in mammals. NESPR expression specificity was confirmed in a cohort of 10,000 tumor samples representing 35 cancer types. In neuroblastoma, high NESPR expression is associated with high stage disease, MYCN amplification and poor patient survival, suggesting NESPR may function as a lineage survival oncogene. To assess the function of NESPR in neuroblastoma, antisense oligonucleotides (ASOs) and siRNA pools (siPOOLs) were used to knock down its expression. While ASOs were capable of reducing both the nuclear and cytoplasmic fraction of NESPR, siPOOLs only reduced the cytoplasmic fraction. Notably, knockdown of the nuclear fraction, but not the cytoplasmic fraction, resulted in a significant decrease in colony formation and cell growth, as evidenced by cell viability assays and real-time cell monitoring. These effects were accompanied by an increase in apoptosis and were validated using independent ASO sequences. RNA-sequencing of ASO-treated neuroblastoma cell lines revealed a significant reduction of several neuroblastoma master regulators including PHOX2B, PHOX2A, DACH11 and ZNF536 while expression of CHD5 was significantly induced. Using 4C-sequencing we could demonstrate a long-range interaction between the NESPR locus and the PHOX2B promoter, suggesting that the nuclear fraction of NESPR acts as a cis-regulator of PHOX2B expression. Silencing of NESPR transcription using CRISPR-interference verified this interaction. NESPR pulldown followed by mass-spectrometry (ChIRP-MS) revealed several chromatin modifiers interacting with NESPR, suggesting NESPR may recruit these factors to regulate PHOX2B expression.

Our results uncover NESPR as a key component in the transcriptional circuit defining neuroblastoma cell identity and suggest that ASO-mediated targeting of NESPR may present a novel neuroblastoma-specific treatment option. In vivo validation of the observed phenotype is currently ongoing.

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Modifying Methylation-Sensitive Binding of MYCN by Targeted DNA Demethylation Using CRISPR/Cas9-TET1 Fusion Proteins

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Amplified MYCN is found in 25% of neuroblastoma cases and is associated with high-risk disease and poor outcome. DNA methylation based-clustering identifies patient subgroups strongly associated with neuroblastoma key clinical and biological features. MYCN-amplified neuroblastomas are characterized by aberrant methylation of regulatory DNA elements being in line with an impact of MYCN on DNA methylation patterns, partly via PRC2 modulation, leading to pronounced transcriptomic and chromatin landscape changes that mediate tumor de-differentiation. Recent data employing DNMT triple knockout mouse ESCs suggest MYCN-binding to be highly responsive to DNA methylation where methylation of the central CpG within the MYCN E-Box motif seems to hinder MYCN-binding.

To elucidate whether MYCN may shape the high-risk neuroblastoma epigenome via deregulating DNA methylation and thereby guiding its own binding profile, we used drug-mediated demethylation in neuroblastoma cell lines and globally assessed MYCN-binding via ChIP-sequencing analysis. High resolution DNA methylation changes were assessed by whole-genome bisulfite sequencing. The consequences of DNA methylation-associated MYCN redistribution were monitored by RNA-sequencing and chromatin mark ChiP-sequencing. With this integrated approach we plan to considerably contribute to understanding of epigenetic patterns in high-risk neuroblastoma and potentially identify candidate genes deregulated by aberrant MYCN E-Box methylation. Preliminary data identified demethylated regions with increased MYCN binding. We established a system for targeted epigenetic editing using CRISPR/Cas9-TET1 fusion proteins in order to validate candidate regulatory elements controlled by MYCN-mediated silencing.

Restoring MYCN-binding by targeted demethylation might allow de-repression of pro-differentiation programs or tumor suppressive targets. Thus, approaches targeting the neuroblastoma methylome may have significant clinical relevance for high-risk neuroblastoma patients.

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Substrate Channeling as a Mechanism for AHCY and MAT2A Synthetic Lethal Interaction with High MYCN

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Amplification of the oncogene MYCN is present in 50% of high-risk neuroblastomas (NBs) and is associated with advanced stages of the disease and poor overall survival. To identify vulnerabilities of this aggressive subset, a MYCN synthetic lethal-siRNA screen has been performed. The screen identified S-Adenosylhomocysteine Hydrolase (AHCY) and Methionine Adenosyltransferase 2A (MAT2A), two enzymes of the methionine cycle, as synthetic lethal interactions with high MYCN expression. To validate these MYCN synthetic lethal candidates, effects of their stable knockdown on viability of MYCN-amplified neuroblastoma cells were assessed. Secondly, this study aimed at elucidating the mechanism underlying MYCN synthetic lethality of MAT2A and AHCY. Therefore, transcriptional and histone modification changes after treatment of MYCN-amplified NB cells with the AHCY inhibitor D9 were analyzed.

Stable MAT2A and AHCY knockdown significantly reduced MYCN-amplified NB cell viability, colony formation and anchorage-independent growth, confirming the synthetic lethal interaction between MAT2A or AHCY and high MYCN. Knockdown of MYCN significantly inhibited MAT2A and AHCY expression and MYCN protein was bound to their promoters, identifying them as direct MYCN target genes. Moreover, high MAT2A and AHCY mRNA expression was associated with reduced overall survival in NB patients.

In this study, the set of genes differentially expressed upon AHCY inhibition using D9 showed a high overlap with genes differentially expressed after MYCN knockdown. Expression of these D9 signature genes specifically identified high-risk neuroblastomas with high MYCN transcriptional activity in a set of 498 primary neuroblastomas. Epigenetically, D9 treatment globally reduced the abundance of all histone marks. However, these epigenetic changes correlated with changes in gene expression. Genes showing a decrease in expression after AHCY inhibition lost activating histone marks, while genes whose expression was induced upon AHCY inhibition showed a reduction in repressive histone marks.

Together our data support the hypothesis that AHCY and MAT2A cooperate with MYCN in transcriptional regulation of MYCN target genes by providing substrates for associated histone modifications. By metabolizing the feedback inhibitor SAH or synthesizing the methyl-group donor SAM, AHCY or MAT2A, respectively, may enable high levels of MYCN-associated oncogenic histone methylation reactions.

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Human Pluripotent Stem Cell-Based Model of Neuroblastoma Reveals Driver and Passenger Mutations

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Next generation sequencing of human neuroblastoma tumors has provided important information on single nucleotide mutations, polymorphisms and chromosomal abnormalities. However, whether genes associated with these mutations are passengers or drivers of tumorigenesis and the stage of development when these mutations occur is unclear. Here, we describe a human pluripotent stem cell(hPSC)-based model of neuroblastoma that can address these issues. We differentiated normal hPSCs toward trunk neural crest cells (NCC), transduced established/candidate drivers of neuroblastoma, and implanted orthotopically into renal capsules of mice. As proof-of-principle, we transduced trunk NCC with established drivers MYCN and/or ALKF1174L. MYCN was sufficient to drive tumorigenesis, while ALKF1174L accelerated MYCN-mediated tumorigenesis. Next, we tested overexpression of EEF2K and KIF15, two candidate driver genes correlated with amplification of MYCN and poor survival. EEF2K, but not KIF15, cooperated with MYCN to decrease the latency of tumorigenesis, suggesting EEF2K as a driver and KIF15 as a passenger gene. Using a recently published protocol differentiating hPSC toward sympathoadrenal (SA) cells, we are now evaluating whether MYCN, ALKF1174L and/or EEF2K can transform SA cells. RNA-seq analysis of resulting tumors will reveal whether cell of origin, trunk NCC or SA cells, influences transformation and/or alignment with human neuroblastoma patient tumors. Thus, we present a hPSC-based model of neuroblastoma that validates novel candidate genetic drivers and enables personalized therapies.

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Alternative Lengthening of Telomeres in Primary Neuroblastoma Specimens – A Genomic, Epigenomic & Proteomic Approach

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Subsets of high-risk neuroblastoma accomplish telomere maintenance by telomerase activation as a consequence of MYCN/MYC induction or TERT-translocation and induction. A third subgroup of high-risk NB tumors uses telomerase-independent mechanisms referred to as alternative lengthening of telomeres (ALT) to elongate telomeres. The focus of this project is to characterize this subgroup of high risk neuroblastoma patients.

ALT-positive tumors were identified by screening an NB cohort of 749 specimens using the presence of C-Circles, which are a reliable marker for ALT activity. Subsequently, C-Circle positive tumors were analyzed using high coverage whole genome sequencing, RNA sequencing, epigenetic analysis and whole proteome analysis.

In total, 9% of the patients were identified as ALT positive. The majority of those was stage 4 tumors and was diagnosed in patients being older than 1.5 years. C-Circle presence was found to be mutually exclusive to amplified MYCN. Telomere content relative to blood control samples was higher in C-Circle positive tumors. Furthermore, ALT-positive tumors exhibited a high frequency of intrachromosomal telomeric insertions. Hotspots regions for recurrent telomeric insertions were found on chromosome 1q, 18q and 19q. 1q42 telomere insertions coincided with a loss of chr1q, whereas the telomeric insertion site overlapped with the chromosomal breakpoint. Mutations in the chromatin remodeler ATRX were the only recurrent events in ALT-positive tumors. However, only 55% of the C-Circle positive tumors showed an alteration in ATRX. Using a LC-MS-MS based whole proteome analysis approach, 6891 proteins could be quantified in our primary neuroblastoma cohort. When comparing the proteomes of ALT-positive tumors with those of other subgroups (TERT-translocated, MYCN-amplified, low-risk), we identified 470 proteins to be significantly up- or downregulated ($p<0.01$; fold change>2). ATRX was among the top candidates to exhibit a lower protein expression in ALT-positive tumors.

The combined analysis of high coverage whole genome sequencing data, whole protein expression analysis and epigenetic profiling deepens the knowledge about the general mechanisms of ALT activation and reveals unknown vulnerabilities and potential therapy targets for this subgroup of high-risk NB patients as well as for ALT-positive tumors form other entities.

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Cell Identity Heterogeneity and Plasticity in Neuroblastoma

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Heterogeneity of cell identity in neuroblastoma has been recently documented by our team (Boeva et al, 2017) and others (van Groningen et al, 2017). We previously showed that cells with different identities can coexist in a same neuroblastoma cell line, with cells expressing noradrenergic factors and cells displaying mesenchymal features. These studies highlighted the opportunity to consider neuroblastoma intratumoral heterogeneity to develop new therapeutic strategies.

In this work we identified a membrane marker distinguishing both identities in different neuroblastoma cell lines. By sorting the two different cell types within the same cell line we highlighted the properties of spontaneous plasticity of neuroblastoma cells by showing that they can transdifferentiate from one identity to the other with time. We also documented this plasticity upon commonly used chemotherapy treatment in vitro. Mesenchymal cells are more resistant to chemotherapy than noradrenergic cells and are selected during treatment in a dose and time-dependent manner. After treatment withdrawal, the initial heterogeneity is re-established. On the other side, we discovered that cells with a mesenchymal identity are more sensitive to Epithelial to Mesenchymal Transition (EMT) inhibitors in vitro.

To further unravel cell identity heterogeneity, we performed single cell RNA sequencing of two heterogeneous neuroblastoma cell lines using Chromium Single Cell 3' Solution (10X Genomics). This strategy allowed us to precisely inspect the heterogeneity of cell identity and to sharply define several groups of coordinated genes differentially expressed among the mesenchymal or noradrenergic cell types. We expect that the analysis of these data will help us to identify the pathways involved in the transdifferentiation from one state to the other one.

Altogether, this work provides new insights into the understanding of cell identity, heterogeneity and plasticity in neuroblastoma suggesting new therapeutic strategies to eradicate both types of tumor cells in neuroblastoma patients.

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Characterization of ALK Downstream Signaling Pathways in Neuroblastoma by Quantitative Phosphoproteomics

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Activating mutations of the ALK receptor are observed in around 8% of neuroblastoma tumors at diagnosis and can also occur at relapse. Whereas ALK is a relevant target for therapies with small-molecule inhibitors in patients with ALK mutated tumors, its inhibition is more difficult to achieve compared to ALK fusion proteins. The characterization of the ALK downstream signaling pathways may reveal novel candidates for targeted therapies. In this study, we used deep quantitative phosphoproteomic analysis to characterize the signaling pathways triggered by activated ALK.

Two neuroblastoma cell lines carrying either the ALK R1275Q or F1174L mutation were labeled with the SILAC (Stable Isotope Labeling by Amino acids in Cell culture) approach in three conditions, i.e., untreated, treated with the agonist monoclonal mAb46 antibody providing ALK activation or treated with the ALK inhibitor NVP-TAE684. Treatments were applied for 30 minutes or 6 hours. Immunoprecipitations with anti-phosphotyrosine antibodies were performed to capture phosphotyrosine peptides and the resulting Flow-Throughs of these experiments were further subjected to titanium dioxide (TiO2) enrichment to capture serine and threonine phospho-peptides. The obtained peptides were analysed using LC-MS/MS high resolution mass spectrometry.

Many phosphosites were modulated upon ALK activation or inhibition. The ALK agonist mAb46 antibody highly increased phosphorylation of several tyrosine of the ALK receptor, including some localized in the autophosphorylation loop. In contrast, NVP-TAE684 treatment resulted in decreased levels of ALK tyrosine phosphorylation sites. These observations validated the quality of our experiments. For further analysis, we considered phosphosites presenting with a fold change > 1.3 between the treated and untreated conditions. In each cell line, ALK modulation resulted in a set of modified phosphosites that were common to the two-time points or specific to one of the two. Comparison of the different conditions in the two cell lines also highlighted common and specific modulated residues. Interesting candidates will be selected for further functional analysis.

Our study provides new insights into the ALK dependent phosphoproteome and will lead to a better understanding of the pathways triggered by ALK activation in neuroblastoma.

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Impact of HDAC Inhibitors on Neuroblastoma Identity and Growth

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Through the characterization of the super-enhancer landscape of neuroblastoma, we recently identified two main types of identity: a sympathetic noradrenergic identity defined by a core regulatory circuitry (CRC) module including the PHOX2B/HAND2/GATA3 transcription factors (TFs) and a neural crest cell (NCC)-like identity driven by a module containing AP-1 TFs (Boeva et al, 2017). We documented growth dependency of neuroblastoma cells with a noradrenergic identity on PHOX2B as well as HAND2 and GATA3. We also showed that NCC-like cells were more resistant to standard chemotherapy. Interestingly the HDAC inhibitor (HDACi) curcumin has been shown to induce a decrease of PHOX2B protein and mRNA in IMR32 cells (Di Zanni et al, 2015). Curcumin treatment also induced c-FOS and c-JUN expression in these cells.

We therefore sought to investigate the effects of HDACi on neuroblastoma cell growth and identity in two noradrenergic neuroblastoma cell lines, i.e., IMR32, SH-SY5Y and in the NCC-like/mesenchymal SH-EP cell line. Treatment with the different HDACi resulted in a rapid cell death after 24/48h of treatment of all cell lines. We validated the effect of the HDACi on H3K27ac levels and documented that they strongly decreased the expression of the PHOX2B/HAND2/GATA3 TFs in IMR-32 and SH-SY5Y cells, at the mRNA and protein levels after 6, 12 and 24 hours of treatment. RNA-seq analysis of SH-SY5Y cells treated with the HDACi panobinostat showed that PHOX2B was the most strongly donwregulated gene and confirmed the decreased expression of HAND2 and GATA3. Panobinostat also induced a decrease of MYCN, DBH and TH expression and an increase of FOS and JUNB expression among others. The RNA-seq experiment for the SH-EP cells treated with panobinostat is ongoing. Interestingly, long term panobinostat treatment has been shown to induce prolonged survival of TH-MYCN mice (Waldeck et al, 2016).

Our data show that the HDACi panobinostat targets the CRC of noradrenergic cells and causes apoptosis in noradrenergic and NCC-like cells. Ongoing experiments are in progress to define which TFs are impacted by panobinostat in NCC-like cells. Since panobinostat kills neuroblastoma cells of both identities, it appears as an interesting molecule to consider for treatment of neuroblastoma patients.

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Hippo-YAP Pathway Activation Favors Neuroblastoma Progression

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Neuroblastomas have been suggested to consist of two distinct cellular phenotypes: the less aggressive sympathetic adrenergic cell type (expressing PHOX2A/B, HAND2 and DBH) and the more aggressive mesenchymal or neural crest-like cell type (expressing YAP1, AP-1 and PRRX1). YAP1 is a transcriptional co-activator contributing to multiple processes, including proliferation, differentiation, migration, epithelial-mesenchymal transition and glucose metabolism. We had previously identified a relapse-specific pattern of Hippo-YAP pathway activation in RNA-sequencing data from paired primary and relapse neuroblastoma samples, which is consistent with reports that the mesenchymal phenotype is more prevalent in relapse tumors. YAP1 may be a key regulator for mesenchymal transition in neuroblastoma cells to promote a progressive and drug resistant cancer cell type. We here aimed to analyze the contribution of YAP1 to oncogenic processes using neuroblastoma cell lines and models. YAP1 expression varied greatly among the 19 neuroblastoma cell lines that we profiled using real-time PCR and immunoblotting, emphasizing the idea of distinct neuroblastoma cell types, inter alia characterized by different YAP1 levels. siRNA mediated YAP1 knockdown significantly reduced viability in 5 of 9 cell lines as well as proliferation without altering the cell death rate or proportion of apoptotic cells in 2 of 4 cell lines (investigated by cell counting, BrdU-ELISA, ATP-based viability assay, Cell Death-ELISA and FACS-based cell cycle analyses), suggesting that a threshold level of YAP1 is needed to maintain cell growth and viability. We stably transfected SH-EP, SK-N-AS and IMR-5 with a Tet-ON system harboring a constitutively active YAP1(S127A). Enforced expression of YAP1(S127A) increased resistance of neuroblastoma cells to chemotherapy and serum starvation compared to control cells (transfected with the empty vector only). Cell viability and proliferation were unaffected by YAP1(S127A) expression under normal growth conditions. Intriguingly, supernatant analyses of YAP1(S127A)-expressing SH-EP and IMR-5 revealed increased glucose consumption rates and lactate production. Our findings delineate a metabolic switch triggered by YAP1-activation, which might enable mesenchymal-type neuroblastoma cells to evade first line treatment and gain resistance against chemotherapeutics and drugs leading to relapse development. Currently, we are investigating a possible contribution of YAP1 to aerobic glycolysis (Warburg effect).

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Identification of BRD3 as a Novel Therapeutic Target in MYCN-Driven Neuroblastoma Involved in Transcription-Coupled DNA Repair

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Introduction: In many tumor types, BRD4 is ubiquitously active, predominantly at super-enhancer regions serving as a target for epigenetic drugging with BET inhibitors such as JQ1. In solid tumors, sensitivity to bromodomain inhibition was mainly observed in MYC(N) driven tumors, like neuroblastoma (NB). Although the initial implementation of JQ1 in clinical practice was successful, responses showed to be transient and therapy resistance hampered in many instances its efficacy. The significance of the other members of the BET subfamily in tumor development has not been extensively studied thus far.

Methods: We performed an integrated cross species in silico analysis to identify candidate epigenetic regulators as targets for novel therapies in neuroblastoma.

Results: By means of a time-resolved transcriptome analysis of TH-MYCN transgenic mice, we first confirmed dynamic regulation of established neuroblastoma oncogenes and tumor suppressor genes. Next, we filtered within the highest upregulated genes for Cancer Gene Census (CGC) genes and identified 21 upregulated CGC genes mainly involved in chromatin remodeling and DNA repair. Finally, after further selection based on expression across multiple tumor types and survival in neuroblastoma patients, BRD3 was identified as the top-ranked candidate. In order to identify non-overlapping functions for BRD3 versus BRD4, we performed label-free mass spectrometry analysis (IP-MS) in IMR-32 NB cells. Pulldown of BRD4 delivered previously described interacting proteins such as several Mediator complex components as well as various members of the SWI-SNF complex. Notably, for BRD3 we identified for the first time several interacting partners involved in DNA repair. Next, we performed stable BRD3 knockdown resulting in significant reduced NB cell viability and colony-forming capacity. We propose that BRD3 may have a function in mediating transcription coupled DNA repair for highly transcribed genes, in particular in MYCN amplified NB.

Conclusion: We identified BRD3 as a candidate novel dependency gene in neuroblastoma associating with the DNA repair machinery putatively involved in transcription coupled repair and suggesting BRD3 as a putative specific drug target in NB cells.

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NANT 2012-01: Phase 1 Study of DFMO and Celecoxib with Cyclophosphamide and Topotecan for Relapsed or Refractory High-Risk Neuroblastoma

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Background: MYC drives polyamine expansion to support its oncogenic functions. Ornithine decarboxylase (Odc) is a direct MYC target that is rate-limiting for polyamine synthesis and is itself amplified in a poor-outcome subset of neuroblastomas. Difluoromethylornithine (DFMO) is an Odc inhibitor with robust preclinical activity via protein translation and immunomodulatory effects. We studied dose-escalated DFMO added to celecoxib (polyamine export inducer), cyclophosphamide and topotecan.

Methods: Patients 2-30 years with relapsed/refractory high-risk neuroblastoma were eligible. DFMO was studied at four dose levels (DL1-3,000; DL2/2A-4,500; DL3A-6,750; and DL4A-9,000mg/m2/day po daily) combined with celecoxib (500mg/m2/daily), cyclophosphamide (250mg/m2/day) and topotecan (0.75mg/m2/day) IV for 5 days, for up to one year with G-CSF support. DFMO pharmacokinetics and biomarkers of ODC regulation (promoter SNP) and polyamine depletion were performed.

Results: Twenty-four patients were accrued; median age 6.8 years. Seven tumors had MYCN amplification (non-amplified=13; unknown=4); 12 had tumor-involved bone marrow at entry. Patients received 124 total cycles (range, 1-17). Dose-levels 1 and 2 used 21d cycles (DFMO given 14/21d). Due to delayed platelet recovery, dose-levels 2A to 4A used 28d cycles (DFMO given 21/28d). Toxicities were predominantly hematologic and fever-related. There were three cycle-1 DLTs (hematologic; anorexia; transaminase elevation) and two DLTs in later cycles (cycle-2 hematuria/BK-virus; cycle-11 transaminase elevation). There were 23 SAEs, 78% fever-related. Eight patients stopped therapy by choice (after 2-15 cycles); two due to DLT; 9 due to PD; 3 completed therapy (CR=1, PR=2; all at dose-levels >4500 mg/m2/day DFMO); and 2 remain on therapy. Median time-to-progression was 19.8 months. Dose-level 4A (9,000mg/m2/day) exceeded tolerability (2/6 with DLT). Dose-level 3A (6,750mg/m2/day) is the RP2D. Steady-state Cmin for DFMO increased by dose-level with median Cmin of 125µM at DL3A, equivalent to DFMO concentrations achieved in preclinical murine studies with 1gm% DFMO in water, and above the exposure needed to inhibit protein translation.

Conclusions: DFMO and celecoxib added to cyclophosphamide/topotecan is tolerable in heavily pretreated neuroblastoma patients, with a RP2D of 6,750mg/m2/day. Achievable DFMO concentrations at this dose have demonstrable bioactivity using in vitro and in vivo preclinical models. A randomized relapse trial of DFMO, irinotecan, temozolomide and dinutuximab is planned within COG.

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A Prospective Study of Expectant Observation as Primary Therapy for Neonatal Suprarenal Masses: A SIOPEN Study

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Objective: To confirm that expectant observation of neonates/young infants <90 days with small adrenal masses would result in excellent event-free and overall survival and to improve knowledge about the natural history and biology of neuroblastoma during infancy.

Methods: A prospective sub-study of patients<90 days, with suprarenal masses<5 cm, no midline extension, nor lymph node/distant spreading was performed at participating SIOPEN institutions, within the LINES protocol (Low and Intermediate Risk Neuroblastoma European Study ClinicalTrials.org:NCT01728155). Initial evaluation included sonography, MRI, urinary catecholamines and MIBG scintigraphy. Sera were collected for MYCN analysis. Observation with US and urine catecholamines (if elevated at diagnosis) during a 48-week period was suggested. Infants experiencing >40% volume increase, L2 progression or metastases were considered events and were fully restaged for appropriate treatment. Surgical resection was performed for patients with >40% volume increase remaining resectable or for patients with residual mass at week 48.

Results: 126 Infants were registered between 7/7/2012 and 14/01/2017: median age 2 days (range 0-87), 38.6% females. Masses were described as cystic (28. 0%), solid (36.8%) or mixed (35.2%). Antenatal diagnosis was evident in 54 cases. Of 119 eligible patients 36 (28. 3%) underwent resection: 17 during observation and 19 at the end of the observation period and 25 proved neuroblastomas (73. 5%). Two surgical complications included hemorrhage and renal ischemia, without fatalities. Ninety-seven sera were collected and analyzed, and all were negative for MYCN amplification, although one tumor at excision after one-year observation was found MYCN amplified. The 1 and 3-year EFS was 88.6% (83.2-94.4, 95%CI) and 86.2% (79.2-93.7, 95%CI), respectively and OS was 100%, with a median follow-up of 27 months. Sixteen events were observed: progression to MS (n=6) or L1/L2 tumors (n=10). Five patients have been enrolled to other LINES groups.

Conclusion: Expectant observation for infants<90 days with diagnosed localized suprarenal masses < 5cm discovered antenatally or postnatal proved safe. Most patients will avoid surgery. Considering that most analyzed tumours were MYCN not amplified, the value of MYCN serum analyses is not definable in this study. Close monitoring is effective, without jeopardizing overall outcome.

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Liquid Biopsies Provide Exosomal microRNAs As Biomarkers of Response to Induction Chemotherapy in High-Risk Neuroblastoma Patients

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Introduction: The response of High-Risk (HR) neuroblastoma (NB) patients to induction-chemotherapy lacks prognostic molecular indicators of sensitivity/resistance. Circulating exosomes represent the bioprint of the tumor cell of origin in liquid biopsies. We investigated the modulation of exosomal microRNAs (exo-miRs) expression in HR-NB-patients in response to induction-chemotherapy.

Method: Exosomes were isolated from plasma samples of 53 HR-NB patients collected before and after induction-therapy. Flow cytometry was used to detect GD2 positive vesicles. Exo-miRs expression was measured by RTqPCR on a 381 miRNA panel. Data analyses included feature selection and pathway analysis.

Results: Exosomes isolated from plasma samples were GD2 positive, indicating that the vesicles are specifically secreted by tumor cells. We analysed the whole exosome population and found that the expression of 18 exo-miRs (fold change > 1.5, P value < 0.05) was significantly modulated in response to chemotherapy, providing evidence that exo-miRs may serve as biomarkers of the induction-therapy. Cluster analysis demonstrated that exo-miR profile can identify two groups of patients potentially reflecting different chemotherapy responses. Pathway analysis results showed that the majority of differentially expressed exo-miRs are predicted to regulate drug efficacy/resistance. Generalized linear models identified three exo-miRs that clearly differentiate poor and good responders. In silico analysis showed that these exo-miRs interact with chemotherapeutics employed in the induction-therapy (Cisplatin/Etoposide). The exo-miRs profile in the middle phase of the treatment is under investigation to identify exo-miRs that are early predictors of response and may allow a timely change in the induction protocol.

Conclusions: We demonstrated that HR-NB plasma samples contain GD2 positive exosomes released by tumor cells. We obtained the proof of principle that exo-miRs can be biomarkers of induction-chemotherapy and indicators of susceptibility/resistance to specific drugs. These results pave the way for the application of exo-miRs in liquid biopsies as circulating biomarkers of response and potentially development of NB targeted treatment.

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Metabolome of Childhood Neuroblastoma: A Pilot Model

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Background and objetives: Neuroblastoma (NB) accounts for at least 15% of cancer-related deaths in children. Previous studies on several cancer types show that metabolomics provides a potentially useful non-invasive screening approach for cancer prognosis and response to treatment assessment. Although the current risk-based treatment approaches in NB has resulted in improved outcome, survival for HR patients remains poor. This study aims to evaluate the use of a metabolomic approach for improving patient´s risk-group classification.

Design and Methods: Plasma samples from 90 NB patients (52 Low-risk and 38 High-Risk) were collected at diagnosis. Metabolomic analysis of plasma samples was carried out by ultra-performance liquid chromatography-mass spectrometry (LC-MS). Pattern recognition and selection of discriminant metabolic features between groups was performed using supervised partial least squares-discriminant analysis (PLS-DA).

Results: The classification performance of the model built using the selected discriminant features was evaluated using the external validation set that included 19 and 16 samples from high and low risk patients, respectively. The metabolic model correctly classified 16 out of 19 patients as a high risk and 15 out of 16 as a low risk validation samples, providing a sensitivity of 84.21% (60.42% to 96.62%, 95% CI) and a specificity of 93.75 % (69.77% to 99.84%, 95% CI). Plasma metabolomic profiles differentiated Low and High-Risk patients at diagnosis.

Conclusions: To the best of our knowledge, this is the first study investigating the predictive power of plasma metabolomic profile on a large cohort of patients with NB. The present results highlight the potential of metabolomic profiling for improving NB risk-group classification strategies.

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Magnetic Resonance-Guided High Intensity Focused Ultrasound in Retroperitoneal Porcine Tissues – A Safety Study for Neuroblastoma Treatment

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Introduction: High-intensity focused ultrasound (HIFU) therapy is used for the ablation of uterine fibroids and in clinical trials for the treatment of prostate, rectal, renal, breast and head/neck cancers. HIFU converges ultrasound energy under magnetic resonance (MR) or ultrasound guidance onto a small volume of tissue to produce temperatures upwards of 57°C leading to tissue destruction. Our study aims to examine the feasibility of MR-guided HIFU treatment of neuroblastoma tumors.

Methods: Given the common retroperitoneal anatomic location and perfusion characteristics of neuroblastoma, these analyses were undertaken in the retroperitoneal organs of Yorkshire pigs. MR-guided HIFU was applied at varying acoustic powers (50-400 Wac) for 20-60s to the adrenal gland, renal vessels, renal parenchyma, and retroperitoneal musculature of 10 pigs (average weight 27kg). After euthanizing the pigs, organs were fixed and stained for gross and histologic evaluation.

Results: HIFU produced temperatures of 43-75°C in vivo. In treated kidneys, lower intensities produced lower temperatures and led to capillary dilatation and congestion. Higher intensities produced higher temperatures and led to frank confluent necrosis and more pronounced tubular injury. Treated renal arteries exhibited tunica intima and internal elastic lamina damage, including ablation of the intima and elastin thickening and fragmentation. Treated adrenal glands were more hemorrhagic compared to controls and showed thermally induced capsular adhesions. There was no evidence of bowel perforation or injury while targeting nearby renal parenchyma or retroperitoneal muscle. Side effects consisted of mild skin reddening and in a few cases, first degree skin burn adjacent to the ribs.

Conclusions: HIFU produces discrete focal tissue damage in retroperitoneal organs, which appears proportional to the applied acoustic power and resultant temperature achieved. There was no evidence of uncontrolled spread of tissue damage or bowel injury. HIFU appears safe and feasible for retroperitoneal tumors. Phase 1 trials evaluating efficacy of HIFU in treatment of neuroblastoma will be guided by rigorous safety margins to minimize damage to nearby organs and normal vasculature.

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Exploring the Contribution of Gene Dosage Effects of 17q Gain on ESC and Neuroblastoma Proliferation

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Introduction: Human embryonal stem cells (hESCs) share similarities to (embryonal) tumors, including a shortened G1/S-phase. Highly proliferative cells undergo replicative stress (RS) which can cause premature aging of ESCs and genomic instability and tumor initiation. MYC(N) overexpression also causes RS due to increased use of replication origins and nucleotide depletion. Neuroblastoma (NB), a MYCN-driven pediatric tumor arising from the sympatho-adrenergic progenitor (SAP) cells, typically presents with a low mutation burden but highly recurrent patterns of DNA copy number alterations including 1q and 17q gain. Chromosome 17q gains have also been reported to arise during in vitro culture in hESC lines, suggesting that dosage effects for 17q gain render proliferative advantages to both ESCs and NB cells through a common mechanism.

Material and methods: We analyzed DNA copy number effects in more than 300 NB transcriptomes and identified multiple dosage sensitive genes implicated in homologous repair and replication fork fidelity. SWGS was performed on hESC cells. RNA-sequencing was performed for differential gene expression analysis.

Results: We present our progress towards a novel ESC derived NB model approach to test our hypothesis for the role of dosage sensitive 17q-genes in ESCs and NB in acquiring a replicative stress resistor phenotype. We obtained a hESC cell line with partial 1q and 17q gain (ESC1q17q) as shown by SWGS. Transcriptome analysis confirmed 17q dosage effects in these cells. In addition, targeted exome sequencing for 300 cancer genes was done to exclude additional tumor driving events. Using the IncuCyte life imaging device, we determined growth characteristics of both the parental and ESC1q17q cells. To test the effects of 1q/17q gain on NB tumor formation, we first generated a protocol to differentiate hESC towards hSAP cells marked by expression of PHOX2B and MASH2. Future work includes transduction of inducible MYCN overexpression and compare the transcriptome of MYCN-ESC versus MYCN-ESC1q17q induced SAPs and perform further analysis of molecular effects of 1q/17q gain on their RS response.

Conclusion: We report on a novel hESC cell line with 1q/17q gain, explored differential gene expression and provide an update on a novel protocol for generating hESC derived NB tumors.

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Mechanisms of Entrectinib Resistance in a Neuroblastoma Xenograft Model

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Introduction: Activation of receptor tyrosine kinases is seen frequently in subsets of many cancer types, including neuroblastoma (NB). Mutational activation of ALK is seen in 8-10% of high-risk NBs, and autocrine activation of TrkB with its ligand (BDNF) is seen in the majority of high-risk NBs. Entrectinib is a novel pan-TRK, ALK, and ROS1 inhibitor that has recently entered phase 1 trials in pediatric relapsed/refractory solid tumors. It has also shown excellent preclinical efficacy in NB xenograft models.

Materials/Methods: Entrectinib resistant cell lines were developed from NB xenograft models that were initially sensitive to entrectinib therapy but later developed resistance. Cell lines were established in increasing concentrations of entrectinib and had >10X increase in IC50. Cell lines underwent genomic and proteomic analysis using whole exome sequencing, RNA-Seq, and proteomic expression profiling. Genomic and proteomic changes noted in this comprehensive approach were confirmed with RT-PCR and Western blot analysis.

Results: There was no evidence of NTRK2 (TrkB) receptor mutation in any resistant cell lines. Inhibition of TrkB was maintained in all cell lines at increasing concentrations of entrectinib. PTEN pathway downregulation and ERK/MAPK pathway upregulation were demonstrated in all resistant cell lines. In individual cell lines, these changes resulted from increased IGF1R or P75 expression, leading to enhanced signaling in individual resistant clones.

Conclusions: Entrectinib resistance in these clones developed from different mechanisms. All clones had similar patterns of gene expression that differed from the parental cell line and that preserved downstream Trk signaling pathways. This was achieved through kinome reprograming using multiple mechanisms, including an increased IGF1R and p75 expression. A better understanding of the mechanisms leading to entrectinib resistance can be used to treat or prevent entrectinib resistance in future phase II/III trials.

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LDHA is Dispensable for The Warburg Effect in Neuroblastoma but is Associated with Aggressive Disease and Constitutes a Therapeutic Target

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Background: Cancers depend on the Warburg effect (aerobic glycolysis) for growth. Lactate dehydrogenase A (LDHA) is thought to be a pivotal component of the LDH tetramer that is crucial for the Warburg effect. LDHA is induced by c-MYC but it is unknown whether it is a target gene of MYCN. Given its presumed pivotal role in the Warburg effect, we aimed to determine whether LDHA is associated with aggressive neuroblastoma (NB) and constitutes a therapeutic target.

Methods: Expression of LDHA mRNA and protein were determined in 651 and 110 NB patient samples, respectively, and correlated to known risk factors and survival. Expression of LDHA in relation to MYCN was measured in NB cell lines and in the TH-MYCN NB mouse model. LDHA was knocked out by CRISPR/Cas9 in human NB cell lines and aerobic glycolysis, clonogenicity and tumorigenicity were determined.

Results: Increased mRNA and protein expression of LDHA and predominant cytoplasmic localization of LDHA protein were markedly associated with known risk factors and decreased patient survival. LDHA expression in NB cell lines was not associated with MYCN copy number and was not induced in NB of TH-MYCN mice. Complete knockout of LDHA in NB cell lines inhibited clonogenicity, tumorigenicity and tumor growth. Surprisingly, lactate dehydrogenase (LDH) activity was not ablated and aerobic glycolysis was not decreased.

Conclusions: Contrary to expectation, LDHA is dispensable for the Warburg effect in NB cells and it not induced by MYCN. Nevertheless, LDHA is associated with aggressive disease in patient NB and targeting LDHA shows preclinical therapeutic benefits. Thus, LDHA maintains NB by mechanisms other than aerobic glycolysis and represents a novel therapeutic target in NB.

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I-123 mIBG SPECT/CT Standardized Uptake Value Quantification for Neuroblastoma

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Introduction: Iodine 123 (I-123) metaiodobenzylguanidine (mIBG) is the functional imaging technique used for staging and response assessment of neuroblastoma.

Semi-quantitative scoring systems are widely available and reproducible techniques for metastatic disease assessment. Uptake intensity is not part of these systems because it is a subjective evaluation and it may be impossible if spot scans are obtained rather than a single whole-body image. Although intensity reduction/increase may be indicative of treatment response/disease progression, this is not reflected in the score.

The addition of SPECT/CT can quantitate radioisotope activity precisely. Standardized uptake values (SUV) for I-123 mIBG can be reported in a similar manner as that for PET/CT imaging techniques. We present here proof of concept of the feasibility of reporting SUV quantification of SPECT/CT imaging.

Methods: Patients with metastatic neuroblastoma were injected with a mean activity of 300 MBq I-123 mIBG and underwent planar whole-body and SPECT/CT imaging at 24 hours post injection. Height and weight were measured at time of injection. Gamma camera system sensitivity was determined using a known activity of I-123. SUV in units of g/ml can be determined from these parameters alone, by manual or automated means. We used a commercial solution, Q.Metrix (GE Healthcare) that applies semi-automatic SPECT and CT segmentation tools to define regions of interest and computes volumes of interest for uptake measurements.

Results: Six patients have been evaluated. 16 lesions were identified (6 soft tissue, 10 bone). Median SUVmean was 4 g/ml (range 1.0 - 24.3) and median SUVmax was 8 g/ml (range 3 - 33.7). In one, disease progression was documented by RECIST criteria although the SIOPEN semi-quantitative score was unchanged. SUVmean increase was 1.9 to 3.1 g/ml and SUVmax increase was 4.9 to 8.6 g/ml.

Conclusions: SPECT SUV values may be a more accurate measure of monitoring response to treatment compared to current planar imaging scoring methods. This technique is worthy of further prospective evaluation within clinical trials, with correlation of standard assessment techniques.

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Dissecting the Immune Heterogeneity of Neuroblastoma Microenviroment in Murine Models to Develop Novel Therapeutic Strategies for High-Risk Neuroblastoma Patients

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Introduction: The presence of tumor-infiltrating T cells and the absence of immunosuppressive elements have been associated with favourable prognosis of high-risk neuroblastoma (NB) patients. Recently a subset of intratumoral dendritic cells (iDC) has been found crucial for anthracycline-induced anticancer immune responses suggesting that it might be exploited to improve NB therapy. Herein, we investigated the modulation of immune infiltrate in NB murine models treated with chemotherapeutic drugs in combination with immune checkpoint blocking antibodies.

Methods: NB cell lines derived from spontaneous tumors arising in the TH-MYCN transgenic mice were engineered to express Ires-Luciferase protein and then injected either subcutaneously or orthotopically into the adrenal grand of syngeneic mice. Mice bearing established tumors were sacrificed and tumor-infiltrating immune cells analysed by flow cytometry.

Results: Tumor microenviroment in murine NBs was characterized by a consistent number of CD45+ immune cells, including T cells, NK cells, NKT cells, macrophages, neutrophils and iDCs. The orthotopic model showed a more aggressive phenotype than the subcutaneous model, resulting in the development of a tumor characterized by an immunosuppressive microenvironment predominantly infiltrated by macrophages, myeloid-derived suppressor cells and T regulatory cells. Treatment with anthracycline-derived drugs resulted in a reduced tumor growth and increase of tumor-infiltrating immune cells. Experiments are currently underway to evaluate the tumor-infiltrating immune cells in tumor-bearing mice treated with anthracycline-derived drugs and immune checkpoint blocking antibodies.

Overall, we characterized a syngeneic murine model of NB suitable to study tumor-infiltrating immune cells following treatments with combined drugs to provide insights for the study of a novel immunotherapeutic approach in NB.

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Cross-Species Transcriptome Analysis Reveals the FOXM1-EZH2 Axis as A Key Component and Drug Target for MYCN Driven Neuroblastoma Development

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Introduction: Neuroblastoma (NB) is a childhood malignancy with high clinical and genetic heterogeneity with poor prognosis for high-risk patients, half of which exhibit MYCN amplification. MYCN driven NBs have been modeled in mouse with morphologic and genomic features closely resembling those observed in human MYCN amplified NBs and thus serve as valid tools for cross-species genomic analysis.

Material and Methods: We applied a previously validated data-mining approach for master regulator identification using murine and human transcriptome datasets for cross-species computational analysis of genome-wide regulatory networks (interactomes) in the context of MYCN-driven tumor formation.

Results: Using the ARACNE algorithm, we first established the interactome in human NB samples and applied time course differential expression analysis to determine patterns of transcriptional changes during murine MYCN-driven tumor development in an established MYCN neuroblastoma mouse model. In a further step, we used the MARINA algorithm and identified the DREAM complex components FOXM1, MYB and EZH2 as important regulators of NB development. Based on these findings, we evaluated FOXM1 as a novel vulnerable target in NB by pharmacological inhibition of MELK, an FOXM1 upstream regulatory kinase, using the small molecule inhibitor MELK-T1. NB cell viability was significantly affected upon MELK-T1 treatment, with prominent downregulation of key FOXM1 downstream targets such as BRIP1, BRCA1 and RRM2. In addition, we are also currently evaluating pharmacological targeting of EZH2 by tazemetostat and the SAH-EZH2 peptide as well as c-MYB inhibition using celastrol and mebendazole as tool compounds.

Conclusion: We propose a model whereby MYCN cooperates with a FOXM1/DREAM complex regulatory network that can serve as a novel vulnerable node for targeted therapy development for NB patients. In addition, additional master regulators including EZH2 and c-MYB may act as additional targets for potential synergistic drug combinations.

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Deep Learning-Based Tool to Analyze I-FISH Spots in Consecutive Sections of Heterogeneously Amplified Neuroblastoma Tumors

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Interphase-fluorescence in situ hybridization (I-FISH) is a robust and powerful method to detect chromosomal aberrations and rearrangements at single-cell resolution. In neuroblastoma diagnostics, FISH is widely used to detect the stratifying MYCN amplification (MNA). Recently, the alternative lengthening of telomeres (ALT) detected by telomere I-FISH has been attracting a lot of attention. While MNA is defined by a ≥4-fold increased number of FISH spots over the reference probe, ALT detection is complicated by highly varying spot sizes and strong intensity differences. The enormous power of I-FISH to resolve intra-tumor heterogeneity (ITH) can be fully exploited by simultaneous quantitative analysis and visualization of spot features in consecutive tissue sections, thereby ensuring efficient quantification of I-FISH spots for diagnostic purposes and allowing insights into the spatial ITH and clonal evolution in neuroblastomas.

We developed an automated tool for I-FISH spot analysis in consecutive sections of neuroblastoma tumors. Multichannel fluorescence images of consecutive tissue sections were aligned using an image registration algorithm. Deep learning algorithms served as the prerequisite for automated image segmentation, a process outlining distinct nuclei and spots within the respective fluorescence images. Features of segmented nuclei and spots were measured at single cell level and loaded into a tool called Image Scatterplot, a powerful method to visualize and further analyze image quantification results. Finally, user-defined regions were selected and nuclear- and spot features were used to obtain region-based statistics in addition to total section information.

As proof-of-principle we analyzed consecutive sections of a heterogeneously MYCN amplified tumor using MYCN-, telomere PNA- and MDM2- I-FISH. The developed image analysis workflow allowed efficient detection of single nuclei and the simultaneous measurement of nuclear morphology features as well as spot count, spot size, spot intensity and mean spot distance of all three I-FISH images at single cell level. Comprehensive visualization in Image Scatterplots and hierarchical gating of genetically distinct regions demonstrated spatial heterogeneity of MYCN- and MDM2- copy-status and allowed robust ALT detection. A semi-automated user-driven image analysis workflow was developed and validated on a use case. These tools will improve diagnostics and allow a better understanding of spatial ITH in neuroblastoma.

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KIR-Ligand Incompatible Allogeneic Cord Blood Transplantation for Relapsed Stage 4 Neuroblastoma in a Multicenter Trial

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Background: The 5-year overall survival rate after recurrence of stage 4 neuroblastoma has been reported to be only 3%, and there are few cases of long-term survival. In the setting of autologous transplantation for patients with high-risk neuroblastoma, it is reported that the survival rate was significantly higher in patients with NK cells missing any KIR (Killer Immunoglobulin-like Receptor) ligands than in patients with matching all KIR-ligands (Venstrom JM et al. Clin Cancer Res. 2009). We conducted a multicenter clinical study of KIR-ligand incompatible allogeneic cord blood transplantation (CBT), which expected antitumor effect by NK cells.

Methods: Sixteen patients with recurrent stage 4 neuroblastoma who underwent KIR-ligand mismatched allogeneic cord blood transplantation from 2010 to May 2017 were included. They were 12 boys and 4 girls, and the median age at transplantation was 6.8 years (range: 3.4 - 10.8 years). Twelve patients achieved the second or third complete response before cord blood transplantation, the remaining 4 had residual diseases. Conditioning regimen consisting of fludarabin, melphalan and low-dose total body irradiation was mainly used for CBT.

Results: The median follow-up period was 3.5 years after CBT, and the 3-year overall survival rate was 56.4% ± 26.7%. Nine patients were progression-free, one survived with recurrence, and six died. Four deaths were caused by progression of neuroblastoma, and two were transplantation-related mortality. In order to access the disease status at CBT, we compared the second response group (n=11) who achieved the second complete response at CBT and the residual disease group (n=5) who had residual disease or second recurrence at CBT. The second response group had significantly lower rate (10.1%) in relapse as compared with the residual disease group (100%), (p=0.005). The 3-year progression-free survival rate of the second complete response group was 70.7% ± 28.0%. There were two long-term survivors more than 5 years after CBT (7.1 and 6.4 years, respectively).

Conclusions: KIR-ligand incompatible allogeneic CBT as immunotherapy could be a salvage treatment option for relapsed neuroblastoma. However, this treatment should be considered when the patient has achieved complete response.

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Dual Inhibition of PLK1 and BRD4 for Treating High-Risk Neuroblastoma

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Inhibiting multiple therapeutic targets is an established strategy to improve the durability of clinical responses to targeted therapies. Resistance to intensive chemotherapy is common among patients with relapsed neuroblastoma, and few molecular therapies have been introduced into protocols for the initial treatment of high-risk disease. Neuroblastoma cells in high-risk disease are often dependent on over-active MYCN. The bromodomain protein, BRD4, has been shown to be required for transcription of MYCN and its target genes, and pharmacological inhibitors have anti-neuroblastoma activity in vitro and in mouse models. Polo-like Kinase 1 (PLK1) is frequently overexpressed in neuroblastomas and regulates MYCN protein stability. Based on these previous observations, we hypothesized that simultaneous inhibition of BRD4 and PLK1 could synergistically target MYCN stability and activity, to enhance anti-tumor activity. To assess this, we evaluated cell viability in response to PLK1 and BRD4 inhibitors. Combining both single agents synergistically inhibited cell viability specifically in neuroblastoma cells overexpressing MYCN. Several kinase inhibitors in clinical trials against other cancers have recently been demonstrated to also inhibit bromodomains at therapeutically relevant potencies and are best classified as dual kinase/bromodomain inhibitors. Using structural activity relationship studies, we generated 49 molecules with dual nanomolar inhibitory activity against BRD4 and PLK1. We screened these dual PLK1-BRD4 inhibitors to assess the impact on cell viability of MYCN-amplified neuroblastoma cells compared with untransformed mesenchymal cells. We identified two dual inhibitors with strong and tumor-specific activities against MYCN-amplified neuroblastoma but not mesenchymal cells, suggesting a wide therapeutic window. To further investigate their effects in MYCN-amplified neuroblastoma cell lines, we analyzed cell proliferation (measured by BrdU incorporation) and cell death (measured by histone release). Dual PLK1-BRD4 inhibitors suppressed proliferation and induced cell death at low nanomolar concentrations. In addition, we evaluated the toxicity of the drugs in vivo. Neither dual inhibitor exerted relevant toxicities in mice. We present a new pharmaceutical strategy to achieve dual synergistic targeting of BRD4 and PLK1 with a single agent.

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A Novel Fetal Human Neuroblast RNA-Sequencing Reference Data Set

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Neuroblastoma tumor cells originate from primitive neuroblasts giving rise to the sympathetic nervous system. As these precursor cells are not detectable in postnatal life, transcriptome profiling for comparative data mining analyses in neuroblastoma is challenging. In 2005, we reported on the isolation of neuroblasts islets from human fetal adrenal glands using Arcturus laser capture microdissection and oligonucleotide microarray based gene expression profiling. Upon initial search for genes differentially expressed in either unfavorable or favorable neuroblastomas versus neuroblasts, MYCN emerged expectedly together with other relevant genes such as ODC1, TOP2A, TWIST1, BIRC5, SOX11 and ALK. Here, we report the generation of a new RNA-sequencing reference data set for human fetal neuroblasts obtained through PALM microdissection followed by RNA amplification, polyA+ RNA-sequencing on 6 neuroblast and 17 high risk neuroblastoma samples. Data were validated using clustering analyses and cross-comparison with the previous microarray expression data. We investigated the expression of recently reported core regulatory circuit transcription factors as well as other genes that emerged from reported studies after 2005, including long noncoding RNAs. This data set will further serve as a reference for future studies including transcriptome validation of human embryonic stem cell induced sympathetic progenitors or in vitro cultured mouse neural crest cells (both used for transformation towards neuroblastoma). This fetal human neuroblast RNA-sequencing reference data set is available for future genomic exploration of human fetal neuroblasts including single cell analysis for transcriptome, open chromatin (ATAC-seq) and epigenetic profiling.

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Post-Consolidation Metronomic Therapy Has Power to Cure Refractory Neuroblastoma

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Background: The metronomic therapy uses low doses of continuously applied chemotherapeutic, anti-angiogenetic, and immunomodulating drugs. We have observed very promising result in relapsed neuroblastoma patients on METRO 2012 protocol (Principal Investigator Prof. Dr. Frank Berthold, University of Cologne, Germany) and therefore we decided to offer metronomic therapy to our patients with refractory disease.

Methods: We used METRO 2012 protocol from February 2013 to December 2017 in 7 patients (3 boys, 4 girls) with neuroblastoma. All patients had INSS stage IV, INRG High Risk neuroblastoma. Average age at diagnosis was 44 months (14-126 months), 3 patients were NMYC positive. Four patients experienced early metastatic relapse of neuroblastoma with average EFS 15 months (7-22 months). Three patients had refractory disease (≤ PR after the end of induction or MIBG Curie score ≥ 3 after 4th cycle od induction chemotherapy). All metronomically treated patients were pretreated by MTD based induction chemotherapy and myeloablative therapy with stem cell support, 6 patients had also I131MIBG therapy. Metronomic protocol METRO 2012 utilize combination of well-known drugs (celecoxib, vinblastine, cyclophosphamide, propranolol and etoposide) in metronomic manner.

Results: Survival in relapsed or refractory neuroblastoma was historically very disappointing at our center. During pre-metronomic time was 2 years OS in relapsed neuroblastoma patients only 5.6%, but from beginning of metronomic era, 2 years OS increased to 57.1%. Patients with refractory disease showed only partial response to MTD based chemotherapy, and usually neither salvage MTD based regimes nor HDTx consolidation could improve their response and almost all patients with refractory disease experienced disease progression in pre-metronomic time. Surprisingly, we could achieve complete remission in 2 out of 3 our refractory patients on METRO 2012 protocol and 1 long term disease stabilization. 2 years EFS/OS in refractory patients in pre-metronomic time was 15.8/15.8%, compared to 2 years EFS/OS 63.5/85.7% in METRO 2012 era.

Conclusion: We can prolong survival in relapsed neuroblastoma patients with metronomic protocol METRO 2012. We can also prevent relapses in refractory disease, if METRO 2012 is used as an early post-consolidation therapy.

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Core Transcriptional Regulatory Circuitries in Neuroblastoma

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Background: Evidence accumulates that epigenetic deregulation, including aberrant DNA methylation in high-risk disease or oncogene activation by enhancer hijacking, plays a prominent role in neuroblastoma (NB). The present study applies a comprehensive approach integrating chromatin modification data with genomic and expression data to elucidate NB subtype specific super-enhancer (SE) landscapes and core regulatory circuitries (CRCs).

Methods: Chromatin immunoprecipitation with subsequent sequencing (ChIP-seq) of histone 3 lysine 27 acetylation (H3K27ac) was used to identify active enhancer elements in 60 NB tumor samples. Additionally, a validation cohort consisting of 24 NB cell lines and two human neural crest cell lines was applied. Circular chromatin conformation capturing with subsequent sequencing (4C-seq) and HiChIP were used to assay for chromatin interactions. ATAC-seq was used to analyze chromatin accessibility and for transcription factor (TF) footprinting.

Results: Unsupervised clustering of 60 NB tumor samples according to H3K27ac signal intensity at the most variable SEs (genome-wide) revealed distinct subgroups of mesenchymal, MYCN-amplified and MYCN single-copy tumors with distinctive SE activity patterns. Calling of CRCs in the 60 NB tumor samples revealed a core set of NB master TFs (CRC TFs), including established NB regulators like HAND2, PHOX2B and MYCN. The gene ontology of the top 50 CRC TFs converges to biological processes like development of neural crest cells, sympathetic and peripheral nervous system. Besides the common NB networks, in-depth analyses further revealed differential NB CRC networks reflecting the above-mentioned subgroups. RNAi-mediated knockdown of corresponding CRC TFs identified subgroup specific responses in cell line models reflecting the respective subtypes. Interactions between promoters and SEs of selected CRC TFs were confirmed via HiChIP, 4C-seq and ATAC-seq.

Conclusion: SE-based clustering and CRC analysis of neuroblastoma tumors reveals subgroups, which were previously unresolved by genetic, expression or DNA-methylation data. The study reveals an association of MYCN amplification with the global SE landscape of primary NBs and suggests a role for MYCN in differentially controlling subsets of CRC TFs and their networks. Specific targeting of the SE-dependent CRC networks may open a therapeutic window for epigenetic drugs, including BET inhibitors, CDK7 or EZH2 inhibition, in NB.

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Integrative Analysis of Whole-Genome Bisulfite Sequence Data Identifies Epigenetic Regulatory Networks of Unfavorable Neuroblastoma

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The clinical spectrum of neuroblastoma ranges from spontaneous regression to rapid progression despite intensive multimodal therapy. Known genetic aberrations only partially mirror this diversity, pointing towards epigenetic involvement in neuroblastoma pathogenesis. Integrating array-derived methylome and transcriptome profiles with ChIP-seq data, we previously identified a largely MYCN-driven deregulation of DNA methylation at both proximal and distant regulatory elements.

To address the regulatory role of DNA methylation in neuroblastoma on an unbiased genome-wide scale, we employed whole-genome bisulfite sequencing (WGBS) in a cohort of 27 primary neuroblastomas including MYCN-amplified, TERT-rearranged ALT-positive and low-risk tumors. An additional cohort of 340 neuroblastomas was analyzed using 450k methylation arrays and expression was assessed in 184 of these tumors via RNAseq. Chromatin mark ChIP-seq was done in selected tumors and cell lines. In vitro demethylation was done via DAC treatment and CRISPR-dCas9-mediated TET1 targeting.

Unsupervised methylation-based clustering separated low-risk from high-risk tumors and MYCN-amplified from non-amplified tumors. Differentially methylated regions, DNA methylation valleys and partially methylated regions were identified for TERT-, MYCN- and ALT-driven subgroups; and integration of RNA expression and ChIP-seq data revealed regulatory DNA-methylation patterns specific for subgroups or common to all high risk-tumors, with the latter largely converging on inhibition of neuronal differentiation. DEXseq and Metilene tools' combined analysis identified a catalogue of genes whose differential methylation is significantly correlated with alternative exon usage in high-risk tumors, indicating that DNA methylation affects both quantity and alternative function of gene products. Employing the Tracing Enhancer Networks using Epigenetic Traits (TENET) approach on WGBS data, we detected a MYCN-specific enhancer network that converged on a limited set of transcriptional activators/repressors and that was largely confirmed in the extended cohort of 450k methylomes. Candidate regulatory sites could be validated by DAC-driven global demethylation and a subset of these is currently evaluated by dCAS9-TET1-driven targeted demethylation.

Together, our analysis of DNA methylation at single nucleotide level, integrated with RNA expression and chromatin modification data reveals previously unrecognized regulatory networks in high-risk neuroblastoma subgroups and pinpoints targets for therapeutic intervention.

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Tailoring Epigenetic Precision Therapy in Neuroblastoma: SGSS05-NS3 As A Potent and Selective Pharmacological Inhibitor of SETD8 That Rescues P53 Functions

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High-risk neuroblastoma (NB) is one of the most aggressive pediatric tumors accounting for 15% of all pediatric oncology deaths, and less than 50% of patients experience long-term survival, despite intense multimodal treatment. The tumor suppressor p53 is rarely (2%) mutated in NB but its functions are diminished. Multiple mechanisms have been identified that attenuate the activity of p53 in MYCN-amplified NB cells, but few affect MYCN-WT NBs. Thus, a major challenge is to identify novel targeted therapies for high-risk NB (HR-NB) patients, specifically for the large fraction (70%) that present with MYCN-WT. Recently we identified SETD8, the H4K20me1 methyltransferase, as a crucial epigenetic regulator of growth and differentiation in NB. In addition to targeting other non-histone proteins, SETD8 monomethylates p53 on lysine 382 (p53K382me1), attenuating its pro-apoptotic and growth arrest functions. Genetic and pharmacological (UNC0379) inhibition of SETD8 impairs NB growth in vivo. Moreover, SETD8 levels are associated with poor prognosis only in MYCN-WT Stage 4 NBs. In order to identify targeted therapy less toxic for HR-NB, we evaluated a more specific SETD8 inhibitor with enhanced activity and selectivity, SGSS05-NS3 (SG3). Our results indicated that in NB cells in vitro treatment with SG3 rescues the canonical p53 functions leading to increases in p53 protein levels and of its target p21 by decreasing p53K382me1, impairing NB cell viability and inducing caspase-dependent cell death at lower IC50 compared with UNC0379 (1µM). Gene expression profile (RNA-seq analysis) confirmed that the most significantly upregulated genes upon SG3 treatment were among the p53 pathway targets. In pre-clinical xenograft NB models, pharmacological SETD8 inhibition by SG3 conferred a significant survival advantage in MYCN WT NB. Our study provides further evidence for targeting SETD8 as a therapeutic strategy in NB, particularly in MYCN-WT subgroup.

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Enhancer-Hijacking Is a Common Mechanism of Oncogene Activation in High-Risk Neuroblastoma

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Background: We have previously shown that genomic rearrangements activate proto-oncogenic telomerase by juxtaposing active enhancer elements to the TERT gene in high-risk neuroblastomas (NBs). Here, we applied a global approach integrating structural variant data with expression profiles and NB-specific enhancer data to systematically identify sporadic or recurrent enhancer-hijacking events in NB.

Methods: Chromatin immunoprecipitation sequencing (ChIP-seq) of histone 3 lysine 27 acetylation (H3K27ac) was used to identify active enhancer elements in 60 NB tumors and 24 NB cell lines. Whole genome sequencing (WGS) profiles were analyzed for structural variants in 107 NB tumors and twelve NB cell lines. Candidate genes were analyzed for outlier expression in matched RNA-seq profiles. Circular chromatin conformation capture sequencing (4C-seq) was used to assay for physical promoter-enhancer interactions.

Results: WGS analyses revealed that chromosomal rearrangements are common events in NB and frequently affect regions known to harbor proto-oncogenes and lineage- specific enhancers. ChIP-seq analyses confirmed that these rearrangements juxtapose active enhancer elements to proto-oncogenes including ATOH1, IGF2BP1, MYC, MYCN and TERT which in turn are upregulated and harbor outlier expression in the cohort as revealed by RNA-seq. Intriguingly, a lineage specific super-enhancer (SE) element on chromosome 4, which is among the most active SEs in the cohort, was involved in hijacking events with several different oncogenes (including all of the above listed except TERT) in five independent samples. Finally, 4C-seq analyses in candidate NB cell lines proofed physical interactions between translocated enhancer elements and promoters of the respective oncogenes in all cases investigated.

Conclusions: The study shows that enhancer hijacking is a common event driving oncogenes in NB. Further it exemplifies that strong, lineage-specific SEs are recurrently involved in hijacking events. The common mechanism of oncogene activation by enhancer-hijacking may open a therapeutic window for epigenetic drugs including BET or CDK7 inhibitors in high-risk NBs

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Immune Microenvironment in Neuroblastoma: Clinical and Therapeutic Relevance

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Tumor-infiltrating immune cells play a key role in controlling tumor progression. The presence of distinct T-cell subsets have been associated with favorable clinical outcome in a wide range of human cancers. Recently, we have shown that tumor-infiltrating T cells have a prognostic value greater than, and independent of, the criteria currently used to stage neuroblastoma. Low-risk neuroblastoma are characterized by a higher number of proliferating T cells and a more structured T-cell organization, which is gradually lost in tumors with poor prognosis. We defined an immunoscore based on the presence of different T-cell subsets that associates with favorable clinical outcome in MYCN-amplified neuroblastoma. We also demonstrated that the combined PD-L1 and HLA class I tumor cell density predicts the clinical outcome in neuroblastoma patients.

To further dissect the immune heterogeneity of neuroblastoma microenvironment, we evaluated the density of infiltrating dendritic cells (iDC), which are crucial for drug-induced anticancer immune response and performed targeted gene expression profiling (Nanostring nCounter Immune panel) in the same cohort of NB samples. Publically available datasets were used to validate primary samples analyses. As for T cells, high density of iDCs was correlated with the presence of tumor-infiltrating T cells, expression of tumor HLA class I and favorable clinical outcome, suggesting that iDCs may be critical for robust tumor control by improving prediction of patient survival.

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Expression of TWIST1 and TWIST2 as Possible Predictive Markers in Neuroblastoma

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Introduction: The embryonic transcription factors TWIST1 and TWIST2 are frequently reexpressed in cancer. TWIST1 expression in neuroblastoma (NB) was previously shown to be associated with MYCN amplified and high MYC expressing tumors.

Material and Methods: A tissue microarray of 72 primary tumors (stage (st)1=12, st2=6, st3=13 st4=36, st4s=5), 25 matched metastases and 44 matched control normal tissues were screened for TWIST1 and TWIST2 expression by immunohistochemistry using anti-TWIST1(Millipore - ABD29, rabbit polyclonal, 1/50) and anti-TWIST2 (R&D Systems - AF6249- sheep polyclonal– 1/200) specific antibodies. A semi-quantitative immunostaining score based on the percentage of positive cells was determined for each tissue sample.

Results: TWIST1 had a nuclear expression in 72% of tumors but was moderately expressed only in neuroblastic cells in with a median score (ms) of 0.84, while TWIST2 had a nuclear expression less frequent in NB (41%) but with a higher score (ms=1.1). However, in normal control tissues, TWIST1 was never expressed while TWIST2 was present in 46% of sympathetic ganglia but with moderate intensity (0.58). Interestingly, TWIST1 expression was significantly higher in tumor associated with poor prognosis: stages 3, 4 versus stages 1, 2 (81% of tumors with TWIST1 expression, ms=1.05, vs 41%, ms=0.42, $p<0.01$); stage 4 versus stage 4S (83%, ms=1.1 vs 40%, ms=0.35, respectively, $p=0.04$); patients >18 months at diagnosis versus less (73%, ms=0.98 vs 29%, ms=0.62, $p=0.045$), and tumors with MYCN amplification versus without amplification (84%, ms=1.18 vs 23%, ms=0.60, $p=0.02$).

In contrast, TWIST2 expression was higher in tumor with better prognosis: stages 1, 2 versus stages 3, 4 (39%, ms=1.22, vs 41%, ms=0.4, $p=0.045$), and in tumor without MYCN amplification versus with amplification (47%, ms=0.84 vs 23%, ms=0.17, $p<0.01$). However, no significant differences in TWIST2 expression was observed between stage 4s versus stage 4, and in relation with age at diagnosis. Moreover, in metastases TWIST1 was frequently expressed (76%, ms=0.95), while TWIST2 expression was uncommon (30%, ms=0.31).

Conclusions: These data highlight an association of TWIST1 expression with unfavorable clinical factors of NB and with metastases, while TWIST2 expression is correlated with tumors of better prognosis.

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The Antibody-Drug Conjugate D3-GPC2-PBD Potently Eradicates Neuroblastoma Patient-Derived Xenografts

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Background: We developed an antibody-drug conjugate (ADC; D3-GPC2-PBD) for neuroblastoma linking a glypican 2 (GPC2) antibody (D3) to the potent DNA damaging pyrrolobenzodiazepine (PBD) dimers (Cancer Cell, 2017).

Methods: To determine the in vivo efficacy of this ADC, we utilized highly annotated neuroblastoma patient-derived xenografts (PDXs). Cohorts of mice (n=9-10) were stratified to treatment with either vehicle or D3-GPC2-PBD at doses of 0.5-3 mg/kg given once or 1 mg/kg given twice weekly x 2 weeks when mean tumor volumes reached 200-300 mm^3. Co-treatment with excess D3-GPC2-IgG1, the efficacy of free PBD dimers, re-treatment of the rare recurring ADC treated tumors, treatment of larger PDXs, and DNA damage and apoptosis assessment by immunohistochemistry (IHC) were also explored.

Results: We first showed that D3-GPC2-IgG1 binds avidly to murine Gpc2 by flow cytometry and that D3-GPC2-PBD is equally potent against murine and human GPC2 in vitro. To date, the D3-GPC2-PBD ADC was tested in four neuroblastoma PDXs with a range of GPC2 expression (Molecules of Equivalent Soluble Fluorochrome [MESF]): CHLA79 (GPC2 MESF=825, MYCN non-amplified, TP53 wild-type), COG-N-421x (GPC2 MESF=3301, MYCN amplified, TP53 wild-type), SKNAS (GPC2 MESF=672, MYCN non-amplified, TP53 mutated) and NB-1643 (GPC2 MESF=1665, MYCN amplified, TP53 wild-type). Treatment with D3-GPC2-PBD resulted in complete and sustained tumor regression in 61% (17/28) of mice treated with 1 mg/kg ADC, 96% (27/28) of mice treated with 3 mg/kg ADC, and 96% (26/27) of mice treated with 1 mg/kg ADC given twice weekly x 2 weeks, including 100% (8/8) larger PDXs (tumor volumes of 600-1300 mm^3). Treatment with free PBD dimers at equivalent molar concentrations had no effect on tumor growth, co-treatment with excess D3-GPC2-IgG1 abrogated a significant amount of ADC induced cytotoxicity, and ADC re-treatment of PDXs that began to regrow after 100 days (n=2) showed complete responses. ADC treated tumors exhibited upregulation of DNA damage (γH2AX) and apoptosis (cleaved caspase 3/cleaved PARP) by IHC. ADC treatment was well tolerated with no discernible drug-related toxicities.

Conclusions: The D3-GPC2-PBD ADC is potently efficacious against a diverse panel of neuroblastoma PDXs. These data support the clinical development of a PBD dimer containing GPC2-directed ADC.

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MYCN Regulates Exosome Protein Cargo in Neuroblastoma

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Despite the optimism raised by discoveries on the molecules and signaling pathways at the base of neoplastic transformation, neuroblastoma still presents a formidable challenge to scientists and clinicians. Amplification of the proto - oncogene MYCN is a key molecular aberration in high-risk neuroblastoma and predictive of poor outcome (Seeger et al, 1985). Although only 25% of total neuroblastomas show amplification of MYCN, there is a prevalence of this aberration in the high-risk form of the cancer. MYCN promotes tumour cell proliferation and metabolism via activation of target genes, by inducing the expression of oncogenic microRNAs and by promoting ribosome biogenesis and protein translation (Eilers and Eisenman, 2008). In this study, we investigated the role of MYCN in regulating the protein cargo of exosomes, vesicles secreted by tumour cells that can be picked up by recipient cells with important functional consequences. Using a switchable MYCN system coupled to mass spectrometry analysis, we have verified that MYCN specifically regulates 152 proteins in the exosomes secreted by neuroblastoma cells. Pathway analysis suggests that the MYCN - regulated proteins belong to 3 main functional groups: a) extracellular matrix-cells interactions, b) glycolysis and c) ribosome biogenesis. In addition, we isolated exosomes from MYCN amplified and non-amplified cell lines, verified their presence by staining with the membrane binding dye PKH67 and visualising them with ImageStream flow cytometry. We analysed neuroblastoma exosomes protein content by Western Blot analysis to validate the results obtained with the mass spectrometry experiment. Proteins relevant to neuroblastoma biology, such as eukaryotic elongation factor 2 and transferrin receptor 1, were confirmed as differentially expressed in exosomes according to the MYCN status. Functional studies indicate that exosomes isolated from MYCN or non MYCN expressing cells confer different signaling, proliferation and metabolic characteristics to recipient cells. In conclusion, we have assessed the presence of MYCN - inducible exosomal proteins in the supernatant of cell lines that could cause “conditioning” of recipient cells in the tumour microenvironment. The profiling of MYC - induced exosomes could lead to the identification of useful biomarkers and new opportunities to uncover vulnerabilities in MYC addicted tumours such as neuroblastoma.

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ALKALs as ALK RTKs In Vivo Ligands in the Neural Crest and Derived Cells

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Background: The receptor tyrosine kinase (RTK) Anaplastic lymphoma kinase (ALK) has a key role in neuroblastoma development and is considered to be an important therapeutic target. ALK is mainly expressed in central and peripheral nervous systems among mammals; however, its physiological role remains still unclear. The recent in vitro identification of ALKAL proteins (previously described as FAM150, AUG) as potential ligands for human ALK and the related LTK RTKs has offered new clues and therefore new questions regarding the role of ALKALs in combination ALK/LTK RTKs during development and tumorigenesis.

Results: In this study we have investigated the role of zebrafish Alkal proteins for activation of the endogenous LTK, which is similar to the human ALK in sequence and domain structure. Zebrafish LTK (DrLtk) controls the development of iridophores, neural crest-derived pigment cells, making Danio rerio an excellent system to study ALK/LTK involvement in neural crest regulation in vivo. In addition, we used Drosophila eye patterning and cell culture-based assays to corroborate our results.
Here we show that zebrafish Alkals potently activate both DrLtk and HsALK, and overexpression of DrAlkals causes ectopic iridophore development, consistent with the loss of iridophores observed in loss of function alleles for DrAlkals.

Conclusions: This work shows in vivo evidence of activation of ALK/LTK receptors by ALKAL proteins, as well as a conserved role for ALKAL proteins in activation of ALK RTKs family in the development of the neural crest.

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A Bicistronic DNA Vaccine Encoding for Tyrosine Hydroxylase and IL-15 Reduces Tumor Progression and Spontaneous Metastasis in Mice

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Introduction: Tyrosine hydroxylase (TH) is involved in catecholamine biosynthesis and highly expressed in NB and therefore a promising target for active immunotherapy approaches. Interleukin-15 (IL-15) represents an attractive adjuvant for DNA vaccination due to its ability to induce cytotoxic but not regulatory T cells. Here, we report the generation and characterization of a bicistronic DNA-vaccine encoding for both TH and IL-15 and its enhanced efficacy to reduce tumor growth and spontaneous metastasis in vivo.

Methods: DNA sequences encoding for human TH and murine IL-15 were integrated into the bicistronic mammalian expression vector pIRES, that enables co-translation of both genes. For optimized presentation of TH-epitopes, the ubiquitin (Ub) DNA sequence was linked upstream to the DNA sequence of TH ensuring enhanced proteasomal degradation and antigen processing. Next, CHO cells were transfected with the generated plasmid for in vitro characterization. TH expression of transfected CHO cells was investigated using western blot analysis. To access IL-15 secretion and bioactivity, an IL-15-specific ELISA and proliferation assay based on IL-15-sensitive CTLL2 cells were performed, respectively. To evaluate anti-tumor efficacy in vivo, A/J mice (n=12) were immunized with the bicistronic TH/IL-15-based DNA vaccine, using attenuated Salmonella typhimurium as oral delivery system, 3× prior and 3× after a lethal challenge with syngeneic NB cells. Plasmids encoding for either TH or IL-15 served as controls.

Results: IL-15-specific ELISA clearly showed a secretion of IL-15 in supernatants of CHO cells transfected with the bicistronic plasmid (230 vs. 0 pg/ml for non-transfected cells). Furthermore, bioactivity of in vitro secreted IL-15 could be confirmed by increased proliferation of CTLL2 cells. Cell lysates of transfected CHO cells showed a specific Ub+TH expression (64 kDa). Importantly, TH-/IL-15-based DNA-vaccination resulted in a reduced tumor progression rate of 45.4% compared to controls (TH 83.3%; IL-15 100%). Moreover, we observed reduced spontaneous metastasis in mice immunized with the bicistronic DNA-vaccine (30.0%) compared to controls (TH 63.6%; IL-15 70.0%), demonstrating efficacy of the new vaccine against NB.

Conclusion: Co-translation of the stimulatory cytokine IL-15 augments reduction of tumor progression and spontaneous metastasis of a tyrosine hydroxylase directed DNA vaccination against NB in mice.

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A Morphometric Study of Lymphatics in Neuroblastoma

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Background: Lymphatic vasculature is a prerequisite for the maintenance of tissue fluid balance and immunity in the body and is now known to play an integral role in the metastatic spread of tumor disease. Nevertheless, its relationship with prognosis in neuroblastoma (NB) is unknown, where a well-defined pre-treatment risk classification still remains unsuccessful in most high-risk patients, thus calling for urgent research on stratification factors and new therapeutic targets.

Aim: We aimed to relate different morphological and density features associated with NB lymph vascularization in order to find any potential role on patients' prognosis, which could therefore be used as a factor to enhance the pre-treatment risk stratification or provide therapeutic targets.

Materials and methods: Two representative cylinders of 333 samples were included in tissue microarrays and stained with D2-40 antibody to specifically detect lymph vessels, which were quantified used the Angiopath® system. Density, as well as shape and size morphologic parameters were calculated for microvascular (three segments: 5-15µm, 15-20 µm, 20-50 µm) and macrovascularizarion (50-200 µm, >200 µm).

Results: A high density of 5-15 µm and 15-20 µm microvascular segments as well as an irregular shape of macrovascular lymph vessel segments were associated with unfavorable histology and metastatic stage, and the risk of belonging to the high-risk pre-treatment group. Additionally, abundant lymph microvascularization (median ≥ 87.88 vessels/mm2) were associated with lower event-free survival (EFS) and overall survival (OS) (p-value 0.021 and 0.073, respectively). Similar results were found for a subgroup of samples with absent lymph vascularization (n=98) (p-value 0.013 and 0.084 for EFS and OS, respectively). Among 126 patients with complete datasets, Cox regression was carried out. Only for EFS, total density microvascularization was retained (hazard ratio 2.403) together with metastatic stage, age (≥18 months) and MYCN amplification variables (hazard ratio 4.703, 3.032 and 2.662, respectively).

Conclusions: The density of different lymph vessel segments and morphologic parameters of shape and size should be taken into account to enhance the pre-treatment risk stratification. Research on drugs targeting lymphatics must be considered.

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Risk-Prediction Based on Post-Induction Bone Marrow Response and Genomic Profile: A Future Way to Stratify Stage M Neuroblastoma Patients?

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Purpose: To evaluate the prognostic relevance of a combined analysis of genomic aberrations and minimal residual disease (MRD) in the bone marrow (BM) of stage M neuroblastoma patients.

Patients and Methods: Accessible BM pairs from diagnosis and post-induction and/or genomic data originating from stage M patients previously enrolled in the HR-NBL1/SIOPEN high-risk neuroblastoma trial were compiled at our center. Disseminated tumor cell (DTC) quantification used an automatic system for highly specific detection of GD2/CD56 positive cells. Information on somatic copy-number aberrations (SCNAs), MYCN amplification, aberrations in ATRX, TERT, and PTPRD genes was generated by high-density single-nucleotide polymorphism (SNP) arrays.

Results: 3-year event-free survival (EFS) of 184 available patients was 39±4% with a 3-year cumulative incidence of relapse (CIR) of 56±6% (median observation time 55.5 months). BM of 83 patients matched for BM-MRD analysis showed BM-MRD negative patients had a 3-year EFS of 67±9% (CIR 39±7%), but only 27±7% (CIR 71±7%) for BM-MRD positive patients ($p = 0.001$). Although there was a correlation with DTC numbers at diagnosis, only the post-induction BM-MRD status-maintained significance for EFS in multivariate analysis (hazard ratio 2.27, $p = 0.031$). Genomic analysis found a higher relapse incidence in patients whose tumors showed a gain or loss on chromosome 1q and aberrations in the TERT and PTPRD genes. Patients with tumors lacking these genomic markers and with BM clearing had the most favorable outcome (3-year EFS 92±7%) whilst those with the presence of two or more of the above mentioned genomic markers and positive BM-MRD fell into an ultra-high-risk group (3-year EFS 0%, $p < 0.001$).

Conclusion: BM-MRD detection after induction chemotherapy significantly correlated with a poor survival in our cohort of stage M neuroblastoma patients. Furthermore, our results provide the first evidence that early disease progression may be linked to certain genomic markers. The combination of BM-MRD monitoring and genomic profiling enables early risk assessment and merits further evaluation.

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Role of SSTR Imaging in Neuroblastomas for Possible PRRT

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Introduction: Neuroblastomas are known to express somatostatin receptors and these are potential targets for future imaging with somatostatin receptor (SSTR) agents. Imaging can be used to assess the feasibility of peptide receptor radionuclide therapy (PRRT) in refractory cases. The purpose of the study was to evaluate and compare the SSTR expression by 99m Tc labelled hydrazinonicotinyl-Tyr3-octreotide (HYNIC TOC), Metaiodobenzylguanidine (MIBG) uptake by 131II MIBG and the metabolic activity of a tumour by 18 F Fluorodeoxyglucose positron emission tomography-computed tomography (FDG PET CT) in diagnosed neuroblastoma cases

Materials and Methods: 99m Tc HYNIC TOC, 131I MIBG and FDG PET CT scans performed in 29 patients were reviewed and compared for concordance (or discordance) of findings. All scans were done within a 2-week time period for each patient.

Results: A total of 23 children with a diagnosis of Neuroblastoma underwent 99m Tc HYNIC TOC, 131I MIBG and FDG PET CT scans. In the rest 6 patients, paired 99m Tc HYNIC TOC and 131I MIBG scans were only done. In 29 patients with paired 99m Tc HYNIC TOC and 131I MIBG scans, 22 (76%) patients showed concordant results. In 7 (24%) patients 99m Tc HYNIC TOC showed more lesions. In 2 patients with positive 99m Tc HYNIC TOC, 131I MIBG was negative. In 23 patients with paired 99m Tc HYNIC TOC and FDG PET CT scans, 19 (83%) patients showed concordant results. In 2 patients, FDG PET CT was positive while 99m Tc HYNIC TOC was negative. In 2 patients with positive 99m Tc HYNIC TOC, FDG PET CT was negative. 131IMIBG showed concordant results in only 13 (57%) patients.

Conclusion: SSTR expression did not correlate with the expression of MIBG or GLUT receptor suggesting an independent expression. SSTR and FDG PET CT showed more concordance than SSTR and MIBG scans. Our study demonstrates that most of the tumors (both primary and metastatic sites) showed a SSTR expression and hence the feasibility of using this receptor for treatment.

SSTR imaging with a PET tracer like68 Ga DOTA Octreotate or SPECT tracer like 99mTc HYNIC TOC appears promising.

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The RRM2-PHF6 Complex Protects Neuroblastoma Cells from DNA Damage Accumulation to Install a Replication Stress Resistance Phenotype

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Introduction: MYCN amplified neuroblastoma (MNA-NB) often present with 1p deletions, 2p gains in addition to MYCN amplification (2p24.2) and 17q gain. Given recent work highlighting the importance of non-mutated dependency genes in other tumor types, we decided to identify copy number driven genes in NB.

Methods: We used a bioinformatics approach based on DNA copy number and gene expression analysis and ranking for prognostic power. Using in vitro functional assays, we tested tumor dependency of candidate genes and responses to targeted drugs.

Results: We identified RRM2 (2p25.1), and CHD5 (1p36) amongst the highest ranked dosage sensitive genes in MNA-NB. For RRM2, we propose three additional levels of expression regulation through combined 1p loss and 2p and 17q gain: (1) direct regulation by MYCN as shown by ChIP-sequencing, (2) CHD5 loss driven upregulation supported by correlation analysis in NB transcriptomes highlighting the previously established regulatory connection for CHD5 and WEE1 and for WEE1 and RRM2, and (3) BRCA1 (17q21.31) controlled RRM2 upregulation as revealed by stable BRCA1 knockdown experiments. Stable RRM2 knockdown leads to attenuated NB cell growth, colony formation capacity, increased apoptosis and induced a FOXM1 (down) and TP53 (up) gene signature. NB cells were sensitive to the direct RRM2 inhibitor triapine and MK-1775 (WEE1 inhibitor) and exhibited increased DNA damage as evidenced by increased yH2AX and p-RPA32 levels. Using mass spectrometry, we identified RRM2 as a robust interaction partner of the 'plant homeodomain zinc finger 6' (PHF6) protein. While loss of PHF6 was reported to render T-ALL cells genomically instable, we observed very high PHF6 expression levels and nucleolar staining in NB cells versus other cancer types. Upon PHF6 knock down RRM2 levels were downregulated, while FOXM1 downstream targets were enriched and a JQ1 signature surfaced. Using a dβh-MYCN zebrafish model we are currently testing the in vivo effects of combined RRM2 overexpression in neuroblastoma tumor formation.

Conclusion: We identified RRM2 as a druggable copy number driven dependency gene in MYCN-amplified NB and propose that PHF6 acts as epigenetic adaptor molecule to facilitate RRM2 dependent DNA repair to protect MNA NB cells from excessive replication stress.

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Liquid Biopsy in Neuroblastoma: ctDNA Analysis for Visualizing Intra-Tumor Heterogeneity and Follow-Up Monitoring

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Intra-tumor heterogeneity (ITH) is a major challenge for the detection of relevant genomic aberrations in primary tumor (TU) biopsies. Hence, there is a yet unmet need for alternative analytic procedures to minimize sampling error in tumor patients. Analyzing cell-free tumor DNA (ctDNA) from more easily accessible patient biomaterial could surmount these challenges. However, in order to apply ctDNA-based results in the clinics, we need to find out how suitable this DNA actually is for identifying relevant genomic aberrations of the TU.

Material and Methods: We compared the genomic results (lcWGS, HD-SNPa) of DNA from TU biopsies, disseminated tumor cells (DTCs) from the bone marrow (BM) and/or ctDNA from peripheral blood (PB) and/or BM plasma, of stage M neuroblastoma patients. In addition, PCR based analysis of ctDNAs was used to follow-up on the MYCN amplification during disease course.

Results: Twelve out of 24 patients showed identical genomic aberrations in the analyzed sources (tumor, DTCs, ctDNA from PB/BM) whereas in the other 50%, besides a high number of concordant somatic copy-number aberrations (SCNAs), discordant SCNAs between the three tissues of origin were found. In the discordant group the mean discordance rate was 5.0 SCNAs per patient with a mean total SCNA number of 14.6 as compared to a significantly lower mean SCNA number of 7.5 in the non-discordant group. In all compartments unique aberrations were found, however, only 3 out of 19 patients showed unique aberrations in the primary TU that were not present in the DTC or ctDNA samples. Only one out of six patients with discordant trio-samples showed unique SCNAs in the tumor while in five patients additional unique SCNAs were detected in the ctDNA from PB and/or BM that were not found in the primary tumor. Furthermore, PCR based detection of MYCN copies in multiple follow-up PB plasma samples provided additional relapse-relevant information.

Conclusion: At diagnosis, circulating tumor DNA and DTCs can provide a more complete genomic picture as compared to the primary tumor. At follow up, detection of gene amplifications allows minimal-residual disease monitoring, providing addition information to high sensitive BM diagnosis especially in non-BM relapse situations.

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Current Strategies for Testing Dosage Sensitive Dependency Genes in the Zebrafish Neuroblastoma Model

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Neuroblastoma (NB) has been proposed as a copy number driven cancer, given the low mutation frequency and highly recurrent copy number gains and losses. It has been shown for other tumor entities that recurrent copy number gains can guide experiments towards identifying dosage sensitive dependency genes. Therefore, the search for such genes, e.g. on 17q, could offer novel therapeutic targets for both MYCN amplified (MNA) and MYCN non-amplified (MNnA) NB. Using a combination of integrated bioinformatic analysis and zebrafish modeling, we provided convincing evidence for a role of the DNA helicase and BRCA1 interacting protein BRIP1 in MYCN driven NB formation (Vanhauwaert et al., in preparation). Despite the success of this approach, we encountered several hurdles that impacted the efficiency of candidate gene testing in this model. First, we encountered low survival of transgenic animals during early stages of development which has been overcome by using rotifers for initial feeding. Second, I-SceI meganuclease-mediated transgenesis ensures integration in one single site in the zebrafish genome and is well suited to generate stable overexpressing transgenic lines but is less efficient for the mosaic approach to test for immediate effects on tumor formation after injection of the overexpression construct. Therefore, we are now testing the TOL2 transposase-mediated transgenic approach, which causes integration of transgenes in several genomic sites and is considered more potent for initial testing of overexpression effects of transgenes in a mosaic approach. Together with an improved method for injection allowing to generate larger cohorts for follow up and increased capacity of our facility, we are now further exploring effects of constructs for stable and regulable overexpression of several of our candidate genes including SOX11, TBX2 and RRM2 as 2p candidates; BRIP1, BIRC5 and further 17q candidate genes; and FOXM1 as putative master regulator. Also, the generation of an animal model recapitulating the MNnA high-risk subtype is a major challenge and could be tackled using zebrafish transgenics. To this end we have prioritized 11q candidates and will evaluate the use of a CRISPR/Cas9 vector system for tissue-specific candidate gene disruption in combination with one or more additional drivers.

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Cross-Talk Between Neuroblastoma Cells and Their Microenvironment at the Metastatic Bone Marrow Niche

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Background: Neuroblastoma (NB) shows a strong tendency to metastasize to bone marrow (BM). Once settled in the BM, tumor cells can acquire chemoresistance, become dormant and hide until they are triggered to proliferate again. This is a likely key factor in disease relapse, which is unacceptably high for NB. Therefore, better understanding of dormancy control is essential to improve NB therapy. In some cancer types, bone metastatic tumor cells create a tumor-protective microenvironment by recruiting and affecting mesenchymal stromal cells (MSCs) that, in turn, have been shown to induce tumor cell dormancy. We aimed to study whether NB-cells influence MSC function in the BM-metastatic niche.

Methods: Diagnostic BM aspirates from patients with metastatic-NB (stage 4) were compared to patients with localized-NB (stage 1-3). We also examined follow-up BM samples of metastatic-NB patients: post-ASCT, at relapse and after treatment for relapsed disease. MSCs were counted by flow cytometry using the classical CD34-, CD45-, CD90+, CD105+ phenotype. To get insight in phenotypical changes within the heterogeneous MSC population, we also analyzed the distribution of subtypes, characterized by surface markers such as CD271 (nerve growth factor receptor) and CD146 (melanoma cell adhesion molecule). Colony-forming unit-Fibroblast (CFU-F) assays were used as a functional method to quantify the frequency of stromal progenitors present in BM samples.

Results: Immunophenotypical analyses revealed that NB patients with BM metastases have a higher frequency of MSCs in their BM than patients without BM metastases, at diagnosis as well as relapse. Of particular interest is a distinctive CD271- CD146+ subtype of MSCs, which appears to be unique for patients with metastatic-NB. Of note, these cells did not express NB-markers, hence are unlikely to be NB-derived. Functionally, NB-primed MSCs showed enhanced colony-forming ability.

Conclusion: Together, these findings suggest that metastasized NB-cells affect MSC in their microenvironment. Further characterization of MSC subtypes within the BM-metastatic niche is ongoing and may uncover tumor-supporting subsets. Ultimately, increasing our understanding of MSC-NB cross-talk could provide targets for potential combination therapies that act on the BM microenvironment to enhance NB-cell chemo-sensitivity.

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FGFR1 N546K is Oncogenic Driver Mutation in Neuroblastoma

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By a recent high-throughput sequencing-based study of 99 neuroblastomas, we have highlighted that there are potentially clinically relevant alterations in target genes present in individual patient. We found the hotspot mutation N546K of FGFR1 in one patient. Recent scientific evidences report an increment of FGFR1 mutated clones in neuroblastoma at relapse and FGFR1 N546K occurrence in diverse tumors: neuroblastoma, glioma,medulloblastoma, angiosarcoma and pheochromocytoma (ID:COSM19176). The study of the role of the low frequency mutations in FGFR1 may offer an opportunity to improve patient risk stratification and survival in high-risk neuroblastoma.
By using two freely available gene expression micro-array data, including respectively 689 (GEO ID: GSE45547) and 102 (GEO ID: GSE3446) neuroblastoma samples, we found that high FGFR1 gene expression was associated with inferior overall survival (P=0.04) and relapse-free survival (P=3.1x10-5). In this study we analyzed the biological role of FGFR1 in neuroblastoma. First, we lentivirally transduced short hairpin RNA against FGFR1 into human neuroblastoma cells (SKNBE2 and SHSY5Y) and verified that the cells FGFR1 depleted show a reduced cell viability and invasion. Therefore we stable transfected wt FGFR1 or FGFR1 N546K in neuroblastoma cells and observed that FGFR1 N546K enhanced cell proliferation and invasion more than wt FGFR1. Upon treatment with AZD4547, a candidate drug predicted to impair wt FGFR1 signaling, we observed that mitogen-activated protein kinase (MAPK) signaling is impaired in both wt FGFR1 or FGFR1 N546K expressing cells but FGFR1 N546K expressing cells show enhanced activation of phosphatidylinositol 3' -kinase(PI3K)-Akt signaling. These evidences highlight FGFR1 N546K might generate mechanisms of resistance to wt FGFR1 inhibitors through Akt pathway.
These data provide evidences that activating mutations of FGFR1 may constitute a novel target for therapeutic approaches in neuroblastoma.

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Post-GWAS Functional Study Identifies Causative Variants at 2q35 Locus Associated with High-Risk Neuroblastoma and Reveals Full-Length BARD1 as Tumor Suppressor

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Background: Genome-wide association studies (GWAS) have previously demonstrated that single nucleotide polymorphisms (SNPs) at the BARD1 locus are associated with the development of high-risk neuroblastoma. However, implications of these genetic findings in providing new insights in neuroblastoma biology remain to be assessed.

Methods: We performed genotype imputation of the BARD1 locus using GWAS data from 556 high-risk neuroblastoma cases and 2575 controls of European-American ancestry. Significant associations were replicated in three ethnically diverse case series: African-American (159 high-risk cases; 2095 controls); Italian (187 high-risk cases; 742 controls); Spanish (39 high-risk cases; 60 controls). To identify putative causal variants, a weighted P-value (wP) based on the annotation of the genome-wide-associated SNPs with H3K427ac ChIPseq and DNAse hypersensitive sites data derived from 32 neuroblastoma cell lines, and alteration of transcription factor binding sites was calculated. In vitro studies were performed in neuroblastoma cell lines following genetic manipulation of BARD1 to assess biological significance.

Results: We identified two independent genome-wide neuroblastoma-associated loci including 138 potential causative SNPs. The most significant SNPs at the two loci independently contributed to neuroblastoma risk (rs17489363: P=1.90x10^-16; wP=50.22; OR:1.72, 95%CI:151-1.96 and rs1048108: P=3.49x10^-10; wP=41.96; OR:0.63, 95%CI:0.55-0.73). SNP rs17489363 replicated in all case series: African-Americans (P=4.25x10^-8); Italians: (P=2.96x10^-8); Spanish: (P=0.03). SNP rs1048108 replicated only in African-Americans (P=0.004) and Italians (P=0.03). Particularly, rs17489363 in the promoter of full-length BARD1 altered binding sites of the transcription factor HSF1 (P=8.6x10-6) and correlated with low expression of full-length mRNA BARD1 in two datasets of 84 (P=0.036) and 73 (P=0.007) neuroblastomas and protein BARD1 in 16 (P=0.027) lymphoblastoid cell lines from healthy individuals.
In SHSY5Y and SKNSH cell lines, upon depletion of full-length BARD1, we observed, an increase of proliferation and invasion cells and an accumulation of DNA damage by increasing of γH2AX phosphorylation. Moreover, DNA damaged cells avoided cell cycle arrest at the G1/S checkpoint and apoptosis through p53Ser-15 phosphorylation disruption and entry in cell cycle faster via downregulation of cyclin B/Cdk1.

Conclusion: We have identified two potentially causative SNPs at the BARD1 locus associated with predisposition to high-risk neuroblastoma and have shown that full-length BARD1 has tumor suppressor functions.

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Participation of Children and Adolescents with Neuroblastoma in Early Phase Trials Within the ITCC Network

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Background: Phase I trials play a key role in the evaluation of novel agents for children with advanced cancer. We aimed to describe the characteristics of neuroblastoma patients in a cohort of children/adolescents phase I trials across eight centres of the Innovative Therapies for Children with Cancer (ITCC) network.

Methods: Retrospective study of patients aged <18 years at enrolment in their first phase I trial between 2000-2014.

Results: Out of 270 patients treated in phase I trials in eight European institutions, 33 had relapsed/refractory neuroblastoma, accounting for 12.2% of all solid tumours and 23.5% of extracranial solid tumours.

Median overall survival (OS)±standard deviation was 13.4±2.5 months for neuroblastoma patients and 6.13±0.5 for non-neuroblastoma patients (p=0.06). The proportion of neuroblastoma patients alive at 90 days was 84.8% and 72.9% for non-neuroblastoma patients (n.s.). In neuroblastoma patients, best response was complete/partial in 4 patients (12.5%) and stable disease in 11 (34.4%) compared to 9.2% and 24.7% in non-neuroblastoma patients (n.s.).

In the analysis of prognostic factors, neuroblastoma patients were younger, had worse performance status at study entry, more metastatic sites, more anaemia, lower neutrophil count & platelets, higher transaminases and lower albumin compared to non-neuroblastoma patients (p<0.05).

Conclusions: 1) Despite poor outcome for relapsed and refractory neuroblastoma, the number of neuroblastoma patients is low, particularly within the context of the number of relapses expected within these centres. 2) The baseline characteristics of neuroblastoma patients treated in dose-finding trials differ from other childhood cancers treated on these trials, showing a higher disease burden, worse performance and organ function. 3) Despite, a meaningful proportion of neuroblastoma patients derive benefit from participation in dose-finding trials showing objective responses or disease stabilisation. Currently, the portfolio of early clinical trials is increasing globally and includes more biologically relevant drugs for neuroblastoma, which could lead to increased number of patients treated and increased patient benefit.

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Deregulated ALK Signaling Impairs Early Sympathetic Lineage Differentiation: The Path to Neuroblastoma?

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Background: Neuroblastoma (NB) is a pediatric malignancy derived from the abnormal differentiation of the sympathetic nervous system (SNS). The Anaplastic Lymphoma Kinase (ALK) gene is frequently altered in NB and ALK-F1174L is described as the most oncogenic driver mutation. Previously published data from our laboratory suggest that ALK activation may impair the neural crest progenitor cell (NCPC) differentiation potential.

Aim: To clarify the impact of ALK deregulation in sympathetic progenitor differentiation.

Methods: Transgenic mice carrying a conditional LSL-ALK-F1174L allele were crossed with Sox10-Cre mice restricting its expression to migrating NCPC. The impact of ALK-F1174L on the development and differentiation of the sympathetic trunk was analyzed by immunofluorescence (IF). Endogenous ALK expression was detected by in situ hybridization (ISH, RNAscope® technology).

Results: Embryos expressing the ALK-F1174L mutation displayed lethality from E12.5 with impaired hematopoietic development. In addition, sympathetic ganglia (SG) formation and differentiation was affected in Sox10-Cre/ALK-F1174L embryos. We first observed an enlargement of the SG with a perturbed architecture and cellular disorganization at E10.5 and E11.5. Then, early sympathetic differentiation, highlighted by the transition from Sox10+/Phox2b- NCPC toward double positive progenitors, and subsequently to Sox10-/Phox2b+ sympathoblasts, was impaired in ALK-F1174L mutant embryos. Indeed, SG displayed a strong increase in the proportion of Sox10+/Phox2b- progenitors in addition to a reduction in the ratio of Sox10-/Phox2b+ neuroblasts both at E10.5 and E11. 5. Third, ALK-F1174L blocked noradrenergic differentiation as revealed by the small proportion of Phox2b+ sympathoblasts expressing Tyrosine Hydroxylase in ALK-F1174L mutants. Moreover, at E10.5, ALK-F1174L strongly increased the proliferation of Phox2b+ progenitors, while no difference was observed at E11.5. Finally, we analyzed the endogenous ALK expression pattern by ISH in Phox2b+ progenitors to determine the onset of ALK expression in neuroblasts of the developing SNS. While ALK mRNA was not detected in Phox2b+ cells at E9.5, an ALK-specific signal was observed starting from E10.5 in the sympathetic trunk.

Conclusion: Our data indicate that ALK-F1174L, the most potent ALK mutation in NB, could play a decisive role in blocking early sympathetic lineage differentiation in addition to enhancing neuroblasts proliferation, both mechanisms being potential crucial elements of NB oncogenesis.

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Mesenchymal Stem Cell Delivery of Therapeutic Molecules as a Novel Gene Therapy Approach for Neuroblastoma

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Neuroblastoma is a childhood cancer with a 5-year survival rates of only about 40%. Drug-resistant neuroblastoma remains a major challenge in pediatric oncology and novel and less toxic therapeutic approaches are urgently needed to improve survival and reduce side effects of traditional therapeutic interventions.

Mesenchymal stem cells (MSCs) are an attractive candidate for cell and gene therapy since they can be easily isolated, manipulated and are not immunogenic. MSCs are attracted by and infiltrate the tumour stroma (Studeny et al, 2004). This feature has been exploited by creating genetically modified MSCs that are able to combat cancer by delivering therapeutic molecules.

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor superfamily that induces apoptosis of target cells through the extrinsic pathway. TRAIL selectively causes apoptosis in malignant cells without affecting normal tissues (Loebinger et al, 2009).

In this study, we investigated whether MSCs engineered to express TRAIL could be used to kill neuroblastoma cells in vitro and in vivo. For this purpose, human mesenchymal stem cells were transduced with a lentiviral vector encoding TRAIL and incubated in a 1:1 ratio with classical or primary neuroblastoma cell lines. Apoptosis was evaluated using Annexin V/DAPI staining and flow cytometry. To assess the killing potential of MSCs-TRAIL in vivo, neuroblastoma cells were injected subcutaneously into the flank of NOD/SCID mice to establish a xenograft tumour model. MSCs-TRAIL cells were labelled using the fluorescent lipophilic dye DiR and delivered via intraperitoneal injections on days 1,7,14 after tumor cell transplantation. Fluorescent MSCs were tracked in vivo using the IVIS imaging system. Our preliminary results demonstrate that MSCs-TRAIL induce apoptosis of neuroblastoma cells in vitro. Neuroblastoma cells can be sensitised to MSC-TRAIL using Bortezomib and MSC-TRAIL are able to home into neuroblastoma tumours when administered intraperitoneally in vivo. We are currently evaluating the anti-tumour activity of MSCs-TRAIL in in vivo experiments. if successful, could pave the way to a new and less toxic therapeutic strategy for this deadly form of childhood cancer.

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Restoration of The Molecular Clock Is Tumor Suppressive in Neuroblastoma

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MYCN activation is a hallmark of advanced neuroblastoma (NB). It is also a potent regulator of metabolic reprogramming that favors NB adaptation to its microenvironment. New connections between MYC oncogenes, molecular clock, and cell metabolism have been recently uncovered. However, little is known about the mechanisms that allow MYCN to reprogram NB tumors. The core molecular clock is the heterodimer CLOCK/BMAL1, which activates circadian gene expression by binding E-box sequences in the DNA. Importantly, the retinoic acid related orphan receptor (RORα) and REV-ERBα directly regulate gene expression of BMAL1, playing key roles in circadian rhythms, metabolism and inflammation. Recently, synthetic ligands RORα agonists (SR1078) have been optimized with great therapeutic potential.

We found that the expression of the main regulators of the clock machinery, the BMAL1 activator RORα and the BMAL1 repressor REV-ERBα, is profoundly altered in MYCN-amplified NB (890 patients). Low RORα and BMAL1 expression and high REV-ERBα expression are strongly associated with MYCN amplification and independently predict poor outcome (RORα OS p= 0.0001). MYCN directly binds to REV-ERBα and RORα promoters. Conditional MYCN overexpression upregulates the expression of REV-ERBα and represses RORα and BMAL1 in NB cells. In contrast, MYCN silencing effectively restores their expression. Importantly, RORα activation via SR1078 inhibits cell survival, promotes apoptosis, and sensitizes MYCN-amplified cells to conventional therapy by restoring BMAL1 expression and oscillation. In addition, both SR1078 and RORα genetic overexpression significantly inhibit tumor growth of MYCN-amplified xenografts by restoring BMAL1 (p=0.013). Furthermore, low-dose SR1078 from 10 days until 5 weeks of age delays tumor growth in TH-MYCN+/+ mice (p=0.004). Interestingly, RORα blocks MYCN-driven transcription of key lipogenic enzymes and de novo fatty acid (FA) synthesis and desaturation (by isotope labeling and GC-MS). Furthermore, culture media supplemented with selected FAs completely rescues cell viability of cells treated with SR1078.

Our data indicate that the molecular clock is profoundly disrupted in MYCN-amplified NB. Its restoration via RORα activation blocks NB tumor growth by restoring BMAL1 and opposing MYCN-driven lipogenesis. Our study is the first exploration of therapeutic strategies for boosting the expression of tumor-suppressing clock genes, such as RORα.

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MYC- and MYCN-Dependent Transcription: Linking Transcription to Ubiquitin-Mediated Degradation

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MYC proteins have global functions in transcription and can act as general amplifiers of transcription. To "amplify" transcription, MYC proteins need to be able to accelerate multiple individual steps of early transcription. To understand how this occurs, we have performed proteomic analyses of MYCN- and MYC-associated proteins. The data show that both MYCN and MYC participate in multiple protein complexes, consistent with effects at different steps of early transcription. Their analysis also shows that MYC function is intimately linked to its turnover by the proteasome system.

For example, both MYC and MYCN form a complex with PAF1, an elongation factor of RNA Polymerase (Buchel et al., 2017; Jaenicke et al., 2016). Binding of MYC to many promoters depends on PAF1. Since PAF1 binds directly to RNA Polymerase, this suggests that MYC binds to promoters when RNA Polymerase is already bound. In the absence of PAF1, RNA Polymerase still exits from the promoter, but its ability to elongate is compromised. MYC turnover by the proteasome correlates with release of PAF1 onto RNA Polymerase. Our data suggest that the recruitment of MYC/PAF1 complex to promoters and the subsequent degradation of MYC transfers PAF1 onto RNA Polymerase to promote transcription elongation.

Analysis of the proteomic data also shows that there is a N-MYC-dependent mechanism that terminates transcription when RNA Polymerase stalls, causing transcriptional repression. This mechanism depends on binding of N-MYC to the ubiquitin-specific protease, USP11, which recruits the BRCA1 tumor suppressor protein to core promoters. USP11 is recruited by N-MYC that is dephosphorylated at T58. Since phosphorylated T58 is recognized by the FBXW7 ubiquitin ligase, our data argue that turnover of MYC also counteracts recruitment of BRCA1 to promote transcriptional activation.

Our data suggest that the regulated turnover of MYC and N-MYC at promoters controls the balance between transcription elongation and termination. We propose that controlling N-MYC turnover using small molecules that target MYC-associated ubiquitin-specific proteases not only controls N-MYC levels, but also allows to selectively control MYC´s transcriptional output.

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Comprehensive Evaluation of Context Dependence of the Prognostic Impact of MYCN Amplification in Neuroblastoma: A Report from the INRG Project

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Background: The negative prognostic implications of MYCN amplification (MYCN-A) in neuroblastoma have long been established. However, the prognostic impact of MNA is not uniform, and the extent to which the prognostic impact of MYCN-A depends upon the context of clinical and biological factors has not been fully characterized. Our objective was to describe the impact of tumor MYCN-A (compared to normal MYCN) on outcome between subgroups of binary clinical/biological factors.

Methods: Using the INRG database, we constructed a series of univariate Cox proportional hazards models of overall survival (OS) to obtain hazard ratios (HR) for MYCN-A within each subgroup and for each clinical/biological factor and calculated the absolute difference in the HR for MYCN-A between the two subgroups of a given factor. In bivariate Cox models, we recalculated the HR of MYCN-A after controlling for each factor, respectively. Analyses were performed in a test cohort and validation cohort, derived by randomly splitting the INRG dataset.

Results: In the overall test cohort (n=6223 with known MYCN status), the HR for death for patients with MYCN-A was 6.3 (95% CI 5.7-7.0; p<0.001). By factor subgroups, the HR for MYCN-A was greatest for age <18 months (HR=19.6); differentiating grade (HR=15.4); non-INSS 4 (HR=12.4); low ferritin (HR=11.3); absence of segmental chromosomal aberration (SCA) (HR=10.7); and absence of 11q LOH (HR=10.3). Age at diagnosis had the largest absolute difference in HRs for MYCN-A between subgroups (absolute difference 16.6; MYCN-A HR=19.6 if <18 months vs HR=3.0 if ≥18 months). HRs for MYCN-A remained significant after controlling for each other factor. Compared to the univariate HR=6.3 for MYCN-A, controlling for 11q status increased the MYCN-A HR to 7.3. Controlling separately for each other factor decreased the MYCN-A HR, with the greatest attenuation seen for LDH (MYCN-A HR=3.1 after controlling for LDH), INSS stage (HR=3.2), and SCA (HR=3.3). Patterns seen in the validation cohort were similar to the test cohort.

Conclusions: MYCN-A has a disproportionate prognostic impact on OS in the context of otherwise more favorable factors, such as young age and low stage. MYCN-A remained a significant prognostic factor after controlling for other clinical and biological factors.

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Measurement of A Panel of Eight Urinary Catecholamine Metabolites by Liquid Chromatography-Tandem Mass Spectrometry at The Onset of NB Disease

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Background/Objectives: Urinary Vanillylmandelic acid(VMA) and homovanillic acid(HVA) are commonly measured in urine of patients with neuroblastoma(NB) at the onset. Their diagnostic accuracy has been proven to be very high in all stages, particularly in 4 and 4S. It has also been demonstrated that VMA/HVA ratio is an indipendent prognostic predictor in patients at diagnosis with localized disease without MYCN amplification.

Other groups have demonstrated that the analysis of a largest panel of urinary catecholamine metabolites can improve their diagnostic accuracy. Nevertheless, few data are currently present on their prognostic significance. Recently Verly at all have demonstrated that 3-methoxythiramine(3-MT) is an independent prognostic biomarker associated with high risk disease and poor outcome.

Design/Methods: The standard method for catecholamines measurement is high pressure liquid chromatography with electrochemical detection(HPLC-EC) that provides high sensitivity but is time consuming. The use of liquid chromatography associated to tandem mass spectrometry(LC-MS/MS) could guarantee very rapid and specific analysis from limited sample volumes and could be useful in the evaluation of a largest panel of targeted metabolites. Recently commercial kits have started to be available. We have analyzed a targeted profile of 8 urinary catecholamine metabolites (VMA, HVA, epinephrine [E], norepinephrine [NE], dopamine, metanephrine [MN], normetanephrine [NMA] and 3-MT) in urine samples of 79 patients with NB at the onset (age 2months-10years) at different stages (17 stage 1-2, 12 stage 3, 34 stage 4 and 16 stage 4S) by LC-MS/MS by using a commercial kit.

The combined use of multivariate statistical analysis and cluster analysis allows to distinguish different stages in a narrow set of metabolites.

Results: Regarding association with prognosis, a statistically significant association could be found only for NMN (p=0.01), MN (p=0.05) and VMA/HVA (p=0.0009). NMN and MN levels were higher at onset in patients that reached a complete or partial remission if compared with patients with relapsed/progressive disease, still on treatment or dead of disease.

Conclusion: NMN and MN are byproducts of the same metabolic pathway starting from NE and having as a final product VMA. This finding confirms the hypotheses that a noradrenergic pathway could be highly expressed in tumors with favorable prognosis.

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Targetable Genetic Alterations in High-Risk NB Patients. A SIOPEN Study

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Background: In high risk neuroblastoma (NB), new treatment strategies are urgently required to improve outcome. We sought to determine the frequency of genetic alterations (SNVs/Indels) in genes considered to be targetable and/or to play a role in oncogenesis in high risk NB at diagnosis.

Methods: Diagnostic NB samples from 401 patients enrolled in the SIOPEN-HR-NBL1 trial were analyzed. Negative controls: 37 normal DNA from healthy donors.

The gene panel consists of 85 genes known to play a role in NB oncogenesis, with 50 genes considered directly targetable.

A targeted sequencing approach (True-seq custom amplicon; TSCA®) was used to sequence exonic regions (target size = 0.35 MB) yielding mean 9Mio raw reads, mean read depth of 2960x per sample and >98.75% at 50x coverage. Variant (SNVs/Indels) and copy number analysis was performed using VarScan2 and HaplotypeCaller tools respectively. European population frequency (0.002) from gnomAD database and 37 normal samples were used to remove background polymorphisms.

Results: Primary variant analysis resulted in a total of 2736 SNVs/Indels with minimum mutant allele frequency (MAF) of at least 5% (MAF below 20%/10% for 84%/60% of SNVs/Indels, respectively). Per NB sample, a mean of 7 SNVs/Indels (95% CI -5.4-8.3; range 0-143) was observed with the most frequent events in known cancer hotspot mutations in the genes ALK(6.2%), TP53(4%), HRAS(1,5%), PTPN11(1,5%) SNVs/Indels with low MAF (<20%) was observed largely in 8 genes (TENM4, CHD7, TNEM2, NF1, SMARCA2, PTPRD, PTCH1, ATM).

Fifty-one percent (206/401) of NB samples harbored at least one pathogenic genetic alteration (COSMIC and/or other predicted pathogenic alteration): 57% had SNVs/Indels in genes considered to be targetable.

Although not statistically significant, a higher number of SNVs/indels were observed in stage 4 versus non-stage 4 tumors, or MYCN-amplified versus non-MYCN-amplified tumors.

No statistically significant difference in the survival of patients with NB displaying higher versus with lower numbers of genetic alterations was observed (>/< 7 SNV/Indels, p= 0,9892).

Conclusion: Distinct targetable genetic alterations could be observed in 57% of high risk NB patients at diagnosis, an important information to take into account when considering introduction of targeted therapy approaches in upfront treatment strategies.

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Gamma Delta (γδ) T Cell-Based Immunotherapy Augments Dinutuximab And Low Dose Chemotherapy to Regress Neuroblastoma Tumors In Vivo

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Background: Most cellular immunotherapies are based on the expansion and engineering of αβ T cells. However, γδ T cells are an attractive immunotherapy candidate because of their intrinsic anti-tumor properties, specifically their role in antibody-dependent cellular cytotoxicity (ADCC) and recognition of stress antigens via the receptors CD16 and NKG2D. We have developed a method for expanding, storing, and genetically engineering γδ T cells and have focused on the use of these cells in neuroblastoma.

Methods: γδ T cells from neuroblastoma patient apheresis products are expanded in serum free medium to >70% of the culture. Patient expanded γδ T cells are equally cytotoxic compared to expansion from normal donors. Our method to freeze cells between days 12-14 of expansion results in retained cytotoxicity by cells pre- and post-freezing with greater than 70% viability post-thaw.

Results: Patient expanded γδ T cells are >90% positive for surface expression of NKG2D and CD16, and cytotoxic against 5 neuroblastoma cell lines. Stress antigens MICA/B, ULBP1, and ULBP2/5/6, which are targeted by γδ T cells, are upregulated in vitro on IMR5 cells for 6 hours after a 1-hour exposure to temozolomide (TMZ). Further, combining γδ T cells expressing CD16 with dinutuximab induces 30% increased neuroblastoma cell death compared to γδ T cells alone and enhances secretion of IFNγ and TNFα. Therefore, to test their in vivo effectiveness, NSG mice harboring palpable IMR5 xenografts (125 mm^3) were administered various combinations of expanded γδ T cells, dinutuximab, and TMZ. Results show γδ T cells alone do not provide an anti-tumor benefit, but tumor regression is achieved when incorporating γδ T cells into dinutuximab and TMZ combinations. This advantage is observed at lower TMZ doses, whereas at higher TMZ doses (>60 mg/kg) chemotherapy dominates the anti-tumor effect.

Conclusion: Expanded γδ T cells augment dinutuximab and TMZ responses in neuroblastoma, allowing for decreased chemotherapy burden without forgoing effectiveness. Ongoing methods to enhance γδ T cell products include transduction with TMZ resistance genes and tumor-homing CARS. Our method to expand unmodified γδ T cells is currently in adult trials, supporting the immediate translational potential for this cellular therapy for neuroblastoma.

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Immunotherapy with Anti-GD2 Antibody ch14.18/CHO±IL2 Within The HR-NBL1/SIOPEN Trial Improves Outcome of High-Risk Neuroblastoma Patients Compared to Historical Controls

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Background: Randomization of immunotherapy versus standard was not possible in the HR-NBL1/SIOPEN trial. In order to explore an impact of immunotherapy on outcome, we used trial patients prior to availability of ch14.18/CHO as a control.

Patients: Trial patients received rapid COJEC, two courses of TVD if needed, surgery, HDT/SCT (BuMel or CEM) and radiotherapy. MRD treatment (MRDT) consisted of isoretinoin alone from 2002 - 2009 (control population, CP) and from 2009 - 2013 with ch14.18/CHO (5 cycles ch14.18/CHO, ± IL2) (immunotherapy population, IP).
Patients (844) were ≤9 months between diagnosis and HDT/SCT, PR or better prior to HDT/SCT and had no progression after HDT/SCT until start MRDT (median time 109 days) (CP 466, IP 378).
Sex, age groups, stage 4 MYCN amplified (MNA) and response prior HDT/SCT were balanced between cohorts. Imbalanced (IP vs CP) were metastatic compartments (MC>1) (80% vs 71%; p=0.0034)), TVD given (12% vs. 32%; p<0.001), MNA localised disease (8% vs. 13% p=0.0019), type of HDT = CEM (8% vs. 45%; p<0.001)), time point of surgery prior day120 after diagnosis (60% vs. 73%; p<0.0001) and radiotherapy given (94% vs. 88%; =0.0034). Median follow up was 5.8y (0.05-13.8y).

Results: The 5y-EFS was 57%±3% for the IP and 42%±2% for the CP (p<0.001). Univariate analysis of risk factors identified no 5y-EFS difference for sex, stage 4 ± MNA and was borderline for radiation given (5y-EFS if yes: 50%±2%, no: 38%±6%, p=0.073). Risk factors with 5y-EFS differences were age, stage, MC>1, delayed surgery, response pre HDT/SCT and type of HDT. However, multivariate analysis identified patients at a higher risk without immunotherapy (p=0.0002, HR 1.573), with CEM (p=0.0029; HR 1.431), response below CR prior MRD (p=0.0043, HR 1.494) and >1MC at diagnosis (<0.001 HR 2.665). After adjustment for age, stage, MC, TVD and pre-MRD response, a benefit for immunotherapy was confirmed in BuMel- (p=0.0066; HR 1,439) with 5yEFS of 56±3% vs 48±3% (IP vs CP) and in CEM-treated patients (p=0.0107; HR 2.334) with 5yEFS of 67±9% vs 35±3% (IP vs CP).

Conclusion: Introduction of ch14.18/CHO immunotherapy achieved a major improvement in outcomes for HR-NBL patients treated within the HR-NBL1 trial.

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MRE11 Inhibition Highlights a Replication Stress-Dependent Vulnerability of MYCN Amplified Neuroblastoma

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High risk neuroblastoma patients are frequently characterized by MYCN amplification. Despite aggressive treatments, these children very often experience consecutive recurrences and fatal outcome, making the search for effective therapies an absolute priority. Since targeting MYCN in the clinical settings cannot be achieved, yet, discovering novel MYCN-associated vulnerabilities might provide alternative strategies for the therapy of MYCN-driven neuroblastoma.

The consolidated notion that MYCN induces replication stress prompted us to explore the possibility to trigger intolerable levels of replication stress-dependent DNA damage to treat MYCN-amplified neuroblastoma.

Previously, we reported that the MRE11 complex, a crucial player in the DNA damage response (DDR) and in the maintenance of replication fork stability, is essential for MYCN-dependent proliferation and survival of neuronal precursor cells.

Now we addressed MRE11 role in human neuroblastoma. Interestingly low MRE11 expression characterizes primary human MYCN-single copy neuroblastomas with bad prognosis, consistent with its established oncosuppressive function. In sharp contrast, high MRE11 expression is associated with bad prognosis and occurs in MYCN-amplified tumors, suggesting it might be required for tumor growth, in this subset. Consistently, either MRE11 knock-down or its pharmacological inhibitor mirin, induced accumulation of replication stress and DNA damage biomarkers (i.e. p53BP1 foci, H2AX phosphorylation), specifically in MYCN-amplified cells. The consequent DDR recruited p53 and resulted in the expression of pro-apoptotic p53 target genes and in p53-dependent cell death, as revealed by p53 loss- and gain-of-function experiments. Encapsulation of mirin in water-dispersible polymeric nanoparticles allowed its use on MYCN-amplified neuroblastoma xenografts. Mirin treatment resulted in a sharp impairment of tumor growth, associated with DDR activation, p53 accumulation and cell death.

In conclusion, our findings show that MRE11 inhibition might be an effective strategy to treat MYCN-amplified and p53 wild-type neuroblastoma and support the idea that targeting the replication stress response to induce DNA damage-dependent apoptosis should be further exploited for the cure of these tumors.

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Pre-Targeted Radioimmunotherapy Using GD2xDOTA(Metal)-Bispecific Antibody hu3F8-DOTA-BsAb Is Highly Effective and Specific for Human Neuroblastoma

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Background: Anti-disialoganglioside (GD2) radioimmunotherapy (RIT) with 131I-m3F8 has shown responses in high risk stage 4 neuroblastoma. Pretargeted RIT (PRIT) can vastly improve the therapeutic index (TI; the ratio of the delivered absorbed dose to tumor versus that to normal tissue, especially of bone marrow) by separating the relatively slow hu3F8 antibody-tumor targeting step from the small ligand radioactive payload which is cleared renally within minutes.

Methods: We built a humanized bispecific antibody (BsAb) that binds to both GD2 and a radioactive payload consisting of the chelator 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) complexed with radiometal. In this DOTA-PRIT we utilize the theranostic radioisotope lutetium-177 (177Lu) which has both ionizing beta-particle and imageable gamma-ray decay products, enabling therapy and treatment monitoring/dosimetry in the same molecule. Built using the IgG(L)-scFv platform (210 kDa MW), BsAb hu3F8-C825 was tested in GD2(+) xenografts where 177Lu was injected chelated to DOTA (MW ~1 kDa). Based on serial biodistribution data, absorbed doses to tumor and normal tissues were calculated.

Results: Optimized highly GD2-specific tumor targeting of 177Lu-DOTA-hapten with TI of 142 and 23 was achieved for blood and kidney, respectively. Dosimetry of a treatment regimen consisting of intravenous administration of three cycles of only 11.1 MBq 177Lu/cycle delivered estimated doses of approximately 3,400 cGy to tumor with <120 cGy to kidney and <20 cGy to blood. In mice with established subcutaneous GD2-expressing tumors (average approximate tumor volume 250 mm3) this regimen was proven curative (total administered 177Lu-activity: 900 uCi or 33.3 MBq/mouse), with 5/5 complete responses and 4/5 showing histological cure at study endpoint necropsy at 28 days post-treatment with no clinical acute and chronic toxicity, and most importantly, no histologic evidence of radiation damage to normal tissues. All non-treated mice showed rapid tumor progression (tumor volume >1000 mm3) and required sacrifice within 12 days. Up to 55 MBq 177Lu/cycle can be safely administered with no organ toxicity.

Conclusion: When extrapolated to humans, this novel 3-step tumor targeting process (injection of naked BsAb, a clearing agent, and then the payload), can be safely administered to deliver a very large dose of radiation to neuroblastoma without harming normal tissues.

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Boosting Natural Killer Cell-Based Immunotherapy of High-Risk Neuroblastoma: New Molecular Strategies

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Neuroblastoma (NB) is the most common extracranial solid tumor occurring in childhood. Amplification of the MYCN oncogene is associated with poor prognosis. Down-regulation on NB cells of ligands recognized by Natural Killer (NK) cell-activating receptors, involved in tumor cell recognition and lysis, may contribute to tumor progression and relapse. We demonstrate that MYCN expression inversely correlates with that of ligands recognized by NKG2D and DNAM1-activating receptors in both human NB cell lines and 12 NB patient specimens. The down-modulation of MYCN is still very challenging and presently JQ1, a BET-bromodomain inhibitor, represents a good candidate. Thus, we treated NB cell lines with JQ1 at subapoptotic doses but we did not appreciated a modulation of activating ligand expression due to down-regulation also of c-MYC, transcription factor known to induce the expression of some activating ligands. Moreover, NB cells showed to be refractory to genotoxic drug-mediated induction of activating ligands due to the abnormal status of some DDR pathway transducers. These data suggested us to explore new molecular strategies aimed to induce the expression of ligands for NK cell-activating receptors in NB.

p53, mutated only in 2% of NB, is a direct transcription factor for ULBP1 and ULBP2. On the other hand, MDM2 inhibits p53 function. Remarkably, both p53 and MDM2 are direct transcriptional targets of MYCN and are co-expressed at high levels in MYCN-amplified NB cells. Indeed, p53 is functionally suppressed by MDM2 in MYCN-amplified NB cells. We discovered that MDM2-targeting mediated by Nutlin-3a, a well-established MDM2 antagonist, represents an innovative strategy to sensitize NB cells to NK cell-mediated killing. Our data show that Nutlin-3a was able to enhance in vitro the expression of activating ligands in NB cells, thus rendering them more susceptible to NK cell-mediated killing, and in vivo in NSG-mice-xenograft model. Moreover, ChIP assay revealed that p53, upon Nutlin-3a treatment, is a direct transcription factor of PVR.
Taken together, these results provide the first demonstration that MYCN acts as an immunosuppressive oncogene in NB cells. The restoration of p53 function, mediated by MDM2-targeting, results as a promising molecular approach to design a new NK cell-based immunotherapy of NB.

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The Development of Complex Genomic Amplification Patterns at Chromosome 12q In Neuroblastoma

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Background: Amplification of genomic regions at chromosome 12q13-15 occurs in neuroblastoma and other cancer entities and may present as extrachromosomal double minutes (DM) or intrachromosomal homogeneously staining regions (HSR). The amplicons cover proto-oncogenes, such as MDM2 and CDK4, and are associated with poor prognosis compared to non-amplified cases. We here aimed to decipher the precise genomic structure of these alterations to gain insight into their genetic evolution.

Methods: We examined seven primary neuroblastoma cases and three cell lines harboring complex genomic amplification patterns along with multiple rearrangements on chromosome 12q. MYCN amplification was present in 6/10 cases. All samples were comprehensively characterized by whole genome sequencing and FISH analyses, and rearrangements were validated by PCR. In addition, large genome maps of the cell lines were generated using Bionano's optical mapping. Rearrangements and copy numbers were integrated and modeled in silico to reconstruct amplification patterns and to infer the order of events and mechanisms behind them.

Results: Amplification of 12q regions occurred both as HSR or DM. Two cell lines harbored neochromosome structures consisting of highly duplicated segments from both chromosome 12 and 2, involving the proto-oncogenes MDM2, CDK4 and MYCN. In the remaining cases, MYCN was rearranged with HSRs and DMs from 12q, or occurred in separate DMs. In HSRs and neochromosomes, the amplified regions were composed of highly fluctuating copy numbers (“firestorms”) intertwined with numerous rearrangements. DMs appeared more uniformly, with fewer rearrangements connected as a loop in a walkable manner. Our data suggest an initial catastrophic event leading to the disruption of one or more chromosomes followed by rejoining of the separated fragments to generate stable loop structures. DMs may undergo further amplification by breakage fusion bridge cycles, resulting in firestorm copy number patterns and abundantly clustered rearrangements. Ultimately, this loop structure may linearize and acquire telomeres, thus resulting in neochromosomes, or integrate into a normal chromosome as HSR.

Conclusion: Amplification of the 12q region shows a complex pattern of copy number changes and rearrangements and is highly variable among patients and cell lines. However, structural similarities allow to infer evolutionary processes underlying these amplification patterns.

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Diagnostic Performance of 124I-MIBG PET over 123I-MIBG Scan for Neuroblastoma

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Background: The metaiodobenzylguanidine (MIBG) scan is one of the most sensitive noninvasive lesion detection modalities for neuroblastoma. Unlike commonly available ^{123}I-MIBG, ^{124}I-MIBG allows high-resolution positron emission tomography (PET). We evaluated ^{124}I-MIBG PET/CT for its diagnostic performance directly compared to paired ^{123}I-MIBG scans with single photon emission computed tomography (SPECT)/CT.

Methods: Prior to ^{131}I-MIBG therapy, standard ^{123}I-MIBG scans (5.2 MBq/kg) that include whole-body (anterior-posterior) planar scans and focused field of view (FOV) SPECT/CT as well as whole-body ^{124}I-MIBG PET/CT (1.05 MBq/kg) were performed. For direct comparison of lesion detection, we evaluated 8 patients who showed at least one ^{123}I-MIBG-positive lesion. Seven patients received ^{123}I-MIBG and ^{124}I-MIBG within 2 weeks, and one patient received both MIBG scans 7 weeks apart. Locations of lesions identified, the number of total lesions detected, and the Curie scores were recorded for ^{123}I-MIBG planar and focused FOV SPECT/CT scans, and ^{124}I-MIBG PET/CT scans.

Results: ^{123}I-MIBG whole-body planar scans, focused FOV SPECT/CT scans, and whole-body ^{124}I-MIBG PET scans found 25, 37, and 87 lesions, respectively. 24 lesions seen by ^{123}I-MIBG planar scans were clearly detected on ^{124}I-MIBG PET/CT. However, there were 1 lesion seen by ^{123}I-MIBG planar scan and 3 lesions seen by ^{123}I-MIBG SPECT/CT, but not clearly detected on ^{124}I-MIBG PET/CT. Most importantly, there were 62 lesions not seen by ^{123}I-MIBG planar scans, and 50 lesions not seen by ^{123}I-MIBG SPECT/CT, but clearly detected on ^{124}I-MIBG PET/CT. In summary, there were significantly more lesions identified on ^{124}I-MIBG PET/CT than those on ^{123}I-MIBG planar scans ($p<0.0001$) and ^{123}I-MIBG SPECT/CT ($p<0.0001$). Also because of this trend, the Curie scores by ^{124}I-MIBG PET/CT were higher than those by ^{123}I-MIBG planar and SPECT/CT scans in 7 out of 8 patients. Finally, since we performed a very low dose ^{124}I-MIBG PET, compared to typical ^{123}I-MIBG administration, the effective dose for ^{124}I-MIBG is approximately twice that of ^{123}I-MIBG despite a large difference in half-lives (100 vs. 13.2 hours).

Conclusion: A superior tumor detection capability by ^{124}I-MIBG PET compared to ^{123}I-MIBG scans was shown in our dataset at an acceptable radiation dose.

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Nucleosome Foot Printing Enables Inference of Expression Profiles in Circulating Tumor DNA From Neuroblastoma Patients

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Background: In neuroblastoma (NB), significant amounts of circulating tumor DNA (ctDNA) enable the detection of tumor cell-specific genetic alterations based on next-generation sequencing technologies in NB, in addition to DNA analyses; expression profiles are also important to detect prognostic signatures indicating resistance or disease evolution. However sequential expression analyses have been hampered as repetitive tumor samples are not readily available. We sought to make use of ctDNA to infer expression profiles in sequential plasma samples. Indeed, it has been shown that gene expression levels are reflected by nucleosome positioning. Differences in nucleosome organization at transcription start sites (TSS) leads to differential clipping of fragments upon ctDNA release. Nucleosome free TSS regions of expressed genes are cut more readily than unexpressed genes, leading to distinct nucleosome footprints and differential coverage upon sequencing, depending on the expression of a given gene in the originating cells.

Methods: To establish nucleosome foot printing and infer expression profiles, we isolated ctDNA from plasma at diagnosis (n=43) and during follow-up (partial/complete remission, n=2; progressive disease, n=5) from 43 NB patients. A target capture was designed to encompass the TSS of 34027 known coding genes (8,7Mb). For each patient, the primary tumor DNA, germline DNA and ctDNA samples were submitted to target sequencing (Illumina HiSeq2500 100x100bp, expected coverage 100x). For 18 patients, diagnostic tumor expression profiles were available as controls.

Results: To predict expression status of individual genes, we trained a machine learning model (SVM) on a set of 1360 housekeeping and unexpressed genes according to the FANTOM5 project. Normalized coverage from -200bp to +200pb with respect to the TSS was used as input. We then performed a cross-validation resulting in a mean prediction accuracy of gene expression of 94%. Preliminary results show an expression of NB-associated genes, unexpressed in blood cells, such as MYCN, ALK, PHOX2B, KIT, RBFOX3, STMN2, SCN3A in 91% of diagnostic samples. For a case with diagnosis/relapse samples we observed a switch from unexpressed to expressed for 1954 transcripts and from expressed to unexpressed for 34 transcripts.

Discussion: Our study develops a new utilization of ctDNA and allows sequential expression analysis in NB.

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The Influence of Surgical Excision on Survival in High-Risk Neuroblastoma Revisited After Introduction Of ch14.18/CHO Immunotherapy in the HR-NBL1/SIOPEN Trial

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Background: The effect of complete macroscopic excision (CME) of the primary tumour on event free survival (EFS) in high-risk neuroblastoma remains controversial. We therefore investigated the influence of CME in patients enrolled on the HR-NBL1/SIOPEN Trial and compared the effect in the pre-immunotherapy era.

Patients and Methods: Eligibility criteria were: inclusion in HR-NBL1/SIOPEN Trial between 2002-2015, stage 4 disease; completion of Rapid COJEC induction with or without two additional courses of TVD; no progression/relapse/death; no prior attempt at resection and complete operation data. Intended therapy following operation comprised: HDT/SCT (BuMel or CEM, after 2011 BuMel), 21Gy radiotherapy to the primary site followed by 13-cis RA and after 2009 ch14.18/CHO antibody (short term infusion) ± IL2. 1504 patients fulfilled these criteria; 737 were treated prior to ch14.18/CHO availability (2002-2009) and 767 in the immunotherapy era. Median observation time was 4.9 years (0.1-14 years).

Results: In the whole group, CME was achieved in 77%, incomplete macroscopic excision (IME) in 21% and 2% were inoperable (0E). Surgical mortality was 0.46% (7/1504). Five-year event-free survival (5y-EFS±standard error) was 39%±2% following CME vs. 30%±3% following IME or 0E (p=0.002). The cumulative incidence of local relapse (CILR) was 0.17±0.01 (CME); 0.31±0.03 (IME) and 0.42±0.10 (0E) (p<0.001). 88% of patients received radiotherapy (78% CME; 21% IME and 1% 0E). In some very young patients and patients with very large primary tumours radiotherapy was omitted. 5y-EFS for patients with CME who received radiotherapy was 44±2%, compared to 31±6% without (p=0.013) and with less than CME and radiotherapy 35±3% compared to 20±1% without radiotherapy (NS). CILR was 0.14±0.01 in patients with CME who received radiotherapy compared to 0.28±0.06 in patients who did not (p=0.005). 5y-EFS was significantly higher (42±2%) for patients enrolled after 2009 compared to before (32±2%) (p<0.001). 5-y EFS for patients after 2009 who achieved CME was 45±2% vs. 36±2% after IME and 26±13% after 0E respectively (p=0.034); compared to 33±2% vs. 26±4% (IME) and 17±11% (0E) (p=0.059) prior to 2009.

Conclusions: In the immunotherapy era, CME in Stage 4 patients who received local radiotherapy resulted in improved 5-EFS.

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TWISTing the Aggressiveness of Neuroblastoma

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Background: Neuroblastoma (NB) is a rare malignancy of childhood associated with dismal outcomes despite aggressive multimodal therapy. The embryonic transcription factors TWIST1/2 are frequently overexpressed in cancer, acting as multifunctional oncogenes.

Aim: Investigate TWIST1 function in NB using in vitro knock-out models.

Materials & Methods: TWIST1 knock-out (KO) was performed in primary NB1-M cells and established LAN-1 (neuronal, N-type) and SK-N-Be2c (intermediate, I-type) NB cell lines via CRISPR/Cas9 technology. The impact of TWIST1 KO on 2D and 3D growth was analyzed by viability and methylcellulose clonogenic assays, respectively. Tumor-initiating cell (TIC) self-renewal properties were measured by the neurosphere assay. SK-N-Be2C genes expression profiling was performed by RNA-seq.

Results: Published dataset analyses using the 'R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl)' revealed that, among several tumor settings, TWIST1 mRNA was highly expressed in NB, and this a) correlated with poor survival in both high and low-risk NB groups, and b) tracked with unfavorable prognostic factors. High TWIST1 mRNA expression was confirmed in NB cell lines, with a significantly higher level in both N-and I-type relative to stromal-type cells. TWIST1 KO in LAN-1 and NB-1 cell lines had no impact on both 2D and 3D growth, while SK-N-Be2c-TWIST1-KO cells displayed a reduction in 3D colony size compared to SK-N-Be2c-TWIST1-positive counterparts. Interestingly, the TIC self-renewal properties of the three cell lines were negatively affected by TWIST1 KO. Transcriptome analysis of SK-N-Be2C-TWIST1-positive and -KO cells revealed that TWIST1 mainly induced genes involved in cell adhesion, positive regulation of epithelial to mesenchymal transition (EMT), migration, proliferation and PI3K signaling, as well as genes involved in nervous system development. TWIST1 is also involved in the upregulation of genes belonging to the adrenergic signature (van Groningen et al. 2017). Finally, gene sets correlated with TWIST1 in NB tumors were also found mostly downregulated in SK-N-Be2C-TWIST1-KO cells, confirming the validity of our findings across in vitro NB models.

Conclusions: Our results indicate a potential role for TWIST1 in supporting NB-TIC self-renewal potential, and suggest that TWIST1 upregulate genes involved in EMT, migration, proliferation and survival, as well as in the maintenance of adrenergic phenotype in SK-N-Be2C cells.

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MYCN-Driven Neuroblastoma Evolves Different Mechanism to Escape Transcription and Replication Conflicts

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MYC proteins are transcription factors that bind globally to active promoters and promote transcriptional elongation by RNA polymerase II (RNAPII). To identify effector proteins that mediate this function, we performed mass spectrometry on MYCN complexes in neuroblastoma cells.

The proteomic analysis shows that MYCN forms complexes with TFIIIC, TOP2A and RAD21, a subunit of cohesin. MYCN and TFIIIC bind to overlapping sites in thousands of RNAPII promoters and intergenic regions. TFIIIC promotes association of RAD21 with MYCN target sites and is required for MYCN-dependent promoter escape and pause release of RNAPII. Aurora-A competes with binding of TFIIIC and RAD21 to MYCN in vitro and antagonizes association of TOP2A, TFIIIC and RAD21 with MYCN during S-phase, blocking MYCN-dependent release of RNAPII from the promoter. Inhibition of Aurora-A in S-phase restores RAD21 and TFIIIC binding to chromatin and partially restores MYCN-dependent transcriptional elongation. Treatment with Aurora-A inhibitors results in phosphorylation of the single-strand DNA binding protein, RPA32, at S33, which is phosphorylated by the ATR kinase in response to replication stress. We propose that complex formation with Aurora-A controls MYCN function during the cell cycle to prevent transcription/replication conflicts (1).

Our findings suggest that MYC-driven tumors are dependent on Aurora-A to avoid transcription/replication conflicts. Our findings open the possibility for rational development of mechanism-based therapies targeting these tumors. We propose that there is a synergism between Aurora-A and ATR inhibition in MYCN-driven neuroblastomas. Currently we are further investigating how treatment with a combination is affecting neuroblastoma cells.

Ref: 1) Büchel et al., Association with Aurora-A Controls N-MYC-Dependent Promoter Escape and Pause Release of RNA Polymerase II during the Cell Cycle, Cell Reports 2017, Volume 21, Issue 12, p3483–3497

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Monitoring of Minimal Residual Disease in MYCN-Amplified Neuroblastoma by Chromosomal Breakpoint Profiling

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About 25% of all neuroblastoma cases show an amplification of MYCN and are assigned to the high-risk group. Nearly 50% of high-risk cases relapse after first-line therapy, which implies the survival of neuroblastoma cells, e.g. in the bone marrow, referred to as minimal residual disease (MRD). Here we show that PCR-based detection of patient-specific breakpoints can be used as a sensitive and specific MRD assay for MYCN-amplified neuroblastoma. We employ a custom neuroblastoma hybrid capture-sequencing approach to recover disease-relevant genomic alterations including the MYCN amplicon sequence from FFPE and fresh-frozen primary tumors. Patient-specific PCR primer are designed from the MYCN amplicon chromosomal breakpoints identified by panel sequencing in order to assess MRD in bone marrow aspirates during or after therapy. As a proof of concept, we successfully recovered all previously identified MYCN-specific breakpoints (n=25) by classical PCR and Sanger Sequencing in ten neuroblastoma cell lines using our approach. To estimate the detection limits of our assay, eight MYCN breakpoints underwent further analysis by RQ-PCR and droplet digital PCR. PCR amplicons were detectable in single tumor cells among up to 106 reference cells. Our assays robustly show high sensitivity, outperforming microscopy-based assessment of residual tumor cells in bone marrow aspirates. Importantly, this assay also detected breakpoints in patient samples (14 breakpoints in 8 primary tumor samples). Individual MYCN breakpoints persisted during the course of the disease up to relapse. Furthermore, we established the parallel detection of MYCN breakpoints and other neuroblastoma-relevant, mutated genes (e.g. ALK, TERT) within one multiplex ddPCR assay. We are currently establishing prospective longitudinal neuroblastoma MRD quantification in bone marrow samples for individual patient follow-ups, with the aim to detect residual neuroblastoma cells before their clinical manifestation in high-risk neuroblastoma. We are providing standardized sample processing and analyses guidelines for future clinical applications, which may support therapy decisions on an individualized basis.

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Detection of Circulating and Disseminated Neuroblastoma Tumour Cells Using the Imagestream Flow Cytometer for Use as Predictive and Pharmacodynamic Biomarkers

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Background: Neuroblastoma (NB) is the commonest extracranial childhood solid tumour. Circulating tumour cells (CTCs) are a useful tumour source for predictive and prognostic biomarkers and may provide pharmacodynamic (PD) biomarkers for new treatments. Our research group is interested in developing MDM2/p53 antagonists as a novel therapy. NB are usually p53 wild-type even at relapse, making them suitable for treatment with MDM2/p53 antagonists.

Objectives: 1) To detect CTCs from blood and disseminated tumour cells (DTC) from bone marrow (BM) from NB patients using the Imagestream flow cytometer (ISx) 2) To use these cells for predictive and PD biomarker studies for novel targeted therapies.

Methods: 40 NB patients, (32 high, 6 low and 2 intermediate risk), 24 at diagnosis, 14 at relapse and 2 at diagnosis and relapse, were recruited. 35 paired blood and BM samples were analysed for CTCs and DTCs, and 5 unpaired blood samples for CTCs using the ISx to detect GD2+ve and CD45-ve NB cells. 5 patient samples were treated ex-vivo for 24 hours with 10µM Nutlin-3 and analysed for p53 and p21 expression compared with DMSO controls.

Results: CTCs were detected in 26/40 blood samples (range, 1-264/ml at diagnosis, 1-39/ml at relapse) and DTCs in 25/35 BM (range, 57-169,635/ml at diagnosis, 112-15,688/ml at relapse). Higher numbers of CTCs (but not DTCs) were found in untreated patients with high risk NB who did not achieve a complete BM response after first line induction therapy versus those that did (n=23, p=0.006). Ex-vivo Nutlin-3 exposure in an untreated patient with >200 CTCs/ml and >6000 DTCs/ml, led to increased p21 and p53 expression in GD2+ve/CD45-ve CTCs and DTCs compared to DMSO controls and GD2-ve/CD45+ve blood and BM cells. Conclusions: This the first study to show that CTCs and DTCs are detectable in NB patient samples at diagnosis and relapse using the ISx. Increased p53 and p21 expression in CTCs/DTCs following MDM2 antagonist treatment may be a useful PD tumour biomarker for early phase clinical trials. Enumeration of CTCs at diagnosis in high risk NB patients with BM infiltration may be a useful predictive biomarker of BM response to induction chemotherapy.

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ASPM is a Novel Risk Factor of Aggressive Neuroblastoma

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ASPM is a microtubule-associated protein, involved in the mitotic spindle assembly during cell division. Biallelic mutations in ASPM are the most common cause of autosomal recessive primary microcephaly (MCPH), a neurodevelopmental disorder characterized by intellectual disability and reduced brain volume due to a proliferation defect of neural stem cells. On the other hand, elevated levels of ASPM during development are associated with hyperproliferation and may therefore have a causative role in embryonal malignancies. We seek to evaluate ASPM as a potential risk factor for neuroblastoma. We investigated the correlation of ASPM levels with clinical outcome in a large, representative neuroblastoma cohort (SEQC cohort, n=498, Zhang et al. 2015). Elevated ASPM levels were significantly associated with adverse clinical outcome and unfavorable clinical covariates (MYCN amplification, INRG high-risk). ASPM levels were also strongly correlated with the expression of key cell cycle regulators (e.g., FOXM1, PLK1) in this cohort, indicating a functional role of ASPM in aggressive, undifferentiated neuroblastoma biology and a crucial function for proliferation. QPCR and immunoblotting indicated generally high ASPM transcript and protein levels in neuroblastoma cell lines. Knockdown of ASPM (via lentiviral transduction) lead to induction of a more differentiated phenotype. Eight days after ASPM knockdown, we detected outgrowth of neurite-like extensions and phalloidin-positive signals in immunofluorescence analyses. Furthermore, using flow-cytometric cell cycle analyses we observed an induction of a differentiation-associated cell cycle arrest and apoptosis. Our results suggest that ASPM may play a functional role in maintaining the undifferentiated phenotype of highly aggressive neuroblastomas. Its function in the spindle apparatus and the functional link to PLK1 and FOXM1 point towards a potential therapeutic value of spindle checkpoint and spindle assembly inhibitors such as the PLK1 inhibitor GSK461364, the FOXM1 inhibitor thiostrepton, or paclitaxel in neuroblastoma.

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Circular RNAs are Expressed in Neuroblastoma Cells

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Circular RNAs (circRNA) are a class of non-coding RNAs that can regulate gene expression. They are present in high numbers in neural tissues and are crucial for neural development and function, and have recently been implicated in cancer pathogenesis. We established an RNA sequencing pipeline to identify circRNAs from total RNA from cells and tissue samples, with circRNA detection built around the DCC tool. As a negative control, we analyzed a published poly-A selected RNA sequencing dataset from 56 primary neuroblastomas. Our method identified only 3 putative circRNAs, either from intergenic or highly repetitive regions, indicating a very low false-positive rate. Detected circRNAs were validated in a panel of neuroblastoma cell lines, using a specific qRT-PCR approach, RNaseR treatment, Sanger sequencing and northern blotting. We applied our sequencing and detection pipeline to the MYCN-amplified IMR-5/75 cell line and identified 959 circRNAs. Circular transcript expression did not correlate with linear transcript expression from the corresponding genes, indicating an independent mechanism regulating circRNA expression in neuroblastoma cells. Among the identified circRNAs are well-characterized circular transcripts from genes, such as CDR1-AS (ciRS-7), HIPK3, and SMARCA5, which were previously shown to be linked to progression in other cancers. We detected circRNAs derived from genes associated with the MYCN amplicon, such as NBAS or DDX1, albeit none from the MYCN gene itself. We also applied our detection pipeline to a recently published total RNA dataset from 39 neuroblastoma cell lines, and identified 1,067 circRNAs candidates, with substantial overlap to circRNAs identified in IMR-5/75 cells. Interestingly, gene ontology enrichment analysis revealed that a significant proportion of circRNAs identified in all 39 neuroblastoma cell lines and our data from IMR-5/75 were derived from genes associated with chromatin modifications (ARID1A, SETD3 and ATRX), suggesting that circRNAs may regulate chromatin in neuroblastoma. We have sequenced ~30 from 90 planned samples from primary neuroblastomas spanning the clinical risk spectrum, and are currently conducting data analysis to identify circRNAs. We present a sequencing and data analysis pipeline to identify circRNAs, and show they are prominently expressed in neuroblastoma cell lines.

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New Approaches to Neuroblastoma Therapy (NANT) Consortium Neuroblastoma Precision Trial

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Background: Patients with high-risk neuroblastoma generally respond to upfront chemotherapy, yet many succumb to recurrent or refractory neuroblastoma (rNB) due to tumor acquired therapy resistance. Understanding tumor specific genomic alterations leading to tumor progression is critical to devising novel targeted therapies. Limited data exists regarding the genetic and immunologic predictive biomarkers in rNB, which can be used to direct targeted therapies.

Methods: Utilizing NANT's established multi-institutional infrastructure and Ashion's GEM™ sequencing platform, biological specimens were obtained from bone, bone marrow (BM), and/or soft tissue of rNB patients to identify subgroups who have potentially targetable genetic (ALK, MAPK pathway, Metabolic-related genes) and/or immunologic (tumor-associated macrophage infiltration, PDL1 expression) biomarkers. These subgroups were defined a priori based on published data. After central pathology review, quality samples with >30% tumor were gene-panel sequenced to identify genetic and copy number alterations. Soft tissue samples were also evaluated for MYCN, MYC, CD163 and PDL1 protein expression by immunohistochemistry (IHC). A NANT Precision Report, containing gene panel and IHC reports, was returned to physicians and patients.

Results: To date, 39 specimens from 41 enrolled subjects (95%) were obtained for evaluation. Twenty-seven (66%) of the samples (BM biopsy n=3 of 8; 38%, Bone Biopsy n=7 of 11; 64%, Soft tissue n= 17 of 20; 85%) had a NANT Precision Report generated. Majority of failures in generating reports occurred at the initial quality control assessment from insufficient tumor percentage in BM or bone biopsies. Actionable genomic results were identified in 19 of 27 (70%) samples. Eighteen of 19 (95%) genomic alterations were within the designated subgroups with 11 in metabolic (40.7%), 7 in ALK (25.9%), 4 in MAPK (14.8%). While ALK and Metabolic subgroups overlapped, MAPK group was exclusive. Six of the 14 soft tissue samples (35%) were identified as immune reactive.

Conclusions: A multi-institution clinical genomics study in neuroblastoma is feasible with high chance of success in obtaining useful samples with guided biopsies, including bone metastases. A substantial proportion of children with rNB have actionable alterations that fit within specific categories and approximately half of rNBs show evidence of immune activity.

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Effects of 13-cis Retinoic Acid and Romidepsin on GD2 Expression for Antibody-based Immunotherapy

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Background: Antibody-based immunotherapy against GD2, a disialoganglioside expressed in neuroectodermal cell membranes, is commonly used in conjunction with retinoid therapy for high-risk neuroblastoma. Previous studies showed improved patient survival when treated with alternating cycles of anti-GD2 therapy and 13 cis-retinoic acid (RA). However, this combination was determined based on pragmatic reasons and the two therapies may not be complementary. Pan-HDAC inhibition has been previously shown to augment anti-GD2 immunotherapy, and HDAC1/2 inhibition has been shown to induce neuronal differentiation in neuroblastoma.

Hypothesis: We hypothesize that RA may inhibit GD2 expression in neuroblastoma, potentially impairing the efficacy of anti-GD2 immunotherapy, and specifically HDAC1/2 inhibition with romidepsin can rescue this effect.

Aims: To test this hypothesis we pursued the following aims: 1) determination of the effects of RA and romidepsin individually and in combination on GD2 expression on neuroblastoma cell lines in vitro, 2) evaluation of the effects of RA and romidepsin on GD2-directed antibody-dependent cell-mediated cytotoxicity (ADCC) in vitro.

Results: We demonstrated by flow cytometry that GD2 decreases in neuroblastoma cells treated with RA, while GD2 increases in neuroblastoma cells treated with romidepsin, and the concurrent use of romidepsin and RA mitigates the downregulation of GD2. Furthermore, these changes in GD2 correlate with the efficacy of NK-cell-directed ADCC as induced by dinutuximab in vitro against neuroblastoma cell lines.

Conclusions: These data suggest that the efficacy of current anti-GD2 immunotherapy could be improved either by the restructuring of the timing of retinoid therapy and/or by the inclusion of HDAC1/2 inhibitors to the regimen. Studies in animal models and using patient-derived xenografts are ongoing at the time of abstract submission.

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Serial Profiling of Neuroblastoma ctDNA Reveals Potentially Actionable Tumor Genetic Heterogeneity and Evolution

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Background: Sequential analysis of circulating tumor DNA (ctDNA) provides a noninvasive method to profile tumor-associated genomic aberrations, heterogeneity, and evolution.

Methods: Peripheral blood from 3 newly diagnosed and 21 relapsed high-risk neuroblastoma patients was serially profiled for ctDNA (n=1-6 samples/patient; total n=66 samples) with the Foundation Medicine FoundationACT (FACT) assay that utilizes hybrid capture-based genomic profiling. Sixty-two genes were sequenced to a median unique coverage depth of at least 3168x and genomic variants were evaluated and compared with temporally-matched tumor/bone marrow sequencing.

Results: Ninety-four percent (62/66) of peripheral blood samples yielded suitable cell-free DNA for sequencing with the FACT assay. At least 1 genomic alteration (short-variant or amplification) was found in 60% (37/62) of samples and 76% (47/62) of samples had a maximum somatic allele frequency (MSAF) >0 (median MSAF=7.7%; range MSAF 0-73%). Forty percent (8/20) of ctDNA genomic short-variants were present in temporally matched tumor/bone marrow samples at diagnosis or relapse (collected within 3 months of each other; n=18 patients). However, 60% (12/20) of detected ctDNA alterations were not found in paired tissue sequencing, including short-variants in ALK, TP53, TERT, NF1, FLT3, and PIK3CA. Only one case with discordant ctDNA/tissue paired sequencing data revealed a tissue unique genomic alteration in TP53. Furthermore, there was 100% concordance between the detection of MYCN amplification (6/6) and ALK amplification (3/3) in paired ctDNA/tissue samples. For example, a newly diagnosed patient with stage 4 neuroblastoma had MYCN amplification noted on both tumor and ctDNA sequencing, however had 3 separate ALK mutations (R1275Q, F1245L, and F1174L) uniquely identified only in ctDNA. Finally, 17 patients had more than one ctDNA sample sequenced (range=2-6 samples collected over 1-13 months). Forty-seven percent (8/17) of patients had alterations emerge or regress across serial ctDNA samples. For example, potentially pathogenic variants in BRCA2, NRAS, CDH1, and ERBB2 appeared in subsequent ctDNA sequencing that were not present in the initial sample. Correlation of these serial ctDNA sequencing data with clinical disease evaluations is ongoing.

Conclusions: The sequencing of ctDNA from neuroblastoma patients demonstrated accurate recapitulation of paired tissue sequencing and also revealed additional potentially actionable tumor-associated genetic aberrations.

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Preclinical Evaluation of BET-Bromodomain Inhibitor TEN-010 as Monotherapy and Combination Therapy in MYC-Driven Neuroblastoma

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MYC signaling is a predominant driver of high-risk neuroblastoma, caused either by amplification of MYCN or by activation of cMYC. The bromodomain and extra-terminal (BET) domain-containing protein BRD4 was reported to cooperate with MYCN in the epigenetic regulation of super-enhancer driven genes in neuroblastoma. The BET inhibitors JQ1 and OTX015 were shown to repress BET/MYCN-mediated transcriptional control and demonstrated antitumoral efficacy in MYCN-driven neuroblastoma. The potent BET inhibitor TEN-010, a structural derivative of JQ1, is currently in clinical trials for various MYC-driven adult tumors. Its efficacy against neuroblastoma is yet to be established. In a preclinical setup, we investigated the antitumoral activity of TEN-010, OTX015 and JQ1 in neuroblastoma cell lines (n=15). We employed an ATP detection assay to assess cell viability 72h after treatment. Seven cell lines were highly sensitive to TEN-010 with IC50 values ranging 85nM to 632nM, five cell lines showed an intermediate response (IC50 values: 1.5-4.8µM), while three cell lines did not show a specific response. Six of the seven highly sensitive lines displayed a decrease in viability by more than 75% at 1µM. The highest sensitivity was observed in cells with high MYCN/MYC activity: TEN-010 showed an excellent effect on MYCN-amplified cell lines IMR5/75 and CHP-134, also on cMYC-activated cell lines, SK-N-AS and SK-N-FI. Consequently, TEN-010 showed a weaker effect in MYCN-non-amplified and low c-myc expressing cell line SH-SY5Y. We obtained comparable results for JQ1 and OTX015. While OTX015 appears effective at lower nanomolar concentrations, our data indicate a higher specificity, and thus potentially a larger therapeutic window, for TEN-010. To increase the therapeutic efficacy and decrease the risk of resistance formation, we turned towards combinatorial treatment options. We assessed combinations of TEN-010 with conventional chemotherapeutics (e.g., etoposide, cyclophosphamide) and with substances interfering with key pathways in neuroblastoma (e.g., sirolimus, volasertib). First results indicate synergistic effects combining BETi with either volasertib or chemotherapeutics, lowering the IC50s into the low nanomolar range. Our results support the notion that BET proteins have crucial functions in MYC/MYCN-driven neuroblastoma and suggest BET inhibition as an effective treatment option that may complement current standard therapy.

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Statistical Framework in Support of a Revised Children's Oncology Group (COG) Neuroblastoma Risk Classification System

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Background: The International Neuroblastoma Risk Group Staging System (INRGSS) was developed through international consensus to provide a pre-surgical staging system, which utilizes clinical and imaging data at diagnosis. A revised Children's Oncology Group (COG) neuroblastoma risk classification system is needed to incorporate the INRGSS and within the context of modern therapy. Herein we provide statistical justification for a revised COG risk classification system and validate outcomes for select cohorts treated with modern therapies.

Patients and Methods: Nine factors were tested for potential statistical and clinical significance in 4,569 neuroblastoma patients diagnosed and enrolled on the COG biology/banking study ANBL00B1 (2006-2016). Image-defined risk factor (IDRF) data was collected during this time interval and therefore both International Neuroblastoma Staging System (INSS) and INRGSS stage could be assigned. Recursive partitioning was performed to create a survival tree regression (STR) analysis of event-free survival (EFS), generating a split by selecting the strongest prognostic factor among those that were statistically significant at each step. The LASSO (least absolute shrinkage and selection operator) was also applied to obtain the most parsimonious model for EFS. These two statistical modeling approaches to risk classify patients were compared. Differences between the two analyses were noted and compared with the original INRG classification.

Results: The overall 3-year EFS and overall survival (OS) were 72.9±0.9% and 84.5±0.7%, respectively. In each approach, the most statistically and clinically significant factors were diagnostic category (e.g., neuroblastoma, ganglioneuroblastoma), INRGSS, MYCN status, International Neuroblastoma Pathology Classification (INPC), ploidy, and 1p/11q. The STR analysis was more concordant with the INRG classification system than LASSO, although both methods showed moderate agreement with the INRG system.

Conclusion: These analyses identified subgroups of patients with significantly different outcomes and provide a framework to develop a new COG risk classification incorporating the INRGSS that may result in changes to the assigned risk groups for some patient subsets.

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Investigating Immunosuppressive Mechanisms in The Tumor Microenvironment of High-Risk Neuroblastoma; An Immunocompetent, MYCN-Driven, Non-Germline GEM Model

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Combining anti-GD2 ganglioside monoclonal antibody with immune-activating cytokines improves survival in high-risk neuroblastoma, a breakthrough for immunotherapy in this disease. However, approaches such as immune checkpoint blockade, anti-GD2 CAR-T cells, and tumor vaccines have thus far shown limited success. While a better understanding of the tumor microenvironment (TME) promises to improve the success of immunotherapy strategies, few immunocompetent models exist for high-risk neuroblastoma. We used a 30-parameter mouse-specific CyTOF mass cytometetry panel to characterize the TME of neuroblastoma arising in mice transgenic for TH-MYCN. The TME of TH-MYCN tumors was dominated by tumor-associated macrophages (TAMs) and devoid of T, B or NK cells; recapitulating the TME of human high-risk neuroblastoma. The TH-MYCN model is highly penetrant only in the 129/SvJ strain however, a challenge to leveraging immunological tools available mainly in the C57/BL6 background.

We therefore created a non-germline GEM model in which primary trunk neural crest cells isolated from C57BL6/J embryos were modified genetically by transducing MYCN (MYCN-nGEMM). Orthotopic transplantation in renal capsules resulted in neuroblastoma with penetrance of 50% and latency ranging from 28 to 120 days. Co-expression of ALKF1174L with MYCN resulted in 80% penetrance and decreased latency. Additionally, and in contrast to the TH-MYCN model, MYCN-nGEMM tumors show frequent liver and spleen metastases. Cell lines could be derived robustly from these tumors, further manipulated in vitro, and serially transplanted with 100% penetrance. CyTOF analysis of MYCN-nGEMM tumors also revealed a highly immunosuppressive TME similar to TH-MYCN tumors, with 80% of immune cells being TAMs, and only 5% T-cells.

In summary, we have characterized an immune-intact model for MYCN-driven neuroblastoma that can easily leverage modern methods to rapidly modify the murine genome, enabling customization of this mouse model for immunological research. We propose this model to understand the immunosuppressive mechanisms of MYCN-driven neuroblastoma tumors and evaluate the potential of combining MYCN-directed therapies with immunotherapy.

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Preclinical Ultrasound and PET/CT Scans in the Hemizygous Th-MYCN Mice

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Positron emission tomography (PET) scans with ^{18}F-fluorodeoxyglucose (FDG) or ^{18}F-fluoro-dihydroxyphenylalanine (FDOPA) distinguish the genomic type and treatment outcome of neuroblastoma (NB) in human. The molecular imaging phenotypes in mouse models of NB are less studied. We aim to investigate the tumor latency, disease progression, and metabolic phenotypes in the Th-MYCN transgenic mouse model of NB by combining preclinical ultrasound and PET imaging. After weaning and genotyping, 53 female hemizygous Th-MYCN transgenic mice of 129/SvJ background were evaluated twice a week using a high-frequency small-animal ultrasound system (Vevo 2100, FUJIFILM VisualSonics, Toronto, Canada; with an RMV-706 transducer at 40 MHz) since age 34.7±4.3 (mean±SD) days. We identified 46 (87%) mice with tumor at a median age of 53 days, arising from the pre-aortic (n = 37; 70%); juxta-adrenal (n = 6; 11%); or thoracic (n = 3; 6%) region. The tumors showed moderate echogenenicity, i.e. kidneys > tumor > liver. During the initial 14 days after tumor detection, the volume of pre-aortic tumors enlarged 78 folds, reflecting rapid progression. After tumor detection, the median time-to-death was 29 days, at a median age of 83 days. Meanwhile, 37 small-animal PET/CT scans using FDG, FDOPA, or ^{18}F-L-thymidine (FLT) were performed in 18 mice with tumor. The FDG, FDOPA, and FLT PET had successfully visualized 16/16 (100%), 11/12 (91.7%), and 0/9 (0%) of tumors, respectively. In 10 mice which were evaluated with paired FDG and FDOPA PET/CT scans, most (6/8 evaluable) tumors showed an “FDG-high, FDOPA-low” uptake pattern, while the uptake of FDG and FDOPA showed an inverse correlation. Surprisingly, although high tumor signal of FLT PET had been reported in a SCID mouse xenograft model of NB, the tumor uptake of FLT in Th-MYCN mice was too low to be discerned. After necropsy, histopathology of the murine tumors showed morphological features resembling poorly-differentiated NB in human. We conclude that preclinical ultrasound provides early detection and longitudinal evaluation of both abdominal and thoracic tumors in the hemizygous Th-MYCN mice, while FDG and FDOPA PET scans further provide in vivo phenotypic information which may correlate with cancer metabolism or differentiation.

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B7-H3 CAR T Cells Mediate In Vitro and In Vivo Activity Against Neuroblastoma Xenografts

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Objective: Immunotherapy has emerged as an effective therapy for neuroblastoma, however new modalities and targets are needed to improve outcomes. Our lab has developed a chimeric antigen receptor (CAR) that targets B7-H3 (CD276), an immune checkpoint molecule overexpressed on many cancers, including neuroblastoma. We hypothesized that B7-H3 would be a good target for CAR based immunotherapy for neuroblastoma.

Methods: Neuroblastoma tissue microarrays of primary patient samples were screened for B7-H3 expression by immunohistochemistry and cell lines were screened using flow cytometry. B7-H3 CAR T cells were tested in vitro by measuring tumor cell killing and cytokine production after coculture with tumor cell lines and in vivo in an orthotopic model of neuroblastoma.

Results: B7-H3 expression was detected by IHC on 82% of 186 screened neuroblastoma patient samples. B7-H3 was expressed at high levels (2+ or 3+) in more than half of these samples (56%). Almost all cell lines screened were homogeneously positive for B7-H3 by flow cytometry.

Retrovirally transduced B7-H3.4-1BB.ζ CAR T cells were co-cultured with three B7-H3 positive neuroblastoma cell lines (SK-N-BE2, KCNR, and CHLA255) and robust tumor cell killing was demonstrated using an IncuCyte assay. Supernatant from the co-cultures was harvested after 24 hours and both interferon gamma and IL-2 production was detected by ELISA.

In an orthotopic subrenal capsule xenograft model of neuroblastoma, mice treated with B7-H3 CAR T cells show significant reductions in tumor growth and prolonged survival compared to those treated with untransduced control T cells. However, the treatment is not always curative.

B7-H3 CAR T cells express high levels of exhaustion markers (PD1, TIM3, and LAG3) when compared to CD19 CAR controls. In order to overcome inhibition from exhaustion, B7-H3 CAR T cells were co-cultured with neuroblastoma cell lines and PD-1 blocking antibody. Nivolumab significantly increased the production of IL-2 and interferon-gamma by B7-H3 CAR T cells.

Conclusions: B7-H3 is expressed on a majority of neuroblastoma samples and appears to be a promising candidate for CAR T cell therapy. B7-H3 CAR T cells demonstrate activity against neuroblastoma xenografts that may be enhanced by the addition of PD1 inhibitors.

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mIBG-Non-Avidity: Correlation with Clinical Features and Prognosis

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Introduction: 123Iod-Metaiodobenzylguanidine (mIBG)-Scintigraphy is an important diagnostic tool in the assessment of neuroblastic tumors. Though the majority of tumors are mIBG avid, patients with mIBG non-avid tumors are found in all risk groups. We correlated clinical features, outcome, histology and specific gene expression features with MIBG-avidity.

Methods: We analyzed a cohort of 2138 patients diagnosed with neuroblastoma and registered in the trials NB97 and NB2004 between 1997 and 2016, with given information on mIBG-avidity in the initial mIBG scans. Expression data of the norepinephrine and monoamine transporter genes NET, VMAT1 and VMAT2 were determined in 532 tumors.

Results: 153 patients (7.2%) had mIBG negative tumors. MIBG non-avidity correlated with INSS stage (localized stages: 10.8%, stage 4S: 8.0%, stage 4: 3.0%; p<0.001), age at diagnosis (<18 months: 6.0%, >18 months: 8.2%; p=0.048), and histology (differentiated neuroblastoma, ganglioneuroblastoma intermixed: 17.0%; poorly differentiated neuroblastoma, ganglioneuroblastoma nodular: 5.8%; p<0,001).

For the total cohort, mIBG non-avidity did not correlate with MYCN status (not amplified 7.0% vs. amplified 7.7%, p=0.58), while a difference was seen in stage 4S (not amplified 6.1% vs. amplified 27.8%, p<0.008) and in stage 4 >18 months (not amplified 2.1% vs. amplified 5.7%, p=0.02).

Negative mIBG scintigraphy (n=23) was correlated with lower NET expression (p<0.001), while neither for VMAT1 (p=0.06) nor for VMAT2 (p=0.13) a significant difference was found.

Outcome was better in patients with MIBG non-avid tumors (3-year-Event-Free-Survival (EFS) 0.79±0.03 vs. 0.63±0.01, p<0.001). This difference was evident in patients >18 months with either localized non-amplified neuroblastoma (p=0.05) or stage 4 non-amplified tumors (p=0.02), but not in patients with amplified tumors (p=0.72) or non-amplified patients <18 months (p=0.32). In addition to stage (HR=2.3, p<0.001), age at diagnosis (HR=1.9, p<0.001) and MYCN status (HR=1.8, p<0.001), multivariable analysis revealed mIBG non-avidity as an independent prognostic marker for event-free survival (HR=0.65, p=0.01), but not for overall survival (p=0.35)

Conclusion: MIBG non-avidity correlated with known prognostic factors (age, stage, histology) and with lower NET expression. In addition, mIBG non-avidity was an independent prognostic marker for better event-free survival but did not prove as independent marker for overall survival.

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Enhanced Intratumoral Delivery of SN38 as a Tocopherol Oxyacetate Prodrug Using Nanoparticles in a Neuroblastoma Xenograft Model

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Background: Currently, <50% of high-risk pediatric solid tumors, like neuroblastoma (NB), can be cured. Many survivors experience serious or life-threatening toxicities, so more effective, less toxic therapy is needed. One approach is to target drugs to tumors using nanoparticles (NPs), which take advantage of the permeability of tumor vasculature.

Methods: SN38, the active metabolite of irinotecan, is a potent therapeutic agent that is readily encapsulated in polymeric NPs. Tocopherol oxyacetate (TOA) is a hydrophobic mitocan that was linked to SN38 to significantly increase hydrophobicity and enhance NP retention. Efficacy of SN38-TOA NPs was compared to the parent prodrug irinotecan (CPT-11) in a NB flank xenograft mouse model.

Results: In TrkB-expressing SH-SY5Y NB xenografts, NP treatment induced complete remissions in all mice for over 60 days, with 100% event-free survival at 110 days, and 60% at 180+ days. Of the remaining survivors, half did not have detectable tumors, and half had stable tumors with volumes less than 0.5 cm3. CPT-11 treatment yielded 0% survival at 75 days. These mice were retreated with NPs, resulting in tumor volume decreases of over 50%, reduced tumor growth rates, and 100% survival at 120+ days. Interestingly, these tumors had a differentiated histology, similar to maturing ganglioneuroblastoma, and were infiltrated with Schwann cells of mouse origin. NP treatment of IMR32 NB xenografts (1.5 cm3) resulted in 100% survival with complete remission for 180+ days.

Conclusion: Compared to conventionally administered CPT-11, NP-based delivery of SN38-TOA as a hydrophobized prodrug achieved higher and more sustained intratumoral SN38 levels, enhancing drug efficacy and resulting in cures. Lower total dose and drug entrapment in NPs during circulation should decrease toxicity. NP-based delivery of a rationally designed prodrug is an attractive approach to enhance chemotherapeutic efficacy in pediatric and adult tumors.

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Alternate Telomere Lengthening (ALT) Neuroblastoma Is a Highly Aggressive Subgroup for Which ATM Kinase Provides a Novel Therapeutic Target

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Telomere maintenance is required for cancer growth and telomerase+ tumors express TERT mRNA. A non-telomerase mechanism, ALT is used by some cancers. Of 110 high-risk neuroblastoma primary tumors 25/110 (22.7%, 95% CI, 15.3-31.7%) were ALT (low TERT mRNA expression (below median) and were positive for ALT-specific telomeric DNA C-circles (13 ATRX-wt., 12 ATRX-mt); 55/110 (50%) had high TERT expression (C-circle-negative), and 30/110 (27%) were low-TERT, C-circle-negative. Overall survival (10 years) was 23.5% (95% CI, 12.8-43.2%) for TERT-high C-circle negative tumors, 24.6% (95% CI, 9.3-65.1%) for ALT tumors, and 72.9 % (95% CI, 58.5-90.9) for C-circle-negative TERT-low tumors. Of 104 human neuroblastoma cell lines and 37 patent-derived xenografts (PDX) we identified 5 ALT cell lines and 5 ALT PDXs. ALT neuroblastoma cell lines had a significantly higher mean IC90 ($P<0.001$) for topoisomerase inhibitors relative to 79 comparator neuroblastoma cell lines. We observed numerous baseline DNA damage foci (>75% co-localized to telomeres) in the nuclei of ALT NB cell lines. We assessed activation of ATM/ATR kinases (involved in DNA damage signaling at telomeres) and observed a marked increase in phosphorylation of ATM kinase and its downstream target CHK2 in ALT but not in TERT+ NB cell lines. Transducing dominant-negative TRF2 (a shelterin protein that blocks ATM at telomeres) into 2 TERT+ p53 non-functional NB cell lines activated ATM at telomeres and induced high resistance to topoisomerase inhibitors. ATM inhibition using shRNA or the ATM inhibitor AZD0156 (adult Phase 1 trial ongoing) in ALT NB cell lines reduced C-circle content in vitro ($p<0.05$) and in vivo ($p<0.05$) and sensitized ALT NB cells to topoisomerase inhibitors ($p<0.01$). AZD0156 enhanced cytotoxicity (> 75% reduction of IC50) of temozolomide+irinotecan (as SN38), in ALT cell lines in vitro. AZD0156 enhanced the activity of temozolomide + irinotecan in 3 neuroblastoma xenograft models (1 PDX), with most mice in complete response at 100 to 150 days compared to earlier progression in mice treated with only temozlomide + irinotecan ($p<0.0001$). Thus, ALT defines a distinct subset of high-risk neuroblastomas with de novo resistance to chemotherapy that can be reversed with AZD0156.

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A Curative Approach for Neuroblastoma Metastatic to the CNS: Safety and Efficacy of Intraventricular 131I-Labeled Monoclonal Antibody 8H9 Targeting B7-H3

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Background: Tumors metastasizing to the central nervous system (CNS) are associated with significant mortality. We tested the toxicity and dosimetry of intraventricular 131I-labeled monoclonal antibody 8H9 targeting surface glycoprotein B7-H3 in patients with primary or metastatic CNS tumors.

Methods: Tumor B7-H3 expression was assessed by immunohistochemistry. CSF flow was determined by 111Indium-DTPA cisternography. 131 patients received 2 mCi tracer of intra-Ommaya 124I- or 131I-8H9 for nuclear imaging followed by a therapeutic injection (10-80 mCi, dose levels 1-8 in 10 mCi increments for phase I patients; expanded cohort 50 mCi/injection) 131I-8H9. Pharmacokinetics were studied by serial CSF and blood samplings over 48 hours. Dosimetry was based on pharmacokinetics and region of interest analyses on serial nuclear imaging scans. Toxicity was defined by the CTCAE v.3.0. 8H9 dosimetry and therapy injections were repeated after 1 month if no serious adverse events or progressive disease ensued. Tumor response was determined by clinical, radiographic, cytologic criteria; overall survival was noted.

Results: 129 patients (93 with CNS neuroblastoma) received 383 injections [median age 5.4 years (1.2- 53.6 years)] Injections were well tolerated and routinely administered in the outpatient setting. Rare self-limited adverse events included grade 1 or 2 fever, headache, vomiting, biochemical elevations in AST or ALT and 1 injection with grade 3 ALT elevation (dose level 3). Although not a dose limiting toxicity, myelosuppression occurred in patients who had received craniospinal radiation and at dose levels 6 and higher (>60 mCi). Of 93 patients treated for CNS neuroblastoma, an improved overall survival was noted compared to survival reported with conventional therapies. At analysis, 48 (52%) patients with CNS neuroblastoma were alive 4.8–152 months (median 58 months) after CNS metastasis, including 56% at 36 months and 29% > 60 months. Interpatient variability for total absorbed dose to the CSF and blood was observed; mean absorbed CSF dose was 104.9 cGy/mCi by CSF sampling, and 2.6 cGy/mCi to the blood.

Conclusions: We conclude that intraventricular 131I-8H9 was safe, permitting favorable dosimetry to the CSF, and in the Phase II expansion demonstrated activity in improving long term remission among patients with CNS neuroblastoma.

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The Functions of EZH2 in N-MYC-amplified Neuroblastoma

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Neuroblastoma is the most common pediatric cancer, accounting for nearly 15% of childhood cancer mortalities. Although remarkable progress has been made in improving the outcomes for children with low and intermediate risk neuroblastoma, high risk disease still retains a survival rate of less than 40%. Neuroblastoma is a solid tumor that arises from aberrant growth of neural crest cells during development of the sympathetic nervous system. One of the most common genetic abnormalities in neuroblastoma is the amplification of the N-MYC oncogene, which is the driver of neuroblastoma. N-MYC encodes a transcription factor that forms large complexes to drive changes in gene expression, thus making drugs that directly target N-MYC technically challenging. One key aspect of a MYC oncogene-driven cancer is the epigenetic dependency for driving gene expression and transformation. A commonly mutated epigenetic complex in multiple cancers is the Polycomb Repressor Complex 2 (PRC2 complex). The PRC2 complex is composed of a group of Polycomb group proteins (PcG) that has methyltransferase activity that epigenetically silence gene expression. In particular, both loss-of-function and gain-of-function mutations have been found in one PRC2 complex component, EZH2, in several cancers. However the role of EZH2 in neuroblastoma is not well understood. A recent study suggested that N-MYC-amplified neuroblastoma is dependent on EZH2. In this study, we used a CRISPR-Cas9 technology to generate clonal EZH2 knockout (KO) cell lines in BE2C, a commonly used N-MYC amplified neuroblastoma cell line. These cell lines are completely devoid of H3K27 methylation due to EZH2 loss. To our surprise, mice subcutaneously injected with either control or EZH2-KO BE2C cells develop tumors at similar rates (p value > 0.05). Furthermore, the EZH2 inhibitor EPZ6438 does not have a significant effect on delaying tumor progression in our mouse model (p value > 0.05). Although there are still many questions that need to be addressed for the exact functions of PRC2 in neuroblastoma, our data suggest that EZH2 might not have a significant role in neuroblastoma growth.

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Exploiting Stromal Schwann Cell Derived Factors for Targeting Aggressive Neuroblastoma

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While aggressive neuroblastomas (NB) are composed of un-/poorly differentiated tumor cells, maturation into benign ganglioneuroma (GN) is hallmarked by tumor cell differentiation and Schwann cell (SC) stroma. Despite SC stroma was early recognized as histo-pathological factor strongly correlating with maturation and favorable outcome, an active role of SCs in favorable NB/GN has long been neglected because of their supposed neoplastic nature. We have previously disproven this concept and demonstrated an active cross-talk of SCs and tumor cells. Based on the reactive nature and plasticity of SCs, these cells could undergo an adaptive response to genetically favorable neuroblastic tumor cells by similar mechanisms as during the nerve injury induced re-programming into repair SCs.

This study aimed at investigating whether stromal SCs in GN adopt similar functions as repair SCs, e.g. the regulation of neuronal differentiation, and whether SC derived factors can be exploited to trigger differentiation and apoptosis also in unfavorable, aggressive NB.

We interrogated our RNAseq data and compared the cellular state of human SCs in the injury (n=5) and the tumor context (n=6). Stromal SCs in GN shared key features with repair SCs in injured peripheral nerves, such as axon re-growth, including a high expression of neuritogens. Next, we studied the effect of SCs on NB cells in co-cultures providing direct or indirect contact for 8 and 16 days followed by multi-color FACS. Increased differentiation and/or impaired proliferation of 2 MYCN-non-amplified and 3 MYCN-amplified NB cell lines were observed independent of direct contact. Also apoptosis was triggered in 2/2 aggressive cell lines. Subsequently, 8 secreted factors highly expressed by stromal and repair SCs have been evaluated for their differentiation inducing ability. Notably, a protein with so far unknown function, epidermal growth factor like 8 (EGFL8), induced differentiation and impaired proliferation of MYCN non-amplified NB cells, similar as NGF, in a dose-dependent manner, was secreted by SCs in vitro and was highly abundant in primary GN.

By analyzing the status of stromal SCs and deciphering SC interaction with NB cells we identified EGFL8 as a new potent neuritogen which could be of therapeutic interest for a subset of aggressive NBs.

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Metronomic Topotecan Induces Senescence and Epigenetically Targets MYCN in Neuroblastoma

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Poor prognosis and frequent relapses are major challenges for patients with high-risk neuroblastoma (NB), especially when tumors show MYCN amplification. In contrast to high-dose chemotherapy, low-dose metronomic (LDM) therapy is a chemotherapeutic concept applying lower doses but administered continuously or at predefined intervals. Functional consequences involve inhibition of angiogenesis, activation of the immune system, targeting of potential tumor-initiating cells and induction of senescence. In MYCN-amplified NB senescence, a state of permanent proliferative arrest, occurs spontaneously or in response to LDM therapy with chemotherapeutic drugs. In this study we investigated the mechanism of action of LDM topotecan (TPT) treatment in low-passage MYCN amplified cell lines in vitro and in vivo. Similar to reports by our group on low-dose hydroxyurea treatment, TPT induces a tumor-inhibiting type of senescence accompanied by changes in the tumor transcriptome and secretome, specifically in MYCN-amplified NB cells. As senescent NB cells showed a reduced MYCN copy number and strongly down-regulated MYCN expression, we investigated the mechanism of MYCN inactivation by genetic interference, a novel semi-automated imaging approach and chromatin immuno precipitation. This showed that upon senescence MYCN is epigenetically silenced and gene copies are recruited to the nuclear periphery where changes in the nuclear lamina composition take place, followed by the packaging of MYCN copies into micronuclei. In a MYCN-amplified NB xenograft model, single agent LDM TPT (0.1 mg/kg/d, i.p., daily over 15 weeks) triggered senescence which was associated with MYCN down-regulation, up-regulation of laminA and the formation of micronuclei. Decrease of MYCN copies was also demonstrated by digital droplet PCR in cell-free tumor DNA (ctDNA) isolated from peripheral blood liquid biopsies of xenograft mice sampled at different time points. Further, LDM TPT treatment improved survival and led to long-term cure (>300 days) in 40% of mice without signs of toxicity.

In this study we have demonstrated the mechanism of MYCN inactivation upon LDM TPT treatment and its preclinical efficacy. Further, we established methods to track MYCN in liquid biopsies, a prerequisite for further clinical testing of this new treatment concept as a maintenance therapy in NB patients with MYCN amplification.

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Role of Histone PTMs in the Epigenetic Silencing of CHD5, a Tumor Suppressor in Neuroblastoma

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Background: Many histone post-translational modifications (PTMs) are associated with the same diverse nuclear functions implicated in cancer development, including transcriptional activation, repression and epigenetic regulation. We examined the role of histone PTMs in the regulation of CHD5, a tumor suppressor gene. CHD5 expression is very low or absent in high-risk NBs, especially those with 1p deletion and/or MYCN amplification. EZH2, a Polycomb group protein and subunit of the Polycomb repressive complex 2 (PRC2), binds to gene promoters and causes histone-3 lysine-27 trimethylation (H3K27me3), leading to transcriptional repression. Recent evidence suggests that MYCN contributes to the regulation of PRC2. We analyzed the global H3K27me3 status, as well as the binding of EZH2 and MYCN at the proximal CHD5 promoter.

Methods: We employed a combination of chemical derivation and high-resolution MS to identify and quantify multiple histone variants and PTMs. ChIP assays were performed using nuclear proteins prepared from NB cell lines NLF, NGP, NBLS and SY5Y. This was followed by immunoprecipitation with antibodies to EZH2, H3K27me3, MYCN and IgG. Bound DNA complexes were analyzed by PCR and qPCR using primers designed at the CHD5 transcription start site.

Results: We analyzed the global histone PTM characteristics of four NB lines and observed a significant difference in the H3K27me3 expression in CHD5 expressing vs. non-expressing NB lines. Furthermore, H3K27 trimethylation was found in CHD5 promoter at -250 bp in NLF and NGP cell lines. Both cell lines showed very low CHD5 expression. EZH2 binding was also found at this site, consistent with H3K27me3 in both NLF and NGP. We also identified MYCN binding to the E-boxes around -250bp and -800 bp of the CHD5 promoter. These three factors (H3K27me3, EZH2, and MYCN) were not found at the CHD5 promoter of the NBLS cell line, which shows high CHD5 expression.

Conclusions: Our proteomic characterizations of the histone modification identified a novel mechanism for the epigenetic regulation in NB of tumor suppressor CHD5. Our data strongly suggest that H3K27 trimethylation by EZH2 contributes to the epigenetic repression of CHD5 expression, and that MYCN binding may also contribute to the regulation of CHD5 expression.

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Relapse Specific Genomic Alterations in UK Neuroblastomas Detected by Whole Exome Sequencing and SNP Arrays of Paired Tumours

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Background: Relapsed neuroblastoma remains a major challenge. Identification of new genetic abnormalities at relapse is needed to predict response to existing targeted agents as well as identify potential new treatment targets.

Aims: To study paired neuroblastoma tumours at diagnosis and relapse to determine the frequency of relapse specific mutations and new copy number abnormalities.

Methods: 38 paired neuroblastoma DNAs from diagnosis and relapse (16 high, 10 intermediate and 12 low risk) were studied, 35 by whole exome sequencing (WES) (Illumina Truseq Rapid Exome Library Prep Kit) to a read depth of 100x including 5 germline DNAs at 30x to subtract variants from matched diagnostic DNAs. 18 pairs were run on SNP arrays (Illumina Infinium CytoSNP-850k v1.1 bead chip) and analysed using Nexus 8 software. Sequence reads were analysed using a GATK/MuTect pipeline. 15 pairs had matched data from SNP arrays and WES.

Results: WES revealed an average of 47 relapse specific missense and stop-gain variants (range 11-176) including ALK in 3 cases. Recurrent variants in ≥ 5 cases in MUC4, MUC17, MUC22, IGFN1 and KMT2C were observed. Germline exome data altered <5% of calls. There was no correlation between detected variants and risk group. In addition, 3 cases with a mutator phenotype resulting in thousands of variants called were identified. Two high risk cases had late relapses spanning 10 and 18 years respectively. 384 copy number abnormalities (CNAs) were detected in 18 pairs with an increase in CNAs at relapse mostly gains rather than losses (277 vs. 95). Chromothripsis was observed in 2 cases (chromosome 11 at diagnosis and chromosome 21 at relapse) in one case and (chromosome 11 at diagnosis and relapse) in the other. No correlation was seen between CNAs and loci of detected variants. MDM2 amplification was detected (at diagnosis and relapse) in 1 case and an intragenic deletion of ATRX at relapse in another.

Conclusions: Mucin genes and pathways may provide new targets for drug development in neuroblastoma. Relapse cases with a mutator phenotype were detected in 9% of cases. WES of paired samples can be accurately analysed in the absence of germline data.

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Targeting MYCN-Driven Fatty Acid Metabolism in Neuroblastoma

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MYCN amplification strongly associates with neuroblastoma (NB) poor clinical outcome. Metabolic reprogramming is emerging as a key function of MYC oncogenes. Although several c-MYC metabolic functions have been characterized, the role of MYCN in NB metabolic reprogramming remains unexplored.

Through global metabolomics profiling (Metabolon Inc.) in both NB cells upon conditional MYCN overexpression and knockdown, and tumors derived from the TH-MYCN mouse model, we found that MYCN profoundly alters lipid metabolism (Random Forest Classification identified lipids as major group separating tumors and normal tissues). Further fatty acid (FA) profiling in NB cells and tumors revealed that MYCN induces the accumulation of monounsaturated FA (MUFA), suggesting increased desaturase activity. FAs can be de novo synthesized (via desaturation from saturated FAs mediated by steroyl-CoA desaturase 1, SCD1) or uptaken from the microenvironment. Through stable isotope labeling and GC/MS analysis, we found that turning on MYCN promotes both FA synthesis and desaturation, and FA uptake. We further demonstrated that MYCN not only directly binds to SCD1 promoter to upregulate SCD1 transcription, but also enhances SCD1 activity. Blocking FA desaturation via SCD1 inhibition suppresses NB cell growth, which can be rescued by supplementation of oleic acid. However, it promotes compensatory FA uptake. Moreover, deprivation of exogenous FAs inhibits NB cell proliferation in both normoxic and hypoxic conditions, suggesting that NB cell growth also relies on FA uptake. We further identified FATP2 as a FA transporter whose expression is consistently upregulated in MYCN-amplified NB patients ($p<0.0001$) and strongly predicts poor survival ($p<0.0001$) in multiple large NB patient cohorts ($n=999$ patients). FATP2 inhibition via grassofermata efficiently inhibits FA uptake, lipid droplet formation, and NB cell survival without affecting normal cells, suggesting a potential selective vulnerability of NB cells. Importantly, combined SCD1 and FATP2 inhibition profoundly inhibits survival of MYCN-amplified cells.

Taken together, our data suggest that MYCN promotes both de novo lipogenesis and FA uptake to support NB growth. They also expose the vulnerability of NB to inhibition of lipogenesis and lipid uptake, and suggest a new therapeutic approach for MYCN-amplified NB.

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Age, Tumor Grade, and Mitosis-Karyorrhexis Index Are Independently Prognostic of Outcome in Neuroblastoma: An International Neuroblastoma Risk Groups Project

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Background: Criteria for COG neuroblastoma risk stratification includes both age and by the age-linked International Neuroblastoma Pathology Classification (INPC) system, confounding the prognostic contribution of age. Previous studies demonstrated the prognostic value of histologic category and individual morphologic features, and these criteria were incorporated in the International Neuroblastoma Risk Group Classification (INRG) system in lieu of INPC. We analyzed the INRG database to determine if these histologic features retained prognostic significance in patients diagnosed between 2003-2016. We also compared risk-group treatment assignment using individual morphologic features versus INPC.

Methods: Cohort 1 patients were diagnosed 1990-2002 (n=2746) and Cohort 2 patients were diagnosed 2003-2014 (n=5012). Associations between established prognostic factors versus MKI and grade of differentiation were assessed by chi-square tests. In cohort 2, Cox proportional hazards models of EFS were used for survival tree regression and multivariable analysis of age, grade, MKI, and diagnostic category.

Results: Undifferentiated grade and high MKI were statistically significantly associated with stage 4 disease, MYCN amplification, diploidy, and age >18mon. The 4 final subgroups of the survival tree analysis ("terminal nodes") of cohorts 1 and 2 were identical: <18mon with low/intermediate MKI; <18mon with high MKI; ≥18mon with poorly/undifferentiated tumors, and ≥18mon with differentiating tumors. In separate multivariable analyses of both cohorts, age, INSS stage, MYCN, ploidy, and grade were independently statistically significantly prognostic. Of the 4821 patients in cohort 2 with sufficient data for COG treatment assignment, the same treatment assignment was made using either INPC or grade plus MKI in all but 7 patients (all > 5 years of age with stage 3 differentiating tumors.)

Conclusions: Incorporating grade plus MKI in lieu of INPC results in nearly identical COG risk-based treatment assignment. Because INPC classifies tumors from patients >5 years as UH, analysis of grade of differentiation in this cohort has the potential to refine treatment stratification, especially for those with stage 3 neuroblastoma. As new criteria are evaluated for future iterations of risk stratification systems, statistical methods should eliminate the confounding of INPC and age by using MKI and grade instead of INPC.

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MYCN-Induced Upregulation of Glutamine Metabolism in Neuroblastoma (NB): A Potential Mechanism of Chemoresistance and Therapeutic Target

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Background: The MYC family proto-oncogenes are emerging as key players of the metabolic reprogramming that is linked to chemoresistance. Importantly, MYC enhances mTOR activity and glutaminolysis to support tumor growth and biosynthesis. However, mTORC1 inhibitors demonstrated limited clinical efficacy as single agents. We previously demonstrated that although temsirolimus (TEM) blocks in vivo NB growth, tumors rapidly re-grow after treatment suspension, suggesting acquired resistance. We hypothesize that activation of glutamine (Gln) metabolism is a key aspect of MYCN-driven tumorigenesis and contributes to NB resistance to mTORC1 inhibition.

Methods: Cell survival and apoptosis were determined in MYCN amplified and non-amplified NB lines upon Gln starvation. mRNA and protein expression of key enzymes of Gln metabolism were assessed in NB cells upon MYCN conditional overexpression and knockdown. Intracellular levels of glutamate were also assessed in these conditions. Intracellular glutamate levels, mRNA and protein expression were then assessed in MYCN-amplified cells upon TEM treatment. Finally, effects of dual mTORC1 and GLS (glutaminase) inhibition (with TEM and CB-839, respectively) were determined in MYCN-amplified and non-amplified cells.

Results: MYCN-amplified cells heavily depend on Gln metabolism for cell survival. Gln depletion induces cell death in MYCN amplified cells. Moreover, MYCN amplified cells display enhanced glutaminolysis compared to non-amplified cells. Interestingly, turning on MYCN induces the transcription of genes involved in glutaminolysis, in particular ASCT2 (a Gln transporter) and GLS1 ($p<0.01$), and silencing MYCN restores their baseline expression. mRNA levels of ASCT2 are also significantly elevated in MYCN-amplified tumors and strongly correlate with poor outcome ($p= 9.3$ e-12, R2 database). Additionally, we found activation of Gln metabolism in MYCN-amplified cells following TEM treatment. TEM upregulates the transcription of GLS2, GDH2, and SLC1A7 ($p< 0.01$), and increases intracellular glutamate levels. Importantly, combined GLS and mTORC1 inhibition profoundly inhibits survival of MYCN-amplified cells.

Conclusions: These results indicate that MYCN-driven NBs rely on Gln as an energy source and upregulate Gln metabolism in response to mTORC1 inhibition. They also indicate MYCN-induced Gln metabolism as a novel potential target in high-risk NB. Ongoing work seeks to elucidate how MYCN-mediated activation of glutamine metabolism contributes both to NB tumorigenesis and therapy response.

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Decreased Dinutuximab-Related Adverse Events by Maintaining Serum Hemoglobin > 10 g/dL in Patients with High-Risk Neuroblastoma – A Case Series

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Background: Immunotherapy utilizing anti-GD2 antibodies against neuroblastoma caries the risk of multiple adverse effects, including pain, neuropathies and capillary leak syndrome. Interventions that mitigate these effects are vital to permit patients to successfully complete this immunotherapy regimen.

Objective: We describe our experience of transfusing red blood cells (RBCs) as needed to maintain a serum hemoglobin of greater than or equal to 10 g/dL in patients while receiving dinutuximab-based immunotherapy and the impact of this intervention on treatment-related adverse events, including ICU transfer, pain, and capillary leak syndrome.

Methods: This is a retrospective case series involving 14 patients treated with dinutuximab for high-risk neuroblastoma from 2011-2016 at Nationwide Children's Hospital in Columbus, Ohio. During 37 of 76 hospitalizations for immunotherapy, RBC transfusions were given to maintain a hemoglobin > 10g/dL (group 1). During the other 39 hospital stays, patients were transfused to maintain hemoglobin of > 7gm/dL or for symptomatic anemia (group 2). We recorded data on pain, capillary leak, length of stay, and ICU transfers/admissions. Two sample t-tests statistically showed the differences between groups 1 and 2.

Results: Respectively, groups 1 and 2 showed differences in mean number of lasix doses (1.11 vs 1.64, p=0.24), mean days with a narcotic drip for pain (3.57 vs 4.28, p=0.03), incidence of capillary leak (20 vs 27, p=0.18), and number of ICU admissions (1 vs 6, p=0.06). Our study showed a mean length of stay of 6.65 and 6.41 days for groups 1 and 2 respectively (p=0.84), and a mean weight change of +1.03kg and +1.01kg (p=0.96).

Conclusion: Patients in whom hemoglobin was kept > 10 g/dL (group 1) had a statistically significant reduction in days with continuous narcotic infusion for pain, and a nearly statistically significant reduction in ICU admissions. Group 1 also had a clinically significant reduction in lasix doses and incidence of capillary leak. Larger studies are necessary to further demonstrate the association between red blood cell transfusions and reduction in adverse effects with dinutuximab. Further studies are also warranted to evaluate for transfusion related immunomodulation to determine the optimal timing of RBC transfusion during immunotherapy.

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High Curability of Brain Metastases Among Infants with Neuroblastoma following Adjuvant Treatment with 131I-8H9 Compartmental Radioimmunotherapy

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Background: Patients with neuroblastoma (NB) <18 months old without tumor MYCN amplification have a favorable prognosis, but development of CNS metastases is typically incurable. We assessed the efficacy of a multimodality regimen incorporating compartmental radioimmunotherapy (cRIT) using intraventricular 131I-labeled monoclonal antibody 8H9 for these patients.

Methods: Patients were treated with combination chemotherapy and/or radiation therapy and evaluated for adequate CSF flow. A 124I-8H9 or 131I-8H9 dosimetry dose with nuclear imaging was followed by a therapeutic injection of 131I-8H9, at a maximum of 2 cycles. Pharmacokinetics were studied by serial CSF, blood samplings nuclear scans. Dosimetry was based on CSF sampling and region-of-interest analyses by serial nuclear scintigraphy. Disease evaluation included pre- and post-therapy MR of brain/spine, 123I-MIBG imaging, and bone marrow testing approximately every 3 months for the first year, then 6 – 12 months annually.

Results: Eighteen patients <18 months at diagnosis developed CNS disease, 12 with MYCNA tumors. Median age at initial NB diagnosis was 7.1 mon (0.5-16 mon) and 23.7 mon (7-106 mon) at the time of CNS event. Patients developed CNS NB at the time of disease recurrence (N=13) or in the setting of refractory systemic NB (N=5). Six patients had multiple parenchymal masses and/or bulky leptomeningeal disease.15 patients were treated with cRIT; 3 patients died of disease before cRIT was given. Twelve of 15 received the multi-modality CNS-salvage regimen incorporating cRIT. Acute side effects were uncommon and generally self-limited. All 12 infants who received the CNS-salvage regimen incorporating cRIT are long term survivors with no recurrence of CNS or systemic NB, mean 6.3 years (1.6-11.8 years) from the detection of CNS disease. Of the 3 patients treated with cRIT without full salvage therapy, 2 died from progressive CNS NB 11 months and 31 months, and 1 from progressive systemic NB 16 months after CNS NB detection.

Conclusions: Infants with CNS NB represent a uniquely curable subset of patients with CNS disease, despite the presence of MYCN amplification, and/or multifocal, extensive bulky metastatic lesions. In this population, cRIT is safe, has favorable dosimetry to CSF, and significantly improves the chance of long term remission.

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Neuroblastoma Stage 4S: Regression Rate and Patients at Risk

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Background: Neuroblastoma stage 4S/MS is a low risk tumor with an overall survival of 65-92%. The natural course consists of initial growth of multifocal disease followed by regression. However, the precise clinical course of the initial growth and regressive phase is poorly described. We performed a retrospective cohort study to describe the clinical course, to identify patients at risk of an adverse outcome and to compare regression rate between treated and untreated patients.

Methods: Charts of all patients diagnosed with neuroblastoma stage 4S/MS between 1972 and 2012 in two Dutch oncology centers were evaluated for therapy, response, outcome and for clinical, metabolic and radiological regression of tumor and liver metastases. Patients with high risk criteria were excluded.

Results: We identified 31 patients. The 5-year overall survival was 84% (median follow up 16.1 years, range 3.3-39). Treatment was given to 55% of the patients. In the first 3 weeks after diagnosis 4 patients died of rapid liver growth and compression. Neonates (infants less than 4 weeks old) were at highest risk of dying: 4/7 (58%) neonates died, while none of the older patients died. During the regressive phase, the primary tumor completely regressed in 69% of patients after a median of 13 months (range 6-73); liver size normalized in 91% after a median of 2 months (range 0-131), and architecture normalized in 52% after a median of 14.5 months (range 5-131). No differences in regression rate were seen between the treated and untreated patients. Late progression to stage 4 was observed in 3 patients, all had persistent elevated catecholamines. Two of three are currently in complete remission.

Conclusion: Neonates are at risk of dying of rapid liver growth. Patients with persistent elevated catecholamines are at risk of progression to stage 4. Radiological regression of primary tumor and liver architecture is slow and takes over 12 months.

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Indolent Stage 4 (ganglio)Neuroblastoma: The Developmental Missing Link Between Stage 4 and 4S Neuroblastoma?

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Background: Disseminated neuroblastoma (NB) is either unfavorable, aggressive stage 4/M or favorable stage 4S/MS characterized by spontaneous regression. Here, we describe a group of patients with a disseminated neuroblastic tumor with bone metastases, but a favorable course of disease. The tumors remain stable or slowly expand. They seem to differentiate, but without regression and they remain metabolically active (urine catecholamine excretion and ^{123}I-MIBG avidity). We describe the symptoms and course of disease and hypothesize how this novel entity bridges a gap between different NB entities.

Methods and Results: Between 1992 and 2014, seven patients, with a median age of 18 months (range 5-47), presented in Dutch centers with a disseminated ganglioneuroblastoma (GNB) or differentiating NB with dissemination to bone (7/7), bone marrow (4/7), lymph nodes (4/7) and the meninges (4/7). All patients, except one, were initially diagnosed as high risk (G)NB patients and treated accordingly with intensive combination chemotherapy. Three patients received additional ^{131}I-MIBG treatment. Response to therapy varied from a temporarily minor response (1 patient), to no response (4 patients) and progressive disease (1 patient). The untreated patient had stable disease. In the six treated patients, the anti-tumor treatment was interrupted and changed to a 'wait-and-see' policy. This resulted in stable disease or slow progression/expansion of the primary tumor and stable disease or differentiation of the metastases. Genetically, all tumors showed favorable patterns without MYCN amplification (6/7; in 1/7 the test failed) or loss of heterozygosity of chromosome 1p36 (3/7; in 4/7 not performed). HVA remained 1-3 times elevated compared to normal levels in 5/7 patients, while VMA normalized in 6/7 patients. All patients are alive and well after a median follow up period of 14.3 years (range 3.8 – 23.2 years).

Conclusion: This cohort consists of stage 4/disseminated (G)NB with a non-aggressive and non-responsive, indolent clinical course despite bone metastases. We hypothesize that these tumors arose at the developmental crossroad of favorable and unfavorable neuroblastoma entities.

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Addition of Activated Natural Killer Cells to Anti-GD2 Antibody Improves Survival and Inhibits Metastasis in a MRD Model of Neuroblastoma

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Purpose: High-risk neuroblastoma (NB) portends a poor prognosis with most patients succumbing to recurrent, metastatic disease. While treatment with anti-GD2 antibody (ch14.18) has improved 2-year event-free and overall survival, 35% of patients continue to progress on therapy. Activated natural killer cells (aNK) have been shown to augment ch14.18 dependent cell-mediated cytotoxicity in vitro, but the combination has not been examined in metastatic neuroblastoma in vivo. We hypothesize that the addition of aNK cells to ch14.18 will decrease NB cell viability and invasiveness in vitro and inhibit metastasis and prolong survival in a metastatic, surgical resection model of minimal residual disease (MRD) in neuroblastoma.

Methods: In vitro, the human NB cell lines (CHLA 136, CHLA 255, SH-SY5Y) were utilized for DIMSCAN cytotoxicity assays and matrigel invasion assays with the following treatment groups: control, ch14.18 alone, aNK alone, and ch14.18+aNK. In vivo, 1 million human NB cells were injected in the renal capsule of NSG mice (CHLA-136 n=40; CHLA-255 n=22; PDX-415X-M-5 n= 33). The primary tumor was resected 7 days post-injection and mice were randomly assigned to 4 treatment arms; control, ch14.18 alone, aNK alone or combination. The mice underwent treatment twice weekly for a total of 4 weeks. Metastases to the liver and bone marrow were assessed by bioluminescence and histopathology. Survival was analyzed using log-rank analysis, metastasis with Fisher's exact test, and in vitro studies with Student's t-test.

Results: In vitro, in all cell lines tested, the addition of aNK cells to ch14.18 led to increased cytotoxicity and decreased invasiveness compared to control and ch14.18 alone ($p<0.001$ for both). In vivo, the incidence of metastases in mice was significantly decreased and overall survival was significantly prolonged in combination treatment mice compared to control in each experiment (CHLA-136, CHLA-255, PDX-415X-M-5 $p<0.05$ for all).

Conclusions: The combination of aNK cells and ch14.18 demonstrates potent in vitro efficacy as well as improved survival and decreased metastasis in a metastatic minimal residual disease model of NB. Infusion of aNK cells in addition to standard immunotherapy may serve as an important adjuvant treatment for children with high-risk NB.

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High-Dose Humanized-3F8 (hu3F8) Plus Stepped-Up Dosing of GM-CSF: Outpatient Treatment, Low Immunogenicity, and Major Responses in a Phase II Trial

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Background: In preclinical and phase I studies, hu3F8 displayed features promising for improving efficacy and quality of life compared to other anti-GD2 antibodies for high-risk neuroblastoma (HR-NB): slow koff in antigen-binding kinetics; enhanced antibody-dependent cellular cytotoxicity (ADCC); substantial but less complement activation which efficiently lyses NB yet causes pain; safe dosing >2.5x higher than standard dosages (100mg/m2/cycle) of dinutuximab and murine-3F8; low immunogenicity; major anti-NB activity; and manageable toxicity and pharmacokinetics supporting 3 doses/cycle (Mon-Wed-Fri), administered outpatient. GM-CSF is well-tolerated clinically, exerts a dose-response effect in ADCC, and significantly improves outcome with murine-3F8 (JCO 2012;30:426).

Methods: In a phase I/II expansion (31/5/2016) (NCT01757626), HR-NB patients in ≥2nd complete/very good partial remission (CR/VGPR) (Group 1), with primary refractory disease (Group 2), or with secondary refractory disease (persistent NB despite treatment for relapse) (Group 3) receive hu3F8 at 9mg/kg/cycle (~270mg/m2/cycle) divided into 3 doses infused intravenously (30 minutes) Mon-Wed-Fri. Subcutaneously-administered GM-CSF begins 5 days pre-hu3F8 in priming doses of 250µg/m2/day, then stepped-up to 500µg/m2/day with the hu3F8 infusions. Response is scored post-cycle 2 and then q10-12wks. Cycles continue monthly x5 after achieving CR/VGPR but are deferred if human anti-human antibody (HAHA) develops.

Results: To date, Group 1 includes 29 patients 0.9-to-17.8 (median 3.3) years post-diagnosis, 2.2-to-24.5 (median 6.3) years old, 25/29 prior-treated with ≥1 anti-GD2 antibody, and status-post 1 (n=18) or ≥2 (n=11) relapses; 12-month event-free survival is 74%. Group 2 includes 17 patients with 15 evaluable for response 5-to-19 (median 6.6) months post-diagnosis, 2.9-to-10.9 (median 5.1) years old, and 9/15 with Curie scores 7-to-23 plus marrow(+); 13/15 (87%) achieved CR/PR. Group 3 includes 25 patients 0.9-to-10.6 (median 3.5) years post-diagnosis, 2.6-to-23.6 (median 6.5) years old, 23/25 prior-treated with ≥1 anti-GD2 antibody, and status-post 1 (n=15) or 2-to-6 (n=10) relapses; 12-month progression-free survival is 55%, and 7/23 (30%) patients evaluable for response achieved CR/PR. HAHA developed in 11/71 (15%) patients; 9/11 HAHA(+) patients were prior-treated with anti-GD2 antibody. Treatment was outpatient, without unexpected toxicities.

Conclusions: Modest toxicity, low immunogenicity, and substantial anti-NB activity support further development of hu3F8 which is proceeding apace and includes a pivotal trial involving US and European institutions.

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Low Frequency ALK Hotspots Mutations in Neuroblastoma Tumors Detected by Ultra-Deep Sequencing: Implications for ALK Inhibitor Treatment

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The ALK tyrosine kinase receptor has been shown to be important in the oncogenesis of the pediatric cancer neuroblastoma. Whereas numerous ALK fusion genes have been reported in different malignancies, in neuroblastoma ALK is mainly activated through oncogenic point mutations and more rarely, through gene amplification. Three hot spot residues (F1174, F1245, and R1275) localized within exon 23-25 encoding the kinase domain of ALK, account for 85% of mutant ALK seen in neuroblastoma.

In a representative cohort of 105 Swedish neuroblastoma cases of all stages and genomic subtypes, these hotspot regions were re-sequenced with >5000X coverage. ALK mutations were detected in 16 of 105 patients (range of variant allele fraction: 2.7%-60%). Mutations at the F1174 hotspot were observed in eleven cases: seven cases with F1174L, two cases with F1174I, one each of the F1174C, and F1174S substitutions. Interestingly the patient harboring F1174S (variant allele fraction 58%) in parallel contained a subclonal F1174I mutation (variant allele fraction 8%). Alterations of the F1245 codon were detected in three cases: two F1245I and one F1245C. ALK mutations were also detected at the I1171 and L1240 codons in one tumor each. No mutations were detected at R1275. Sanger sequencing of all ALK mutated samples could confirm ALK status for all samples with variant allele fraction above 15%. Four of the samples with subclonal ALK mutation fraction below this would have gone undetected relying on Sanger sequencing only.

No distinct mutation spectrum in relation to neuroblastoma tumors of the genomic subtypes numerical only, other segmental, 17q-gain or MYCN-amplified could be detected although there was a relative paucity of ALK mutations among 11q-deleted tumors.

Our results indicate the presence of ALK point mutations at subclonal levels that risk being undetected when employing Sanger sequencing only. As activating ALK mutations mark patients for treatment with emerging small molecule inhibitors targeting ALK, early and sensitive detection of ALK alterations are of high clinical importance. This study shows the utmost importance of ultra-deep sequencing in clinical practice, highlighting the potential and the importance of serial samplings for therapeutic decisions.

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Late Relapses after Anti-GD2 immunotherapy for High-risk Neuroblastoma (HR-NB)

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Introduction: Antibody-mediated anti-GD2 immunotherapy is standard-of-care for HR-NB in first remission with reported long-term overall survival (OS) of 40-60%. Most patients relapse <2 years after starting immunotherapy. There is scant information on patients with later relapses.

Methods: All HR-NB patients treated with m3F8+GM-CSF in first CR/VGPR after high-dose chemotherapy and surgery on one of 4 MSKCC protocols (clinicaltrials.gov NCT00002560, NCT00072358, NCT01183429, NCT02100930) from 1998-2015 were evaluated. Patients requiring second-line therapy to achieve CR/VGPR were termed ultra-high-risk (UHR-NB). Surveillance testing with CT/MRI chest/abdomen/pelvis, 123I-MIBG scans and bone marrow aspirates+biopsies was mandated q3 months for 2 years after initiating immunotherapy. Further testing schedule was individualized. Characteristics of patients relapsing >2years post-immunotherapy initiation (LR) were compared with those relapsing earlier (ER). Statistical analysis was performed using Chi-square or Kaplan-Meier methods.

Results: At a median follow-up of 38 months post-m3F8+GM-CSF, 109 of 255 (43%) patients relapsed. Median time-to-relapse was 10 (range 0.9-50.3) months. Fourteen (13% of relapses) patients had LR: 9 between 2-3 years and five >3years after immunotherapy. Factors predicting ER included MYCN-amplified (58% and 21% in ER and LR respectively; p=0.01) and UHR-NB (28% versus 7%; p=0.08), but not autologous stem cell transplant (39% versus 44%; p=0.4). CNS relapses always occurred early (24% versus 0%). Both ER and LR were primarily detected with surveillance scans: only 3/14 LR patients were symptomatic at relapse. Salvage therapy was less successful in achieving second CR in patients with ER vs LR (58% vs 70%; p=0.02). Achieving second CR was highly prognostic for OS both from time of immunotherapy initiation and first relapse (p<0.001). From time of first relapse median progression-free survival was 9.7±1.2 and18±5.4 months for ER and LR respectively; respective median OS was 26±3.3 and 26.6±2.9 months. LR patients had significantly improved 5-year OS from time of immunotherapy compared to ER (83±11% vs 37±5%; p=0.007) though not from relapse (p=0.1).

Conclusion: Most LR patients have non-MYCN-amplified NB and do not relapse in CNS. LR usually occurs between 2-3 years post-immunotherapy, suggesting that routine surveillance scans be extended to 3 years. Attempts to achieve second CR after relapse are beneficial to both ER and LR.

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Expression- and Immune- Profiling of Neuroblastoma Associated with Opsoclonus Myoclonus Ataxia Syndrome (OMAS) to Identify Features of Auto- and Tumor-Immunity

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OMAS is a devastating neuroimmune disease affecting 2-3% of children with neuroblastoma, causing ataxia, myoclonic jerks, and disordered mood/behavior. Paradoxically, tumor related outcomes are excellent; survival rates among children with OMAS exceed 90%. The OMAS causative antigen is unknown and the nature of the immune process is poorly understood, with few reliable markers. We hypothesize that the distinctive immune response in OMAS patients is responsible for superior neuroblastoma-tumor outcomes, and that deeper understanding of immune response in OMAS may provide insights into mechanisms of effective anti-neuroblastoma tumor immunity.

We have undertaken a molecular study of a large cohort of OMAS neuroblastoma samples collected as part of a Children's Oncology Group clinical trial for OMAS therapy. Tumor, blood and CSF were collected at time of diagnosis from fifty-four children enrolled in the study. We used RNAseq to compare gene expression profiles of OMAS neuroblastoma to low- and high-risk neuroblastoma without OMAS, to identify differentially expressed or novel antigens in OMAS that may drive anti-neuronal reactivity or improve anti-tumor immunity. B and T cell gene signatures dominate the differentially expressed genes, corresponding to increased tumor infiltration in OMAS associated samples. We also identified several intriguing candidate genes that are differentially expressed in OMAS, which we are testing for recognition by OMAS patient serum antibodies. HLA typing of this patient cohort will enable identification of novel peptides with predicted HLA-specific immunogenicity, as well.

In parallel, we are searching for distinctive features of OMAS immunity, which may be linked to improved tumor outcomes. We used T cell receptor beta chain sequencing of tumor genomic DNA to profile tumor-infiltrating lymphocytes in OMAS neuroblastomas and control neuroblastomas. We predicted a more clonal, antigen driven response in OMAS patient samples. A pilot set of OMAS and control neuroblastoma samples revealed no clonal dominance in OMAS, but rather diverse repertoires, suggesting that the anti-tumor response may not be dominated by the OMAS associated antigen. Analysis of OMAS T cell repertoires, and B cell repertoires to assay antibody diversity, clonality, and sharing in OMAS neuroblastoma using the full cohort and controls are ongoing.

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Fenretinide (4-HPR), Via NOXA Induction, Enhanced Activity of the BCL-2 Inhibitor ABT-199 in High BCL-2-Expressing Neuroblastoma PDX Models

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Despite intensive treatment, many patients with high-risk neuroblastoma still die of the disease. The anti-apoptotic BCL-2 family of proteins protects cancer cells from apoptosis, with neuroblastoma mainly depending on MCL-1 and BCL-2 for survival. Therefore, modulating anti-apoptotic proteins may provide an effective therapy against neuroblastoma. Fenretinide, a synthetic retinoid formulated in Lym-X-Sorb, has shown multiple complete responses in a phase 1 neuroblastoma clinical trial. ABT-199 is a specific BCL-2 inhibitor with an FDA approved indication for CLL with 17p deletion. 4-HPR+ABT-199 is synergistically cytotoxic in 29 out of 32 tested neuroblastoma cell lines, with stronger synergism in ABT-199-sensitive versus ABT-199 -resistant cell lines ($p<0.0001$). ABT-199-sensitive cell lines express higher BCL-2 protein compared to ABT-199-resistant cell lines ($p<0.001$), suggesting that BCL-2 is a biomarker of ABT-199 and 4-HPR+ABT-199 activity in neuroblastoma. 4-HPR+ABT-199+ketoconazole (a CYP3A4 inhibitor used to increase 4-HPR plasma concentration) significantly ($p<0.0001$) improved the event-free survival of mice relative to single agents in a high BCL-2-expressing patient-derived xenograft (PDX) established from progressive disease but not in a low BCL-2-expressing PDX. In matched-pair cell lines (established at diagnosis (DX) and relapse (PD) from 10 patients), we observed that the BCL-2 expression in the DX line was consistent with that of the PD line from the same patient. Our data suggest that BCL-2 expression at diagnosis and relapse are consistent, enabling assessing BCL-2 expression at diagnosis to identify patients likely to respond to 4-HPR+ABT-199. In a pair of PDX models established at DX and PD from the same patient, the PD PDX was resistant to cyclophosphamide+topotecan compared to the DX PDX (median survival 71 days for the DX and 37.5 days for the PD model), but both DX and PD PDXs were responsive to 4-HPR+ABT-199+ketoconazole (median survival 83 days for the DX and 101 days for the PD model). 4-HPR induced NOXA (a pro-apoptotic protein that inhibits MCL-1) expression in multiple neuroblastoma cell lines. NOXA knock-down abolished the synergy of 4-HPR+ABT-199 while overexpressing NOXA significantly enhanced ABT-199-single-agent activity. Thus, 4-HPR, via induction of NOXA, enhanced ABT-199 activity, and the combination is well-tolerated and highly active against high BCL-2 expressing neuroblastoma PDX models.

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High Oct4 Expression Significance in Neuroblastic Tumors

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Background: The octamer-binding transcription factor 4 (Oct4) is a stem cell marker associated with cancer progression and aggressiveness due to its role in cellular pluripotency regulation. In neuroblastic tumors (NBTs), Oct4 is linked to cancer stem cell phenotype but its expression pattern and its clinical significance is still unknown.

Aim: To define how Oct4 is expressed in NBTs and determine its association with different prognostic factors and the survival rate of patients with NBTs.

Materials and methods: Nuclear Oct4 expression was automatically analyzed in 563 NBTs included in tissue microarrays using Pannoramic Viewer (NuclearQuant module) and validated by pathologist analysis. According to the median percentage of Oct4 positive cells (Oct4+), cases were classified in low (0% to 8.67%) or high (8.70% to 58.44%) and depending on their negative cellularity, cases were divided in poor (248 to 2,596 negative cells) or rich (2,609 to 7,731 negative cells). SPSS Statistics was used to carry out the statistical analysis with $p<0.05$, performing Kaplan-Meier curves to assess event free survival (EFS) and overall survival (OS) of patients with different Oct4 expression in their NBTs. A multivariate analysis was done to determine which parameters independently predicted EFS and OS.

Results: 185 cases (33%) presented high Oct4 expression. The percentage of high Oct4+ cases was higher in NBTs with poor negative cellularity (20.6% versus 12.1%) and in neuroblastoma and nodular ganglioneuroblastoma histological categories with undifferentiated neuroblasts (25.0%) rather than those with poorly differentiated neuroblasts (17.1%) or with differentiating ones (11.4%). No Oct4 expression was found in ganglioneuroma cases (n=18). A statistically significant association was found between high Oct4+ tumors and patients older than 18 months, metastasis, segmental chromosomal aberrations, MYCN amplification and worse EFS and OS. A Cox proportional hazards procedure determined that tumors with high Oct4 expression almost quadruplicated the patient's death hazard.

Conclusion: Oct4 could be considered as a therapeutic target when NBTs present high Oct4 expression with poor negative cellularity, neuroblastoma/nodular ganglioneuroblastoma histological categories and low differentiated neuroblasts.

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Spinal Canal Invasion in Peripheral Neuroblastic Tumors – Report From The Siopen Prospective Study Registry

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Background/Objectives: Spinal canal invasion (SCI) occurs in 10-15% of children with peripheral neuroblastic tumors (PNTs). These patients have favorable clinical and biologic features at diagnosis and better survival probability but are prone to develop late neurologic and orthopedic sequelae. The question of their optimal treatment has not been solved yet. This prospective SIOPEN Study Registry aims to collect data on these patients with the aim 1) to increase the knowledge on natural history of PNTs presenting with SCI, 2) to evaluate the combined effects of different risk factors on their outcomes, and 3) to ultimately develop common treatment guidelines.

Design/Methods: This SIOPEN Study intends to collect clinical, pathological, biological, therapeutic and follow-up data on symptomatic and asymptomatic patients with PNT and SCI. A minimum of 150 patients are expected. Type and severity of symptoms were to be reported according to age adjusted, CTCAE, FLACC, pain scores and ASIA scales.

Results: From June 2014 to December 2017 83 patients have been registered from 11 countries. Median age is 12 months, M/F ration is 36/47. The most common histology is NB poorly differentiated and only 7 % of the patients had MYCN amplification. Forty-five (60%) patients were symptomatic. Symptoms include: 34 (44%) motor deficit, 24 (31%) pain and 2 (3%) bladder & 3 (4%) bowel dysfunction (one patient could have more than one symptom). Forty-nine (66%) patients have L2 disease. Patients were treated according to the SIOPEN / institutional protocols. Median follow up is 12 months. After 2 months from diagnosis 66 % of evaluable symptomatic patients with motor deficit had improvement/disappearance of their symptoms. Interim evaluation showed no apparent difference in outcome in relation to type of therapy.

Conclusion: This is the first cooperative study designed aiming for data collection for evaluating the combined effects of different risk factors and neurologic functioning and orthopaedic outcomes of PNT's patients presenting with SCI. We encourage pediatric oncologists to participate in this prospective study.

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Implementation of Personalized In Vitro Neuroblastoma Models in The Dutch Individualized THERapy (iTHER) Program for Children with Relapsed Tumors

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Background

To improve the treatment options for childhood cancers, we have initiated the individualized THERapy (iTHER) program in the Netherlands. This program aims to determine the presence of actionable molecular aberrations in untreatable relapsed or refractory tumors in children and is performed in close collaboration with the German INFORM program. Currently, over 40 pediatric cancer patients, including 11 neuroblastoma, have been analyzed in the iTHER trial. Tumors have been profiled by whole-exome sequencing, low-coverage whole-genome sequencing, RNA sequencing, gene expression profiling and methylation profiling. In line with the INFORM results (>300 cases), analysis of the profiling data by the local molecular tumor boards showed that individualized treatment advices could be given for about 50% of the patients. To improve the treatment options for the remaining 50% of the children with incurable cancer, standardized ex vivo drug testing is currently being integrated in the iTHER program for several tumor types including neuroblastoma.

Results

To this purpose we generate tumor-derived organoid cultures from all relapse neuroblastoma patients that are included in our precision medicine program. These tumor organoids are subsequently subjected to high-throughput robotics screening using a 170-compound library, containing all regularly used cytotoxics/cytostatics and all targeted compounds in (pre)clinical development for pediatric cancer treatment. As a reference, the same compound library is tested in a panel of >20 classical neuroblastoma cell lines and will be tested in healthy organoids. In addition, literature searches have been performed to link ex vivo observed efficacies to clinical relevant plasma concentrations. Screening of the first neuroblastoma organoid cultures with 0.1 nmol/L-10 µmol/L of the compounds on the library plate yielded PARP inhibitor talazoparib and CHK1 inhibitor prexasertib among the most effective targeted inhibitors for neuroblastoma.

Conclusion

Clinical implementation of the iTHER precision medicine program with organoid drug screening can improve the predictive value of clinical trials with targeted compounds. Eventually, this should lead to the availability of more effective and less toxic treatment options for children with incurable cancer.

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Investigating the Role of Musashi 2 in Neuroblastoma Tumorigenesis

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Background: Patients with high-risk neuroblastoma endure an extremely intense, multidrug treatment regimen and yet, approximately half of them die, thus mandating a better approach to this cancer. Our laboratory is investigating the overarching hypothesis that RNA binding proteins (RBPs), which play key roles in balancing self-renewal, differentiation, and cell proliferation are deregulated in neuroblastoma, and drive oncogenic signaling networks. Therefore, RBPs may constitute novel targets for therapeutic development. In this work, we focus on Musashi 2 (MSI2), an RBP that has been shown to play a critical role in the maintenance of stem cell populations and in the formation of aggressive tumors, notably in acute myeloid leukemia and in colon cancer. The role of MSI2 in neuroblastoma, however, has not been assessed and is the focus of this investigation.

Methods: To test this hypothesis, we used in silico analysis of neuroblastoma gene expression datasets annotated with clinical parameters. Additionally, we used shRNAs to manipulate transcripts of interest in neuroblastoma cells and measured effects on cell proliferation, cell cycle status, and apoptosis.

Results: Gene expression profiling revealed that MSI2 is robustly expressed in neuroblastoma cell lines, tumors, and patient derived xenografts, within the MYCN and non-MYCN amplified context. Immunoblotting in a representative panel of lines confirmed the expression of MSI2 at the level of protein. High MSI2 expression was associated with higher stages across multiple independent datasets and was correlated with worse survival. Depletion of MSI2 using four independent shRNAs was successfully confirmed via Western blotting and led to 2 to 3-fold decreased proliferation across four cell lines that was due in part to increased apoptosis.

Conclusions: MSI2 is robustly expressed in multiple models of neuroblastoma and appears to drive increased proliferation and survival. Current studies are using gene expression profiling to identify key downstream networks of interest. Further understanding the role of MSI2 in neuroblastoma will provide a foundation for designing strategies to target this RBP for therapeutic effect.

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Nuclear Patterns of High Chromosome Unstable Neuroblastic Cells Through Digital Pathology

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Introduction: Presence of high chromosomal instability in tumor genomes can be reflected in nuclear morphological changes. Image analysis techniques are helpful to better characterize the complex interplay of colors observed in histological sections and provide a basis for subcategorizing different neoplasms based on nuclear features.

Aim: Analysis of telomere dysfunction (TD) and perinucleolar compartment (PNC) in a cohort of neuroblastoma with high chromosome instability (HR-NB) to define nuclear patterns, given features as fluorescence or stain intensity, size and number of signals, in a semi-quantitative color-based manner using digital microscopy images.

Material and Methods: A cohort of HR-NB were included in a TMA and nuclear features were quantified and characterized from digital microscopic images. TD were analyzed in seventeen HR-NB by interphase quantitative fluorescence in situ hybridization (IQ-FISH) using a telomeric probe (Dako) and Telometer software (Image J). Standard deviation (SD), fluorescence ratio, area and number of signals per nucleus were calculated. PNC prevalence, size and number per cell were analyzed by immunohistochemical staining (SH54, Axxora) and Image Pro-Plus in sixteen HR-NB. Morphometric data were associated with SNPa tumor profiles (Affymetrix) and clinical data.

Results: Analysis of IQ-FISH fluorescence parameters resulted in two tumor groups, the largest group (9/16) with longer telomeres and presence of ALT mechanism, both defined by the presence of high intensity, area of telomere sign and high SD intensity values that suggest heterogeneity in cell clones. Patients in this group showed reduced EFS and OS. Three SH54 staining patterns were observed in the eight positive tumors, ranging from weak and diffused nucleoplasm staining without presence of PNC (2/8), presence of 2-5 small PNC/cell with prevalence of 20-50% (3/8) to punctate medium and strong nucleoplasm stain, with presence of some large PNC and prevalence of 50-90% (3/8). The latest group showed the highest number of genetic amplifications and SCAs of the cohort, with presence of chromothripsis in two biopsies.

Conclusion: Nuclear semi-automatic quantification and characterization by color-based software allows the identification of nuclear patterns, associated with genomic and clinical prognostic markers in HR-NB.

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PBX1 Interacts with the Epigenetic-Modifying CHD5-NuRD Complex to Promote Differentiation in Neuroblastoma

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Objectives: Neuroblastoma presents in patients as a spectrum of disease, ranging from low-risk disease that may spontaneously differentiate to high-risk disease that is much more aggressive and variably responsive to differentiating agents like 13-cis retinoic acid. We previously showed that PBX1, a cofactor of HOX proteins, promotes differentiation in neuroblastoma and has increased expression after treatment of neuroblastoma cells with 13-cis-RA. However, the mechanism by which PBX1 promotes differentiation is unclear. Nucleosome remodeling and deacetylase (NuRD) complexes work as chromatin-modifying epigenetic regulators, and these complexes include HDAC1 and HDAC2 and a member of the chromodomain-helicase-DNA-binding (CHD) family. CHD5 has been previously demonstrated to be found in the NuRD complex in neuroblastoma, where it also functions as a tumor suppressor by promoting differentiation, and CHD5 expression also increases with 13-cis retinoic acid treatment. PBX1 has been shown to bind and redirect HDAC1 and HDAC2 in other tissues. We hypothesize that PBX1 directly interacts with the CHD5-NuRD complex through HDAC1, HDAC2, and/or CHD5 to alter histone acetylation and promote spontaneous and retinoid-induced differentiation in neuroblastoma.

Methods: Retinoid sensitive and resistant neuroblastoma cells lines were grown in the presence of 13-cis-RA or vehicle. Interaction between proteins was demonstrated through proximity ligation assay (PLA) and co-immunoprecipitation (Co-IP). Effects of PBX1 expression on histone acetylation were performed by western blot.

Results: PLA demonstrated that PBX1 co-localizes with subunits of the NuRD complex including CHD5 and HDAC1 in neuroblastoma cell lines. Nuclear localization of PBX1 and subunits of the NuRD complex increases with treatment of 13-cis-RA. Co-IP revealed binding between PBX1 and components of the NuRD complex, including CHD5, and these interactions increase after treatment with 13-cis-RA. Modulation of PBX1 expression is associated with alterations with histone 3 lysine 4 acetylation, as shown by western blot.

Conclusion: PBX1 interacts with the CHD5-NuRD complex to promote differentiation in neuroblastoma, suggesting that differentiation is avoided in neuroblastoma by modulating this pathway.

Significance: This study is the first to identify a direct interaction between PBX1 and a NuRD complex. Elucidating the mechanisms of neuroblastoma differentiation may allow for improved treatment selection in this disease.

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Integrated Analysis of Tumor Microenvironment and Copy Number Alterations in Neuroblastoma

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Background: With the advent of omics technologies and digital pathology, an unprecedented amount of data in neuroblastoma (NB) has been generated. However, the analysis of such large-scale datasets is challenging and has not led to effective therapies, especially in high-risk (HR) NB. Therefore, it is essential to integrate this complex data to best refine patient stratification and pinpoint genetic factors that play critical roles in NB.

Purpose: Computational integration of digital pathology data in combination with genomic data obtained from NB in order to identify aggressiveness patterns.

Methods: The following information from 149 NB was integrated using the arrayMap repository (http://arraymap.org): i) clinicobiological data; ii) digital pathology of immune cells infiltration and patterns of vascularization using Panoramic Viewer v.1.15 software (3DHistech Ltd) and Angiopath® system; and iii) segmental chromosome aberration (SCA) diagnosed by SNPa (Affymetrix/Illumina).

Results: A positive association between a high infiltration of macrophagic cells (CD68+) and 11q deletion was found (p=0.01). Besides, in cases with low infiltration of dendritic cells (CD11c+) 7q gain region was the most frequent atypical SCA (12/15). The most relevant morphometric feature associated with survival was high perimeter for lymphatic vessels of 5-15 µm (5-year OS 36.3±0.074% vs 80.6±0.71%, p=0.007; median perimeter ≥27). In these patients age ≥18 months (p=0.002) and SCA tumor profile (p=0.029), specially with 11q deletion (40 out of 98, p=0.011), were frequently found. In cases with low width for lymphatic vessels of 15-20 µm 11q deletion was less frequent (62% vs 43%, median width <9.36 µm)) while gain of 7q was more frequent (80% vs 45%, p=0.014).

Conclusions: Integration of the NB data obtained from digital pathology and genomic analysis into arrayMap repository has found patterns of the tumor microenvironment related to SCA and has detected potential driving factors that would define the switch to aggressiveness in high-risk NB.

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Patients with Localized Neuroblastoma and NMYC Amplification: A Report from the HR-NBL1/SIOPEN Trial

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Purpose: To analyze survival rates, patterns of relapse and prognostic factors of patients with localized NMYC-amplified (MNA) neuroblastoma included in the first international SIOPEN high-risk trial (HR-NBL1).

Methods: We analysed data from patients with localized MNA neuroblastoma, any age, included in the HR-NBL1/SIOPEN trial (NCT01704716) from 2002 to 2014. Primary endpoint was 5-year event-free survival (EFS). Secondary endpoints were overall survival (OS), cumulative incidence of relapse (CIR), and non-relapse mortality (NRM). Prognostic factors assessed were age, histology, primary tumour site, LDH, ferritin and high-dose chemotherapy (HDC) regimen.

Results: Among 2587 patients, 238 had localized MNA neuroblastoma. The median age at diagnosis was 2.05 years (range 0.09-9.1), and 97 (41%) were females. The primary tumor site was abdominal in 205 patients (86%), thoracic in 15, pelvic and cervical in 11 and 7 patients, respectively. Seven over 101 patients had favorable histology. All received Rapid Cojec as induction chemotherapy, with complete response in 12% of patients, partial response in 81%, stable and progressive disease in 5% and 2%, respectively. HDC consisted in Busulfan-Melphalan and Carboplatine-Etoposide-Melphalan in 162 and 41/203 patients, respectively. Complete surgery and radiotherapy were performed in 157/206 and 175/199 patients, respectively. Maintenance treatment with anti-ganglioside2 antibodies was administered in 45/238 patients.

Median follow-up was 6.8 years (range 0.5-15.0). Five-year EFS and OS were 64%±3% and 70%±3%, respectively. None of the assessed prognostic factor had a significant impact on survival. Sixty-eight patients experienced a recurrence with a median delay of 1.1 years (25-75th: 0.6-1.4 yrs). The 5-year CIR was 31%±3%. Recurrences were "localized only" in 28/68 patients, combined and "metastatic only" in 16 and 16 patients, respectively. Eleven treatment-related deaths were identified, with a 5-year NRM of 6%±2%.

Conclusions: In spite of a better outcome than other high-risk patients, the treatment of patients with localized MNA neuroblastoma still needs to be improved. Further biological analysis may help to better stratify these patients in the future. The early timing and the pattern of recurrence have to be taken into account to design future strategies for these specific patients.

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Functional Synergy Between Sense-Antisense lncRNAs at 6p22.3 Locus Determines Neuroblastoma Susceptibility Via USP36-CHD7-SOX9 Gene Regulatory Axis

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Neuroblastoma (NB) is one of the most common extracranial solid tumors in children and it accounts for 10% of all pediatric cancer mortality. NBs derived from the neural crest cells often found in adrenal medulla and sympathetic ganglia, where improper differentiation of developing sympathetic nervous system leads to tumor formation. Trait associated loci often map to noncoding genome that encode lncRNAs. However, the functional role of trait-associated lncRNAs in disease etiology is largely unexplored. Genome-wide association studies (GWAS) have uncovered several NB risk loci that show strong association to increased risk of NB development and aggressive disease. 6p22.3 is one such locus harbors a cluster of SNPs, associated with increased risk of neuroblastoma. This locus also contains several lncRNAs such as NBAT1, CASC15-003 and CASC15-004.

We show sense/antisense lncRNA pairs CASC15-003 and NBAT1 (6p22lncRNAs), which map to 6p22.3 locus, are tumor suppressors and their higher expression in neuroblastoma patients correlate with good prognosis. CpG methylation and a high-risk neuroblastoma associated SNP at the 6p22.3 locus contribute to the differential expression of 6p22lncRNAs. Loss of functional synergy between 6p22lncRNAs in neuroblastoma cells results in an undifferentiated state which is maintained by a common gene regulatory network, including SOX9 located on 17q region frequently gained in neuroblastoma. 6p22lncRNAs regulate SOX9 expression by controlling the stability of its upstream activator CHD7 via modulating the cellular localization of another 17q candidate and ubiquitin specific protease USP36.

Our study highlights the significance of trait-associated lncRNAs in NB pathogenesis. These trait-associated 6p22lncRNAs act as better independent prognostic markers alongside the existing biomarkers in the risk stratification in clinical setting. More importantly, our study unravels a new mode of lncRNA mediated gene regulation where 6p22lncRNAs act in co-operation to regulate the cellular localization of their common interacting protein USP36 and affecting the stability and expression of downstream genes CHD7 and SOX9, respectively. Thus, this regulatory nexus between 6p22.3 and 17q regions may lead to novel therapeutic strategies for NB treatment.

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Analysis of The Prospective Minimal Residual Disease Monitoring Study In GPOH-DCOG High Risk Neuroblastoma

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In a retrospective study, we previously demonstrated minimal residual disease (MRD) detected by real-time quantitative PCR (qPCR), to correlate with survival. Here we report the prospective study in high risk neuroblastoma (NB) patients treated in NBL2004 (GPOH) and NBL2009 (DCOG). We studied the prognostic value of the level of NB-specific mRNA at diagnosis, the clinical significance of clearance of NB from the BM, after 2 courses and at the end of induction-therapy.

Methods: Serial BM samples from 280 high risk NB patients at diagnosis and at set time points during treatment were prospectively collected at Dutch and German centers between 2009 and 2017. qPCR was performed using five NB-markers: paired-like homeobox 2b (PHOX2B), tyrosine hydroxylase (TH), dopa decarboxylase (DDC), cholinergic receptor alpha 3 (CHRNA3) and growth-associated protein 43 (GAP43). BM infiltration was defined as described previously (Stutterheim; van Wezel). Kaplan-Meier and a multivariable Cox regression model, with age, MNA and level of infiltration were used to study event-free survival (EFS) and overall survival (OS).

Results: Level of BM infiltration at diagnosis was associated with EFS (p<0,001) and OS (p<0,001). The adjusted hazard ratio for patients with BM infiltration >10% at diagnosis was 2,23 [95% CI1,39-3,57] and 2,41 [95% CI1,38-4,21] for EFS and OS, respectively. Early BM clearance or <0,1% infiltration after 2 courses did differ significantly in terms of EFS but not OS, compared to BM infiltration >0,1%. Persistence of BM positivity after induction therapy was associated with a significant poorer outcome. EFS and OS at 5 years for the negative, <0,1% and between 0,1 and 10% were 55% (95%CI: 40-70), 26% (13-38) and 20% (1-45); 66% (50-81), 47% (33-62) and 30% (16-58), respectively.

Conclusion: We show that high levels of BM infiltration at diagnosis are associated with a poor outcome. Furthermore, that fast response as determined by BM clearance or low BM infiltration after 2 cycles of induction therapy is associated with better EFS but not OS. Finally, BM clearance at the end of induction therapy is significantly associated with better EFS and OS.

References: Stutterheim et al. Clin Chem. 2009;55
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Summary of the Second Neuroblastoma New Drug Development Strategy (NDDS) Meeting

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Background: There is an urgent need to develop new therapies to improve cure rates and reduce toxicities of children and adolescents with neuroblastoma. The Neuroblastoma NDDS initiative started in 2012 with the aim of elaborating a strategy to accelerate and improve the development of targeted drugs for neuroblastoma.

Methods: In September 2017, a workshop organised by ITCC, SIOPEN and GPOH and supported by Neuroblastoma UK of European and US experts in neuroblastoma biology and drug development, representatives from pharma, parent organisations and regulators was held with the aim of enhancing collaboration, identifying effective strategies to target ATRX, TERT and MYCN, and evaluating and prioritising targets. Data relating to 40 targets were reviewed.

Results: TERT, ALT and BRIP1 were considered high priority targets but have limited available new drugs and efforts should be directed towards developing drugs for these targets. RAS/MEK, BCL-2, MDM2, BRD4, polyamine pathway, aurora kinase, CDK4/6, CDK9, CHK-1, CDK7, mTORC1/2, BIRC5, WEE1 and telomere function (with 6-thio-dG) were also considered high priority targets and have drugs available. These include drugs already evaluated in early clinical trials in neuroblastoma (e.g. aurora A kinase), drugs with early trials just opened (e.g. mTORC1/2, CHK1 or MDM2) or drugs with early trials not yet commenced (BRD4, CDK9, CDK7, BIRC5 inhibitors and 6-thio-dG). Opening early phase trials with these agents should be high priority. For these, efforts should concentrate in speeding up paediatric early trials and enhancing the preclinical data package for neuroblastoma. Inhibition of CDK9, CDK7, BRD4, mTORC1/2, polyamine pathway, aurora Kinase and ATR are potential strategies to target MYCN. Combination strategies of immunotherapy with targeted therapies should also be pursued.

Conclusion: The meeting should act as an initiator of regular European - US communication, dialogue and planning between all stakeholders. Greater emphasis in establishing optimal combinations at early stages of development is required. Future work should include a clearer definition of prioritisation tools and the “pre-clinical” package for evaluation of new drugs in neuroblastoma. Improved planning and agreed prioritisation of drugs with biologically driven hypotheses will lead to a faster and improved drug development process for neuroblastoma.

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The Epigenetic Mark 5-Hydroxymethylcytosine Identifies Activated Oncogeneic Pathways and Serves as a Biomarker for Risk Classification in Neuroblastoma

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Background: 5-hydroxymethylcytosine (5hmC) is an epigenetic modification associated with gene activation. In adult cancers, 5hmC signatures serve as robust biomarkers of cancer and outcome. Recently, Dr. He invented a novel, highly sensitive chemical labeling technology (nano-hmC-Seal-Seq) allowing characterization of genome-wide 5hmC using small quantities of tumor DNA or cell-free DNA (cfDNA). We hypothesized 5hmC plays a key role in regulating genes expression driving neuroblastoma phenotype, and that 5hmC profiles are prognostic of outcome in neuroblastoma.

Methods: 5hmC was quantified by nano-hmC-Seal-Seq using 100ng of DNA from locally banked, FFPE diagnostic neuroblastoma tumors. Fragment counts were called using featureCounts and normalized with DESeq2, which also identified genes with differential 5hmC between risk groups. Gene Ontology pathway analysis of differential 5hmC genes was performed. 5hmC patterns were compared to gene expression in publically available tumor microarrays.

Results: We profiled 5hmC in 15 high-risk, 11 intermediate-risk, and 27 low-risk tumors. Low-risk tumors had increased 5hmC peaks per sample compared to high-risk tumors (140,062 vs. 79,727, p=0.014). Hierarchical clustering of differential 5hmC genes identified two patients with MYCN-amplification with unexpectedly good outcome and one patient classified as low-risk who relapsed. In the high-risk tumors, increased levels of 5hmC were identified in 200 genes that were enriched for embryonal pathways. Analysis of public expression data from the 709 patients in E-MTAB-1781 showed that 76 of the 200 genes with increased 5hmC in high-risk tumors had high levels of expression in tumors from patients who died (poverlap<1E-5). Conversely, low-risk tumors had increased 5hmC in 128 genes enriched for pathways of neuronal differentiation. Of the 128 genes with increased 5hmC accumulation in low-risk tumors, the expression data revealed that 60 of these genes were more highly expressed in patients who were alive (poverlap=0.03).

Conclusions: 5hmC profiles differentiate neuroblastoma risk groups and may refine risk classification at diagnosis. RNAseq analysis of the 53 profiled tumors is ongoing to confirm the correlation between 5hmC and gene expression. Additionally, 5hmC profiling is being conducted using cfDNA isolated from blood samples collected from patients with neuroblastoma and healthy controls to test the feasibility of comprehensively assaying 5hmC in liquid biopsies.

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Novel MHC Antigens in Neuroblastoma and Development of Tumor-Specific T-Cells

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Methods: To test the feasibility of adoptive cellular therapy in neuroblastoma, we purified MHC and eluted bound antigens from 8 patient-derived xenograft (PDX) and 8 primary patient tumors, and characterized peptide sequences by MS/MS. We inferred HLA types from exome sequences for these tumors using PHLAT, and subsequently obtained predicted MHC binding affinities for eluted peptides using NetMHC. Antigens derived from differentially expressed genes (determined by comparing 153 NB cases to the GTEx database of normal tissue gene expression) were compared against a database of MHC presented peptides from 190 healthy tissues, resulting in the discovery of 265 neuroblastoma antigens not previously observed in healthy tissues.

Results: We identified 265 ligands presented on common MHC alleles and not observed in healthy tissues. We then prioritized 6 antigens (PHOX2B, TH, CHRNA3, IGFBPL1, GFRA2, and HMX1) as lead candidates for adoptive T-cell therapy based on binding affinity predicted to elicit T-cell response, level of differential expression, lack of expression in healthy tissue, commonality of HLA allele in patient population, relative abundance, biological relevance to neuroblastoma, and recurrence across tumors. We validated these antigens using synthetic peptides and have synthesized peptide/MHC dextramers to screen for antigen-specific CD8 T-cells. Using HLA-matched donors, we have identified rare populations of neuroblastoma antigen-specific CD8 T-cells. These cells were sorted and their T-cell receptors (TCRs) were sequenced in a single-cell manner to identify matched alpha/beta pairs. We then cloned these constructs into bicistronic T-cell-specific expression vectors and have generated early evidence of antigen-specificity in the cloned TCRs, which we are currently validating these using functional assays.

Conclusions: We have shown that neuroblastoma cells present a significant number of tumor-specific MHC antigens, many of which are derived from genes critical to tumor survival including six antigens which we expect will be potent and specific clinical targets. We have found tumor-specific CD8 T-cells in healthy HLA-matched donors and sequenced the TCRs of these cells, which we are currently pursuing in preclinical development.

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The Hippo Pathway Protein YAP Mediates Resistance to MEK1/2 Inhibition in Neuroblastoma

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Background: Compared to matched diagnostic tumors, relapsed neuroblastomas harbor an enrichment in mutations in the RAS-MAPK signaling pathway. The MEK1/2 inhibitor trametinib has shown tumor growth delay in neuroblastoma preclinical models, but no tumor regressions. Recent studies have implicated the Hippo pathway transcriptional coactivator protein YAP in relapsed neuroblastoma as well as trametinib resistance in other cancer models. We hypothesized that increased YAP transcriptional activity is a mechanism of MEK inhibition resistance in RAS-driven neuroblastomas.

Methods: We selected several RAS-driven neuroblastoma cell lines due to high YAP expression, including NLF (biallelic NF1 inactivation) and SK-N-AS (NRAS Q61K). NLF and SK-N-AS cells were treated with the MEK inhibitor trametinib (20 nM) over a 72-hour period, and changes in YAP protein expression and cellular localization were observed via immunoblot. YAP protein expression was modulated in NLF cells by siRNA-mediated knockdown and CRISPR-Cas9 genetic knockout. Effects on survival, proliferation, signaling, and phenotypic changes were observed with and without trametinib treatment by CellTiter-Glo, RT-CES, IncuCyte, and immunoblot.

Results: Trametinib caused a near-complete reduction in the levels of pYAP, but not total YAP, in both NLF and SK-N-AS cells after 72 hours, indicating an increase in active YAP in the cells. Nuclear extracts confirmed that the active YAP protein translocated from the cytosol to the nucleus, where nuclear YAP expression increased two-fold in response to 72 hour trametinib treatment. Transient knockdown and genetic knockouts of YAP in untreated NLF cells had a modest effect by decreasing proliferation by 20% ($p<0.01$), while combinatorial inhibition of MEK and YAP signaling caused a significant reduction in cellular proliferation by 70% ($p<0.001$), as measured by real-time sensing and cell viability assays.

Conclusion: Prolonged trametinib exposure causes a marked nuclear enrichment of active YAP, and co-inhibition of MEK and YAP in neuroblastoma models effectively reduces proliferation and survival. We have pinpointed a potential mechanism of trametinib resistance in RAS-driven neuroblastomas to support future development of a novel combination therapy for neuroblastoma patients.

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Advanced Network Analysis of Gene Expression Data to Unravel Oncogenic Dependencies in MYCN Amplified and Non-Amplified High-Risk Neuroblastomas

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Neuroblastoma (NB) has been regarded as a predominantly copy number driven disease. Low risk NB are characterized by whole chromosome imbalances with very few mutations. In 10% of high risk cases activating ALK mutations are found together with low frequency mutations affecting RAS and neuritogenesis pathways and chromatin remodelers. Half of these are MYCN amplified (MNA) with frequent 1p-deletion, 2p- and 17q-gain while the remainder shows no evidence of MYCN amplification (MNnA) with common 3p- and 11q-deletions as well as 17q gains. To what extent both high risk entities have a different underlying gene regulatory network is still a matter of debate.

To study this further, we applied advanced network analysis methodologies to recently established gene expression and copy number data sets for large series of primary human NB. Firstly, we identified and ranked dosage sensitivity genes for MNA and MNnA NB cases and selected the top 5% most significant genes for both MNA and MNnA separately. These top ranked genes consequently formed the central hubs in a network analysis on which an in-house curated gene interaction network was projected. The perturbed pathways in high risk NB patients were then characterized and compared within and between both high-risk groups. We explored the immediate neighborhood for top ranked dosage sensitive genes in both MNA and MNnA cases for related pathways and gene sets by applying a random walk-with-restart model to obtain a probability score of finding a random walker connecting functionally related genes. This score expresses how closely these top ranked genes are related to any of its pathway peers, and by ranking those relationships we can assess the relative importance of the pathway. We will present detailed results of our analysis and they can deliver novel insights into MNA versus MNnA major driving perturbed gene networks and cellular functionalities.

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Proteomic Analysis Reveals Novel Kinome Profile In ALK-Driven Neuroblastomas

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The Anaplastic Lymphoma Kinase (ALK) has been positioned as the major tractable oncogene product for targeted therapy in neuroblastoma (NB). Mutations are found mostly at three hotspots (F1174, F1245 and R1275), with the F1174* and F1245* mutations conferring broad resistance to crizotinib. We have recently demonstrated that lorlatinib, a novel ALK inhibitor, overcomes de novo resistance and exerts unprecedented activity in patient-derived xenografts (PDXs) harboring F1174L or F1245C mutations. The objective of our work was to identify differences in kinome reprogramming upon ALK inhibition with crizotinib and lorlatinib. We hypothesize that identification of the targets through which lorlatinib mediates its superior anti-tumor effects will provide insights into the mechanisms by which full-length ALK inhibition abrogates tumorigenesis, activates compensatory pathways, and uncover novel targets to overcome resistance. We used Multiplexed Inhibitor Beads coupled with Mass Spectrometry (MIB/MS) to quantitatively measure kinase activity dynamics on a proteomic scale. Three PDX models harboring F1174L (COG-N-453x), R1275Q (NB1643) and R1245C (Felix) were treated with either crizotinib (100 mg/kg/day) or lorlatinib (10 mg/kg/day) for 2.5 days or 6.5 days prior to MIB/MS analysis. Lorlatinib more potently inhibited ALK and preferentially inhibited a series of other kinases. Additionally, kinases involved in G2/M transition including PLK1, CHEK1 and aurora kinases were significantly inhibited by lorlatinib, suggesting that this multi-kinase inhibition is responsible for the robust anti-tumor activity observed in vivo. We interrogated the role of SHP2, a ubiquitously expressed tyrosine phosphatase recently implicated in the proliferation of kinase-driven cancers and shown to have a direct role in the G2/M checkpoint. Inhibition of ALK with lorlatinib as well as shRNA knockdown in SY5Y (ALK F1174L, PTPN11 T507K) and NB1643 showed decreased levels of pSHP2. Depletion of SHP2 using CRISPR constructs significantly inhibited cell proliferation in SY5Y, suggesting that SHP2 plays a crucial role in ALK driven NB. Additionally, knockdown of ALK in SY5Y downregulated PLK1 expression and arrested cells in G2/M phase. Moreover, lorlatinib treatment also inhibited pSHP2 and PLK1 and immunoprecipitation of ALK showed an interaction with SHP2. Whether SHP2 is regulated primarily downstream of ALK or jointly through alternative or compensatory signaling networks warrants further investigation.

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Risk Factors for Complications of Surgery for Neuroblastoma

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Objective: To determine the prognostic value of clinical and biological risk factors for adverse surgical outcome in children with neuroblastoma.

Methods: Retrospective multi-center cohort study including children who had resection of the primary tumor site for neuroblastoma in The Netherlands between 1998 and 2014. Surgery related adverse events were documented. Intra-operative complications were stratified into minor or major complications. Post-operative short-term complications were classified using the Clavien-Dindo classification (CD 1-2 and CD 3-5). Univariate logistic regression was calculated to study the effect of the following risk factors on adverse surgical outcome: stage, risk group, MYCN amplification, pre-operative MIBG therapy, extent of resection (complete, ≥95% or <95% resection), surgeon experience (≥50, <50 resections) and presence of encasement at time of surgery.

Results: Intra-operative minor complications were found in 19% of children, with no risk factors associated. Intra-operative major complications were found in 30% of children, Odds Ratio (OR) for risk factors associated was: INSS stage III (OR 6.76 (2.30-19.85)); INSS stage IV (OR 7.44 (2.85-19.45)); High-Risk disease (OR 2.40 (1.40-4.08)); MYCN amplification (OR=3.23 (1.77-5.88)); Extent of Resection ≥95% (OR 4.46 (2.45-8.13)); Extent of Resection <95% (OR 4.66 (2.32-9.35)); Encasement (OR 5.34 (3.00-9.49)).

Post-operative Clavien-Dindo 1-2 complications were found in 33% of children, risk factors associated: Extent of Resection ≥95% (OR 1.88 (1.08-3.29)); Extent of Resection <95% (OR 2.13 (1.10-4.11)); Encasement (OR 1.68 (1.00-2.82)).

Post-operative Clavien-Dindo 3-5 complications were found in 14% of children, risk factors associated: Extent of Resection ≥95% (OR 3.55 (1.54-8.14)); Extent of Resection <95% (OR 4.96 (2.03-12.27)); Encasement (OR=2.26 (1.10-4.65)).

Post-operative unintended PICU support was necessary in 6% of children, risk factors associated: Extent of Resection <95% (OR 5.07 (0.95-11.69)); Encasement (OR=3.80 (1.18-12.29)).

Post-operative unintended return to the operating theater was necessary in 6% of children, with no risk factors associated.

Conclusion: Less then complete resection and encasement are significantly associated with most intra- and post-operative complications. INSS Stage III and IV, MYCN amplification and High-Risk disease were significantly associated with major intra-operative complications only.

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Clinical Impact of ALK Mutations and Coexisting Genomic Aberrations in Neuroblastoma

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Introduction: Somatic ALK mutations have been reported in 8-10% of neuroblastoma (NB). The largest published report on clinical correlates included 126 patients enrolled on COG protocol ANBL001 (Bresler et al. Cancer Cell, 2014). Few others detail clinical characteristics of patients with ALK-mutated NB.

Methods: With IRB approval, information on patients with ALK mutations treated at Memorial Sloan Kettering Cancer Center (MSKCC) for whom long-term follow-up was available, was retrospectively analyzed. ALK sequencing was performed either by whole genome sequencing (35x depth), targeted exome sequencing (>500x depth on MSK-IMPACT or Foundation One platforms), or hotspot ALK mutation profiling(Sequenom). Statistical analyses were performed using Chi-square or Kaplan-Meier methods.

Results: Seventy-six (36 female; 40 male) patients with a median age at diagnosis of 2.6 (range 0.3 to 68.8) years were identified. Fifty-five (72%) and 21 (28%) tumors were analyzed before, and at relapse respectively. Seventeen (22%), 5(7%) and 54(84%) of patients had low, intermediate and high-risk disease at diagnosis, respectively; 17 (22%) had locoregional and 59 (78%) stage 4 disease, respectively. ALK mutations included amino acids F1174 (53%), R1275 (30%), R1245 (8%) and others (9%). Coexisting MYCN-amplification was noted in 27 (36%), and ATRX aberrations in 10/55 (18%). F1174 mutations were more common in high-risk patients and coexistent with MYCN-amplification ($p<0.05$ for both). All high-risk patients received high-dose induction chemotherapy and surgery, 27 (50%) achieving complete remission. ALK mutation site was not prognostic ($p>0.3$) for progression-free (PFS) or overall survival (OS) both in the entire cohort and in the high-risk subgroup. Neither co-existing ALK mutations and MYCN-amplification nor co-existing ALK and ATRX mutations were prognostic for PFS or OS ($p>0.2$ and $p>0.1$ for both, respectively). The coexistence of these mutations remained prognostically insignificant even when only F1174-mutations plus MYCN-amplification were considered ($p>0.2$). Germline ALK mutations (G1128A) were detected in 2/36 patients (siblings) tested. Twenty-four patients received ALK inhibitors as monotherapy, with transient response detected in 1/23 (4%) evaluable patients.

Conclusions: Somatic ALK mutations were noted across all stages and risk groups of NB. ALK mutation site and its concurrence with MYCN-amplification were not prognostic. Single-agent ALK inhibition was therapeutically ineffective for all mutations.

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Curative Targeting of CNS Neuroblastoma Micrometastases Using the Optimal Therapeutic CSF Dose Delivered by Compartmental Radioimmunotherapy (cRIT)

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Background: The administration of tumor-targeting monoclonal antibodies into the cerebrospinal fluid (CSF) compartment (cRIT) delivers high doses of radiation to tumor cells in the CSF. cRIT with 131I-8H9 targeting B7-H3 has been successful in controlling neuroblastoma metastatic to the central nervous system with few long-term toxicities. We now report the correlation of tissue and radiographic dosimetry with clinical outcome.

Methods: Patients with CNS neuroblastoma received 2 mCi tracer of intra-Ommaya 124I- or 131I-8H9 for nuclear imaging followed by a therapeutic injection (10-to-80 mCi for phase I and 50mCi for expanded phase II studies) of 131I-8H9. Dosimetry was based on serial CSF and blood samplings over 48 hours and on regions of interest analyses by serial PET or SPECT imaging. CSF dose was calculated as cGy/mCi and as total cGy delivered. A Mantel-Cox analysis was performed to assess the prognostic significance of: the number of injections received, total mCi 131I-8H9 delivered, and total dose of 131I-8H9 delivered to the CSF.

Results: 93 patients with CNS neuroblastoma received a total of 188 tracer and therapy injections. 46 patients (50%) received a single therapy injection; 47 patients (50%) received 2 therapy injections. Mean total therapy dose 131I-8H9 received was 67.2 mCi (19.6-104.9 mCi). 36/55 (65%) patients assessed for total CSF dose delivered received a total CSF dose >2100 cGy, including 16 who received only 1 therapy dose. Interpatient variability for total absorbed dose to the CSF was observed; mean total absorbed CSF dose was 3368.8 cGy (range 677-13143 cGy) by CSF sampling. There was no statistical difference in survival among patients receiving >50 mCi 131I-8H9 or receiving >2100 cGy to the CSF by CSF sampling. A trend towards improved survival was noted for patients receiving 2 131I-8H9 therapy injections although not statistically significant (p=0.08).

Conclusions: Although interpatient variability for total absorbed dose to the CSF was observed, overall survival appeared to be superior with 2 therapy injections 131I-8H9. It appeared that a tumoricidal dose >2100 cGy to the CSF can still be delivered by a single injection 131I-8H9 cRIT.

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Impact of Extent of Resection on Survival and Local Recurrence for High-Risk Neuroblastoma

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Objective: To evaluate impact of extent of resection on survival and local recurrence in patients with High-Risk Neuroblastoma.

Methods: Retrospective multi-center cohort study including all high-risk Neuroblastoma patients who had resection of the primary tumor site between 1998 and 2014.

For all patients (n=155), as well as for the patients in the current protocol DCOG-NBL2009 (n=62), 5-year overall survival (OS) and 5-year event free survival (EFS) were estimated for complete, ≥95% and <95% resection. 5-year cumulative incidence of local progression (CILP) was estimated for complete and incomplete resection.

A multivariable Cox regression model was estimated to study the effect of age at diagnosis (<18 months vs > 18 months) and MYCN amplification on OS, EFS and CILP for all resection groups.

Results: Extent of resection in the total cohort: Complete in 49%, ≥95% in 33%, <95% in 17%.

Extent of resection in the DCOG-NBL2009 cohort: Complete in 44%, ≥95% in 34%, <95% in 22%.

In the total cohort, 5-year EFS was 26% (15.1-35.9), 24% (11.9-35.0), 36% (18.0-53.3); OS was 26% (15.0-36.2), 29% (16.0-41.1), 50% (31.1-68.3) for complete, ≥95% and <95% resection respectively.

CILP was 22% (12.3-31.9) and 17% (8.2-24.7) for complete and incomplete resection respectively.

In the DCOG-NBL2009 cohort, 5-year EFS was 34% (13.1-53.9), 32% (12.4-51.2), 36% (10.6-60.8); OS was 38% (15.5-57.7), 39% (18.4-60.3), 64% (39.2-89.3) for complete, ≥95% and <95% resection respectively.

CILP was 22% (3.3-40.4) and 8% (0.9-17.5) for complete and incomplete resection respectively.

Multivariable Cox regression models did not show any significant association between prognostic factors and EFS, OS and CILP for both cohorts.

Conclusion: For the total cohort as well as the DCOG-NBL2009 cohort, no significant difference was seen in OS, EFS and CILP for complete, ≥95% or <95% resection. No statistical significant association for EFS, OS and CILP with any of the prognostic risk factors was found.

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Dosage Sensitive FOXM1 Target Genes on Chromosome 17q Are Part of a Functional MYCN/FOXM1/AURKA Regulatory Network in Neuroblastoma

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Introduction: Aggressive neuroblastomas (NB) present with low mutation rates but highly recurrent segmental chromosome imbalances, including 17q gain as most frequent aberration in both MYCN amplified (MNA) and non-amplified (MNnA) high-risk cases. As shown for other tumor entities, identifying dosage sensitive dependency genes is a valid strategy to identify novel druggable targets. We report on our ongoing efforts to identify putative copy number driven dependency genes in NB using extended primary tumor datasets.

Material and Methods: We performed an integrated bioinformatic analysis based on DNA copy number, gene expression and prognostic impact in a novel and larger cohort of >300 primary neuroblastomas and subsequent further data mining.

Results: BRIP1 (BRCA1 interacting protein), previously shown by us to suppress MYCN driven replicative stress and causing in vivo accelerated MYCN driven NB formation, was confirmed as top ranked candidate 17q dependency gene. Correlation analysis for BRIP1 in three independent primary NB data transcriptome sets showed enrichment for FOXM1 targets. Using FOXM1 knock down and pharmacological inhibition, we confirmed BRIP1 as FOXM1 activated target in NB cells. Intersection analysis for the MNA and MNnA ranked dosage sensitive dependency genes with FOXM1 targets and differentially expressed genes in MYCN transformed mouse neural crest derived NB cells revealed 6 common genes, all located on 17q: BRCA1, BIRC5, TOP2A, KPNA2 and PRR11 together with BRIP1. Interestingly, AURKA, itself a FOXM1 target and a potential DNA repair modulator, is functionally connected to four of these genes: KPNA2 is a negative regulator of TPX2 which stabilizes AURKA; MYCN driven transcriptional pause release is controlled by AURKA through competing with TOP2A for MYCN binding; BIRC5 is stabilized by AURKA and AURKA regulates BRCA1 mitotic functions. Further functional assays confirmed NB dependency on high BRCA1 levels for growth and survival. Combined FOXM1 and AURKA inhibition is ongoing to test for synergistic effects.

Conclusion: Taken together, our data suggest that 17q gain may act as an amplifier for dosage effects for multiple FOXM1 target genes that install a MYCN overexpression tolerance state, amongst others through interaction with AURKA, that may be targetable with novel drug combinations.

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Alternative Lengthening of Telomeres Occurs Simultaneously with MYCN Amplification but Only in Heterogeneously MYCN Amplified Neuroblastomas

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Homogenous MYCN amplification (homMNA) and alternative lengthening of telomeres (ALT) are both mutually excluding key events in the evolution/progression of high-risk neuroblastomas. However, in case of heterogeneous MNA (hetMNA, only a sub-population of tumor cells shows MNA) we have previously described the occurrence of ATRX intragenic deletions (ATRXdel) which is always associated with the ALT phenotype. In this study, we scrutinized the co-occurrence of MNA and ALT in hetMNA neuroblastomas.

Twenty-seven hetMNA tumor specimens were analyzed by I-FISH applying MYCN/2q and pan-telomere PNA probes on frozen sections, FFPE-thin sections and tumor touch imprints. DNA was extracted from the same material and analyzed by HD CytoScan SNParray and WGS. RNA-seq data were available for 53 tumors (31 homMNA, 5 hetMNA, 17 nonMNA).

From 27 patients with hetMNA tumors, 10 patients were >18 months at diagnosis. ATRXdel as well as the ALT phenotype occurred exclusively in the patient age group >18 months. 3/10 tumors showed ATRXdel and 7/10 the ALT phenotype. Moreover, these tumors frequently showed multiple somatic copy-number aberrations (SCNAs). In 4/4 of these tumors investigated by simultaneous MYCN/pan-telomere I-FISH, telomere signals typical for ALT were not only found in non-amplified tumor cells but also in those with MNA. The mean MYCN expression of 5 hetMNA tumors corresponded to 17 nonMNA neuroblastomas and was significantly lower as compared to the expression in homMNA neuroblastomas. In case of homMNA, neither ATRXdel (0/67) nor the ALT phenotype (0/23) was detected.

This so far unrecognized phenomenon of the co-existence of MYCN amplification with ALT in identical tumor cells is restricted to hetMNA tumors of patients >18 months and occurs preferentially in tumors with multiple SCNAs. The latter is rather untypical for homMNA. The high frequency of the ALT phenotype in this hetMNA patient subgroup is currently not explainable. HomMNA tumors, in contrary, never show ALT positivity. Thus, there is apparently no transition from ALT positive hetMNA tumors to homMNA tumors. We hypothesize that a suppressed MYCN expression in the MYCN amplified ALT positive cells is at least partly responsible for this fact.

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Shallow Whole Genome Sequencing Allows Successful Copy Number Profiling of Formalin-Fixed Paraffin-Embedded Material in Neuroblastoma Patients

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Introduction: Neuroblastoma is typically characterized by the presence of copy number alterations (CNA). Presence of segmental CNA is associated with an unfavourable outcome, while presence of numerical CNA is associated with a good prognosis. Therefore, copy number profiling is mandatory in the diagnostic work-up of every neuroblastoma patient at diagnosis. The amount of available tumor material is unfortunately limited, and hence fresh or fresh frozen material is often not available. In contrast, Formalin-Fixed Paraffin-Embedded (FFPE) material is always available since this is part of routine clinical practice. Genomic profiling of FFPE material using arrayCGH analysis is however not very successful, especially due to the thorough fragmentation of the DNA.

Material and Methods: In this study, 10 neuroblastoma FFPE samples were selected for which also fresh tumor tissue was available. DNA from FFPE was extracted using the Qiagen FFPE tissue kit. Subsequently genomic profiling using shallow depth sequencing (sWGS) was performed with a mean coverage of 0.4x on both fresh and FFPE material. Image analysis was done using an in-house developed software tool Vivar and results of the two analysis platforms were compared.

Results: Comparison of genomic profiling both on fresh and FFPE material showed that FFPE can be used to obtain a reliable genomic profile. Whole chromosome alterations, as well as large and small segmental alterations and also MYCN amplification could be readily detected. This study demonstrates that FFPE material can be used as a source for genomic profiling and opens up opportunities for retrospective as well as prospective studies in the neuroblastoma field.

Conclusion: This study demonstrates the feasibility of the use of FFPE material for genomic profiling using sWGS. Since FFPE is always available in routine clinical practice, this could offer additional opportunities to study genomic alterations in retrospective as well as prospective studies.

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Lead Time from Surgery to HDT/SCT Might Impact Survival in High Risk Neuroblastoma

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Aim: Investigation of the local relapse rate (LRR) and outcomes in high-risk neuroblastoma in a single center following the HR-NBL1/SIOPEN protocol.

Methods: Patients with high risk neuroblastoma included in the HR-NBL1/SIOPEN Trial and surgically treated at the Karolinska University Hospital during a 10-year period between 2006-2015 were included. 18 patients met the inclusion criteria. 17/18 had metastatic disease at diagnosis and 9/18 had MYCN amplification. Treatment included Rapid COJEC induction, two TVD courses if unsatisfactory metastatic response, surgery with an intent of complete macroscopic excision, HDT/SCT with BuMel and 21 Gy radiotherapy to the primary site followed by 13-cis-RA. Two patients received taylored targeted therapies, but no antibody therapy was given.

Results: Median time from diagnosis to surgery was 117,5 (90-521) days, from surgery to HDT 25 (14-47) days and from surgery to radiotherapy 98,5 (62-175) days. Complete macroscopic excision (CME) was achieved in 13/18 cases (72%), an incomplete macroscopic excision (IME) was achieved in 4/18 cases (22%). In all of these cases >90% of the tumor volume was removed, corresponding to a gross total resection rate of 94%. In one case the tumor was deemed inoperable after two attempts at surgery. 16/18 (89%) patients received 21 Gy radiotherapy to the surgical field. After a median follow-up of 3.7 (0.7-10.9) years, 13/18 (72%) patients are alive without evidence of disease. 5 patients have died of recurrent disease. All deaths have been due to metastatic disease and the LRR is 0% so far. Interestingly, in patients with MYCN amplification, 6/9 (67%) are alive without evidence of disease after a median follow up of 4.7 (0.7-10.9) years.

Conclusion: The combination of surgery and systematically given 21 Gy radiation to the surgical field has been effective in controlling local disease even in the presence of MYCN amplification in this small cohort of patients. We speculate that the relatively short lead time from surgery to HDT might have a positive impact on survival.

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Neuroblastoma Risk Assessment and Treatment Stratification with Hybrid Capture-based Panel Sequencing

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Neuroblastoma risk assessment and therapy stratification is based on assessment of a combination of molecular and clinical covariates. The GPOH NB2004 trial protocol made use of patient age, presence of metastases, and FISH cytogenetic analyses for the detection of MYCN amplifications and loss at chromosome 1p36. Only few genes are recurrently altered in neuroblastoma (i.e., MYCN, TERT, ATRX, and ALK). It has been demonstrated, however, that genes of the RAS and p53 pathways as well as telomere maintenance mechanisms are frequently affected by genomic alterations in high-risk neuroblastoma and relapsed tumors. Importantly, we observed that clinical phenotypes are likely driven by respective genetic alterations affecting exactly these processes. Hence, we propose to implement early detection of such alterations into diagnostic routine.

We have investigated targeted cancer panel sequencing as a diagnostics approach in comparison to current routine diagnostics. Our approach employs a hybrid capture-based technology for high-coverage sequencing (>1000x) of 55 selected genes and NB-relevant genomic regions (chr. 1p36, 2p25, 11q, 17q). The assay design allows for the detection of nucleotide changes, structural rearrangements, and copy number alterations. In a retrospective screen, data obtained by targeted sequencing from 20 primary neuroblastomas were compared with corresponding data from 30x whole genome sequencing and FISH results acquired during routine diagnostics. We observed high concordance for risk markers identified in routine cytogenetic diagnostics, i.e., panel sequencing detected all 9 MYCN amplifications and 7 of the 8 chromosomal changes involving 1p36 loss. Additionally, relevant SNVs (e.g., mutations in ALK, HRAS, NRAS, in 5 patients) and structural rearrangements involving the TERT locus (in 4 patients) were identified with the cancer panel assay and confirmed by the WGS data.

Our results warrant further evaluation of this assay in a prospective study. This panel sequencing approach may be implemented into routine diagnostics as a single assay which covers all essential covariates for initial classification, as well as provides the detailed genomic information required for i) considering targeted treatment approaches (such as ALK inhibition), and ii) setting up PCR-based monitoring of minimal residual disease (e.g. based on MYCN breakpoints).

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The Importance of Segmental Chromosomal Abnormalities in Children > 18 Months with Unresectable, Localized Non-MYCN Amplified Neuroblastoma and Unfavourable Histology

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Background: Localized unresectable non-MYCN amplified (non-MNA) neuroblastoma with unfavourable histology in patients > 18 months of age is classed as intermediate risk in the UK/Europe and treated with induction chemotherapy, surgical resection, local radiotherapy and 13 cis retinoic acid. In the US & Canada these patients are treated as high risk with single myeloablative chemotherapy and stem cell rescue (MAT).

Aims:
1) To report the number of children with localized, unresectable, unfavorable histology, non-MNA neuroblastoma since the closure of the previous trial (NB2006), their treatment and outcome.
2) To identify risk factors that may help future treatment stratification.

Methods: A retrospective study of UK Paediatric Oncology Principal Treatment Centres was conducted to identify relevant patients diagnosed between 01/01/07 and 31/12/14. Demographic, biology treatment and outcome data were collected

Results: 40 cases were identified from 18 centres age 1.5-16.4 years, mean 4.3 years. Segmental chromosomal abnormalities (SCA) were present in 24/35 with results, 68%, of patients (most often 17q gain, 11q and 1p loss) SCA were present in 91% patients > 5 years and 58% in patients < 5 years (chi squared p = 0.09). Overall 3-year survival was 75% and progression free survival 62.5%. Follow up ranged from 1.3-9 years (mean 3.9). Of 15 patients who relapsed 10 (67%) died. 3/5 relapse survivors are alive with disease. The relapse rate in those < 5 years of age at diagnosis was 29% compared with 58% in those ≥ 5 years (log rank p = 0.118). The relapse rate in those with SCA was 54% compared with 18% in those without (log rank p = 0.056). Of 10 patients who died, 9 had SCA and 4 were ≥5 years at diagnosis. Median length of survival from diagnosis in those who relapsed was 28 months (9-68) and from relapse 16 months (5-52).

Conclusion: Relapse is more common in older patients and those with SCA and is often unsalvageable. We recommend intensification of therapy with MAT in patients > 5 years and or those with SCA.

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Single-cell RNA Sequencing of Neuroblastoma: A Pilot Study

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Background: Neuroblastoma is a remarkably heterogeneous disease. Recent sequencing studies of paired primary and recurrent tumors have demonstrated considerable clonal evolution at the time of recurrence. Minor cell populations may evolve during the course of treatment. However, bulk sequencing studies show "averages" of genomic and transcriptomic aberrations across millions of cells. This approach does not allow for unbiased studies of neuroblastoma tumor and stromal cell populations. The aim of this pilot was to establish a protocol for sample processing and library generation that allows studies of tumor heterogeneity and microenvironment at single-cell, whole-transcriptome level.

Methods: Fresh neuroblastoma samples were collected from four patients. Two tumors were high-risk, recurrent NBs; two were low-risk NBs. After mechanical and enzymatical tissue dissociation, cells were suspended in DMSO/FBS and stored at -80 degrees Celsius for a period of approximately two months. After thawing, cells were stained with DAPI as a viability marker and sorted by FACS. Single-cell libraries were prepared using the 10x Chromium Single-cell 3' v2 protocol and sequenced on the Illumina NextSeq platform. Library quality was assessed using the Agilent 2100 BioAnalyzer. Sample demultiplexing, alignment, and gene expression quantification per cell was performed using the cellranger pipeline. Gene expression analysis and visualisation was performed using the R pagoda2 package.

Results: A total of 17,705 transcriptomes from individual cells were successfully sequenced across the four samples. In each sample, approximately 50% of all reads were confidently mapped to the transcriptome (range: 48,9% to 55,2%). The mean number of reads per cell ranged from 16,734 to 145,789 across the four samples. In preliminary analyses, we identified several cell types, including tumor cells with developing neural gene signatures, B cells, T cells, macrophages, NK cells, mast cells, smooth muscle cells and stromal cells.

Conclusions: We conclude that our current protocol generates data suitable for further bioinformatic analyses significant for enhanced neuroblastoma single-cell understanding.

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Long Term Outcome of Observation Strategies in Patients with Localized Infant Neuroblastoma

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Background: In 2008, we reported on observation strategies in patients with localized infant neuroblastoma and showed that overall survival (OS) and survival without progression to stage 4 (metastasis-free- survival, MetFS) of patients with observation only were not different from the outcome of patients receiving front-line chemotherapy or undergoing front-line resection. We were interested in the long-term outcome of these patients.

Methods: We analysed the long-term outcome of the previously published cohort (J Clin Oncol 2008, 26(9), 1504-1510) with special focus on late progressions and late sequelae. Infants with localized neuroblastoma without MYCN amplification registered in the trials NB95-S or NB97 were included. In case of tumor associated symptoms, patients were treated with chemotherapy (n=57), otherwise, they underwent resection (n=190) or were observed without frontline cytotoxic treatment (n=93).

Results: The median follow up was 10.2 years. Since publication in 2008, only one further progression was reported: one patient of the observation group developed metastases five years after diagnosis without evidence of progression in a minor residual of the primary and is in complete remission nine years after progression. One patient of the observation group treated with chemotherapy for local progression died from second leukemia. No further late deaths were reported, especially not for patients with previously reported metastatic progression. The outcome of the groups was comparable with respect to overall survival (10-year-OS chemotherapy group: 0.95±0.03, resection group: 0.98 ± 0.01, observation group: 0.96 ± 0.02; p=0.41) and to survival without progression to stage 4 (10-year-MetFS: chemotherapy group: 0.92±0.04, resection group: 0.96 ± 0.03, observation group: 0.97 ± 0.0; p=0.24).

Significant late effects were observed in all three groups. Not surprisingly, as intraspinal involvement often led to cytotoxic treatment, scoliosis was reported more often in the chemotherapy group (11/57; resection group: 6/190; observation group 3/93, p<0.001) as was hearing impairment (chemotherapy group 9/57; resection group: 5/190; observation group 5/93; p=0.001). No difference was found with respect to renal damage (chemotherapy group 3/57; resection group: 4/190; observation group 5/93; p=0.28).

Conclusion: The analysis of long term follow up confirmed the favorable risk-benefit-ratio of observation strategies for patients with localized infant neuroblastoma.

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10-year Therapeutic 131I-MIBG Experience in Neuroblastoma

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Purpose: Jubilant DraxImage's (JDI's) 10-year experience in providing 131I-MIBG for therapeutic usage under Expanded Access and other FDA reviewed protocols was analyzed with a focus on pediatric populations. Since 2007, JDI began producing therapeutic 131I-MIBG for investigational use in response to a request from the New Approaches to Neuroblastoma Therapy (NANT) consortium. 131I-MIBG is being provided for clinical trials, both under its own IND and under Investigator-Sponsored INDs, for use in neuroblastoma, pheochromocytoma and paraganglioma.

Method: Data collected between 2007 and 2017 were analyzed. These data were derived as part of the clinical drug supply process. 131I-MIBG doses were aggregated yearly and categorized into four age groups, six dose ranges and by the number of treatments received over that period for each patient.

Results: Of the initial cohort (418 patients), 191 patients (168 pediatric and 23 adult) received 131I-MIBG through JDI Expanded Access. This analysis focuses on the 168 pediatric Expanded Access patients where 54% of the patients were aged between 6-11 years at the time of first treatment. Fifty percent (50%) of the doses requested ranged between 100-400 mCi, with the median patient age of 6.5 years. By comparison, in the 401-800 mCi dose range the median age was 7.5 years. During this period of analysis, 138 patients received one dose, 29 received 2 doses and 1 patient received 3 doses. The interval between doses was two months for most patients.

Conclusion: Jubilant DraxImage has 10 years experience in providing 131I-MIBG to US-based institutions for therapeutic use under Expanded Access protocol for defined patient populations in relapsed/refractory neuroblastoma. The analysis revealed that the majority of patients (168/191) were pediatric. This analysis also provided insights into age distribution, dose ranges and the number of patients who received multiple doses.

The decade of expanded access availability and investigator-published results have significantly contributed to advancing the therapeutic understanding of 131I-MIBG in neuroblastoma. This, in turn, is providing the necessary insights for future investigations with combination therapies and/or earlier intervention with 131I-MIBG.

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Mutational Signatures in High-Risk Neuroblastoma Patients

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Background: Mutational processes generate unique patterns of base substitutions generally referred to as mutational signatures. Mutational signatures can give insights into fundamental mutational processes underlying somatic mutations in tumors and have been shown to provide a starting point from which to evaluate therapeutic options. Neuroblastoma is clinically heterogeneous ranging from tendency of spontaneous regression to frequent formation of metastases. Earlier studies have identified several recurrent genetic alterations, including mutations in genes such as ALK and ATRX, as well as chromosomal rearrangements such as MYCN amplification, 11q deletion and 17 gain. Despite recent progress in the treatment of high-risk neuroblastoma, patients in this subgroup still have a poor prognosis, underscoring the need to further stratify patients in order to help inform clinical decision making.

Methods: Here, we sought to characterize high-risk patients and their underlying pattern of aberrations by conducting whole genome sequencing of 30 neuroblastoma patients. For each tumor/normal pair, the mutational landscape comprising single nucleotide and structural variants including copy number alterations was investigated. Non-negative matrix factorization was used to decompose single nucleotide variants to discover mutational signatures.

Results: De novo mutational signature analysis identified two mutational profiles (signature A and signature B). The landscape of signature A revealed a strong bias towards C>A substitutions. Out of 13 patients with a clear dominance of signature A, 12 patients were classified with MYCN amplification or 11q deletion. Furthermore, 5 out of 6 patients with a main contribution of signature B were metastasis or relapse cases.

Conclusions: Our results suggest that what is currently considered to be the typical neuroblastoma signature is mainly present in primary high-risk patients whereas the mutational landscape is altered in more advanced stages of the disease. To better understand the connection between the identified signatures and the high-risk subgroup, we are currently investigating the genome wide landscape of the observed C>A substitutions.

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An “Atavistic” View of Neuroblastoma Malignancy Revealed by a Phylostratigraphic Approach

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Neuroblastoma (NB) is the most common extracranial solid tumor of childhood. It originates from neural crest tissues of the sympathetic nervous system which fails to terminally differentiate when sympathoadrenal neural crest progenitors reach their final destination site, neural ganglia and the adrenal medulla. During their developmental program, normal neural crest cells are remarkably very motile and invasive, and assume, when delaminated from the neural tube, an ameboid-like phenotype.

Given the absence of a heavy mutational load at lower stages of the disease, NB can be considered at least initially a “gene expression-based” type of cancer, in which transcriptome alterations are responsible for the onset of the disease. To identify transcriptome profiles relevant for the malignancy of NB, we performed differential analysis of expression data of 709 well-annotated neuroblastoma samples grouped by staging and risk classification. We prioritized those genes whose expression variation is consistently associated with increased risk and higher stage of the tumor. We then performed on these genes functional enrichment and interaction network analysis, identifying co-expressed gene groups and protein pathways involved.

We finally annotated these prioritized, co-expressed genes following a phylostratigraphy approach, which classifies genes by their time of evolutionary emergence. We found that genes conserved in unicellular eukaryotes (ancestral genes) are highly enriched in clusters of increased expression following progression, while multicellular-level genes, with more recent emergence, undergo mostly an opposite trend. This “atavistic” view, proposing NB as a disease in which neural crest cells reacquire a unicellular-like pattern of expression downregulating the gene network essential for integrating into a tissue, is especially impressive given the unique nature of these cells.

We provide several validations of this concept, and we discuss this new perspective under the light of new NB therapeutic approaches.

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Current Strategies for Testing Dosage Sensitive Dependency Genes in The Zebrafish Neuroblastoma Model

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Neuroblastoma (NB) has been proposed as a copy number driven cancer, given the low mutation frequency and highly recurrent copy number gains and losses. It has previously been shown for other tumor entities that recurrent copy number gains can guide experiments towards identifying dosage sensitive dependency genes and therefore the search for such genes, e.g. on 17q, could offer novel therapeutic targets. Using a combination of integrated bioinformatic analysis and zebrafish modeling, we provided convincing evidence for a role of the DNA helicase and BRCA1 interacting protein BRIP1 in MYCN driven NB formation (Vanhauwaert et al., in preparation). Despite the success of this approach, we encountered several hurdles that impacted the efficiency of candidate gene testing in this model. First, we encountered low survival of transgenic animals during early stages of development which now has been overcome by using rotifers for initial feeding. Second, I-Sce1 meganuclease-mediated transgenesis ensures integration in one single site in the zebrafish genome and is well suited to generate stable overexpressing transgenic lines but is relative less efficient for the mosaic approach to test for immediate effects on tumor formation after injection of the overexpression construct. Therefore, we are now testing the TOL2 transposase-mediated transgenic approach, which would cause integration of transgene in several genomic sites and is considered a more potent approach for an initial testing of overexpression effects of transgene in a mosaic approach. Together with an improved method for injection allowing to generate larger cohorts for follow up and increased capacity of our facility, we are now further exploring effects of constructs for stable and regulable overexpression of several of our candidate genes including SOX11, TBX2 and RRM2 as 2p candidates; BRIP1, BIRC5 and further 17q candidate genes; and FOXM1 as putative master regulator. Also, the generation of an animal model recapitulating the MNnA high risk subtype is a major challenge and could be tackled using zebrafish transgenics. To this end we have prioritized 11q candidates and will evaluate the use of a CRISPR/Cas9 vector system for tissue-specific candidate gene disruption in combination with one or more additional drivers.

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Low Expression of the P53 Regulated lncRNA NBAT1 Provides Chemotherapeutic resistance in Neuroblastoma

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Introduction: Neuroblastoma (NB), an extracranial childhood embryonal tumor, is thought originate from undifferentiated neural crest progenitor cells of the developing sympathetic nervous system. The tumor arises mainly in the adrenal medulla or paraspinal sympathetic ganglia in the abdomen, chest, pelvis and bones. NB is a highly heterogeneous tumor presents itself from good prognostic, low-risk and spontaneously regressing tumors to high-risk, chemo-resistant tumors with poor clinical outcome. High-risk tumors often characterized by non-random chromosomal alterations such as MYCN amplification, 1p deletion and 17q gain. Despite improvements in the treatment of high-risk tumors with multimodal chemotherapy, the current event free survival rate of high-risk patients stands at <40%, thus necessitating the requirement for novel therapeutic options. Recent evidence implicated long noncoding RNAs (lncRNAs) in cancer development and progression, including NB, and also they have been considered potential therapeutic targets in the treatment. We have previously shown that lncRNA NBAT1, located at the NB-specific risk associated locus 6p22.3, shows decreased expression in high risk NBs and its decreased expression contributes to the NB development by promoting cell proliferation and preventing differentiation of the NB cells.

Results: In the current investigation we show that downregulation of NBAT1 in NB cells leads to resistance to chemotherapeutic drugs whereas its overexpression sensitizes NB cells. Interestingly, treatment of genotoxic drugs induces NBAT1 expression in a p53-dependent manner, and also, the p53 dependent target gene expression is compromised in the NBAT1 depleted cells, which is consistent with the reduced p53 enrichment over its target genes. Importantly, the activation of ATR-Chk1 pathway also occurs in the NBAT1 depleted cells. Growth of the NBAT1 depleted xenografts could be reversed following MDM2 inhibitor Nutlin3a treatment.

Conclusions: Based on our in vitro and xenograft studies, we propose that lower expression of NBAT1 in high-risk NBs may provide chemotherapeutic resistance. Our study highlights importance of studying lncRNA expression signature to predict therapeutic outcome in NBs. Drugs activating P53 could provide novel therapeutic option, where P53 related pathways are dampened by decreased lncRNA expression.

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Arid1a is a Haploinsufficient Tumor Suppressor that Collaborates with Mycn in Neural Crest Derived Tumors

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A major barrier to advancing targeted therapy in Neuroblastoma (NB) is deciphering which genes, buried in the large segmental chromosomal gains and losses that characterize high-risk NB, are essential to NB initiation and/or maintenance. Though 30% of high-risk NBs are MYCN amplified with 1p36 deletions, the 1p36 tumor suppressor that collaborates with MYCN is unknown. To tackle this problem, we used a model derived from primary neural crest cells (NCCs), progenitor cells of the sympathetic nervous system, that are isolated from mouse trunk neural tube explants. CRISPR/Cas9 screenings of multiple 1p36 candidate tumor suppressors in primary NCCs showed that simultaneously deleting two chromatin-remodeling factors, Chd5 and Arid1a, initiated tumorsphere formation, but failed to generate tumors. Next, we asked if these genes collaborate with Mycn. We overexpressed Mycn and flank injected a mixed population of NCCs containing three combinations of CRISPR/Cas9 mediated deletions: 1) both Chd5 and Arid1a; 2) each gene alone; 3) the 1p36 syntenic region. This mixed population reduced tumor latency by ~50 days compared to controls. Whole-exome sequencing revealed all tumors selected for Arid1a loss over Chd5 or 1p36 loss. This suggests Arid1a loss confers the greatest advantage during Mycn driven tumorigenesis. To further delineate the role of Arid1a, we used an Arid1a floxed mouse model to generate primary NCCs. We recombined the floxed sites ex vivo, overexpressed Mycn, and flank injected mice. We found that heterozygous loss of Arid1a significantly ($p=0.002$) decreased latency relative to control tumors, while homozygous loss was no different than controls, defining Arid1a as an obligate haploinsufficient tumor suppressor and confirming collaboration with Mycn. Next, RNA-sequencing and gene enrichment analysis showed Twist1 and downstream targets that regulate metastasis were significantly increased in heterozygous and homozygous null tumors. Furthermore, heterozygous tumors have a pronounced PRC2 signature, while homozygous null tumors have a strong stem signature. These results suggest Arid1a is an important 1p36 obligate haploinsufficient tumor suppressor, that collaborates with Mycn to transform NCCs. Hopefully, untangling pathways altered in this model will advance therapeutic treatments in high-risk NB patients with 1p36 LOH and MYCN amplification.

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Neuropeptide Y Stimulates Neuroblastoma Cell Motility Via its Y5 Receptor and RhoA Pathway

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The mechanisms underlying neuroblastoma (NB) dissemination are unclear. Consequently, treating metastatic NB remains an unsolved clinical problem. Neuropeptide Y (NPY) is a sympathetic neurotransmitter highly expressed in NB and released from undifferentiated tumors. Previously, we have shown that NB cells expresses NPY Y2 and Y5 receptors (Y2R and Y5R), which control NB cell proliferation and survival in an autocrine manner. However, our clinical data implicated also the NPY/Y5R axis in NB metastasis. In NB patients, elevated release of NPY is associated with metastatic disease and poor outcome, while within tumor tissue Y5R is preferentially expressed in angioinvasive NB cells. Thus, the goal of our study was to determine the mechanisms by which NPY/Y5R pathway contributes to NB metastasis. We have found that overexpression of Y5R in CHO-K1 cells led to activation of an essential cytoskeleton regulator, RhoA, which is tightly controlled in a spatial and temporal manner during cell migration. RhoA activity markedly increased upon NPY stimulation, while Y5R antagonist blocked this effect. Y5R and active RhoA co-localized in CHO-K1/Y5R transfectants, as well as SK-N-BE(2) and SK-N-AS NB cells that endogenously express Y5R. Both proteins were also undergoing concurrent changes in subcellular localization during cell migration. In NB cells migrating as single cells, both Y5R and active RhoA accumulated in the trailing and leading edges of the cells, while in cells migrating collectively their expression was elevated in those present at the front of the moving colony. The role of NPY/Y5R pathway in NB cell migration was confirmed by Transwell assay, in which NPY significantly increased spontaneous migration of SK-N-BE(2) cells and this effect was completely blocked by a Y5R antagonist. Altogether, our data support the role of the NPY/Y5R/RhoA pathway in regulating cellular motility, and along with results of our clinical studies implicate its essential role in NB dissemination. Further studies are required to determine if other functions of the NPY/Y5R/RhoA pathway contribute to metastasis and validate Y5R as a potential therapeutic target. Importantly, Y5R expression increases in chemoresistant NBs, promoting their survival. Hence, the NPY/Y5R axis may be particularly important in dissemination of relapsing tumors.

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Targeting B7-H3 (CD276) in Neuroblastoma: Generation and In Vitro Evaluation of Different anti-CD276 Antibodies and Immunocytokines

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Introduction: Targeting disialoganglioside GD2 with monoclonal antibodies (mAbs) significantly improves survival in high-risk neuroblastoma. However, GD2 expression can be heterogeneous and alternative or additional target antigens might improve immunotherapy. We evaluated the use of B7-H3 as a potential target and generated and evaluated different anti-B7-H3 mAb constructs for their ability to elicit antibody-dependent cellular cytotoxicity (ADCC).

Method: The abilities of various anti-B7-H3 mAb constructs and the GD2-specific mAb CH14.18 to mediate ADCC were compared in vitro in cytotoxicity assays using the RTCA xCELLigence system. Target cells: Neuroblastoma cell lines expressing high levels of B7-H3 but variable levels of GD2 (LAN-1, Kelly, SH-SY5Y). Effector cells: Human expanded NK cells. Derived from a murine anti-B7-H3 clone (HEK5-1B3), five additional mAb constructs were engineered: (1) chimeric mAb fused to human IL-2 (cHEK5-IL2), (2) cHEK5-IL2 produced in rat myeloma YB2/0 cells to create a low-fucose version (cHEK5-IL2-LF), (3) chimeric and Fc-optimized (SDIE) w/o fusion (cHEK5opt), (4) cHEK5opt fused to human IL-2 (cHEK5opt-IL2) and (5) cHEK5opt fused to human IL-15 (cHEK5opt-IL15). Results: All anti-B7-H3 constructs were able to elicit ADCC. Specific lysis of LAN-1 (high expression of both GD2 and B7-H3) was calculated after 36 hours and at an effector-to-target ratio at 5:1: targets + effectors w/o mAb 24%, parental HEK5-1B3 29%, cHEK5opt 44%, cHEK5-IL2 76%, cHEK5opt-IL15 85%, CH14.18 90% and cHEK5opt-IL2 97%. Similar results were obtained using SH-SY5Y as target cell line, which lacks GD2 expression, making CH14.18 ineffective. Indeed, compared to targets + effectors w/o mAb, specific lysis was not enhanced with CH14.18 (45% and 40%, respectively) but drastically increased using the cHEK5opt-IL2 instead (99%). Since production of cHEK5-IL2-LF just recently started, a direct comparison with all other constructs was not possible but first results suggest an activity comparable to that of cHEK5opt-IL2.

Conclusion: B7-H3 has been demonstrated to be a suitable alternative or additional target antigen for neuroblastoma. Fc-optimized mAbs and immunocytokines targeting B7-H3 might be an additional or alternative approach for immunotherapy of neuroblastoma.

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Modulation of Innate Immune Sensing Pathways in Neuroblastoma by MYCN

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Background: While immune checkpoint blockade therapies have revolutionized the treatment of some adult cancers, they have been largely ineffective in pediatric tumors, including neuroblastoma. Improved therapies will require a better understanding of the molecular basis of immune escape in these tumors. Poor prognosis MYCN-amplified neuroblastomas have lower PD-L1 mRNA levels and lower transcriptional markers of T-cell infiltrates than non-amplified tumors. We sought to investigate the role of MYCN in modulating inflammatory signaling pathways, focusing on innate immune sensing pathways.

Methods: We treated MYCN-amplified and non-amplified neuroblastoma cells lines with a variety of inflammatory stimuli including interferons and agonist of toll-like receptors and other pattern-recognition receptors. We determined the response by measuring phosphorylation of STAT1, STAT3, and IRF3; change in mRNA expression of NF-κB and interferon response genes; and the change in activation of an NF-κB reporter construct. We then used non-amplified cell lines with inducible MYCN to identify the effects of acute MYCN activation on the response to these inflammatory stimuli.

Results: We found that all cell lines tested generated an inflammatory response to treatment with exogenous interferon. However, while some non-amplified cells lines were able to generate inflammatory responses to agonists of TLR1, TLR2, TLR3, TLR4, and TLR6, non-amplified lines were universally insensitive to these stimuli. Acute MYCN activation decreased responsiveness to these stimuli. Focusing on a single pathway, we found that TLR3 was more highly expressed in responsive cell lines and that MYCN activation acutely decreased its expression.

Conclusions: MYCN-amplified neuroblastoma cell lines are insensitive to several innate immune stimuli. Further experiments will seek to determine the mechanism of pathway inactivation as well as its significance in creating an immune-poor microenvironment.

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Exosome Encapsulated miRNA Mediate Drug Resistance and Disease Progression in High-Risk Neuroblastoma In Vitro

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Background: The acquisition of multidrug resistance is a major impediment to successful treatment of high-risk neuroblastoma. Interactions between tumour cells, the tissue microenvironment and neighbouring cells act as powerful determinants of disease initiation, progression, treatment response and patient prognosis. Exosomes containing bioactive molecules including RNA and proteins, in part, mediate these interactions. Increased exosome levels in the tumour microenvironment can drive tumour growth, progression, metastasis, and drug resistance by reprogramming non-cancer cells and chemotherapy naive cancer cells to be more pro-tumorigenic.

Cisplatin-resistant cell models (KellyCis83, SK-N-ASCis24, CHP212Cis100), which were developed and characterised in our lab demonstrated cross resistance to numerous chemotherapeutics, genomic and epigenomic changes as well as altered protein expression and enhanced invasive potential in vitro. Exosomes derived from these drug resistant neuroblastoma cells could potentially confer a resistant phenotype to non-tumour cells and chemotherapy naive tumour cells, driving tumour progression, with exosomal miRNAs acting as predictors of chemotherapy responsiveness.

Methods: We assessed the ability of drug resistant cell line derived exosomes to promote cell growth in vitro. Kelly sensitive cells demonstrated increased colony forming ability when treated with exosomes from drug resistant cells (KellyCis83 p=0.0006). These cells exhibited an even greater colony forming ability when treated with exosomes from the highly invasive SK-N-ASCis24 cell line (p=0.0001). Drug resistant KellyCis83 exosomes significantly increased resistance to cisplatin in Kelly cells. MiRNA profiling of exosomes from drug resistant neuroblastoma cells identified common altered miRNAs between resistant cell lines: miR-17-5p, miR-19-3p, miR-20-5p, miR-106a-5p, miR-526b-5p, miR-320a-3p and miR-191-5p. The identification and validation of these miRNAs in clinical samples could aid in identification of biomarkers for drug response in neuroblastoma and allow for stratification of patients for personalised treatment. GO analysis revealed enrichment of genes implicated in the induction and regulation of programmed cell death (enrichment score 3.74 and 4.18 respectively), and those involved in cell cycle regulation (enrichment score 2.97). KEGG pathway analysis demonstrated significant enrichment of multiple targets in TGF-β signalling and m-TOR signalling pathways, involved in neuroblastoma pathogenesis.

Conclusion: These results indicate that exosomes derived from drug resistant neuroblastoma cells can reprogramme the recipient cell machinery, driving drug resistant phenotypic changes.

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Long-term Outcome of Dumbbell Neuroblastoma in Children Under One Year Old: Results of the INES-FU-SCI – A SIOPEN Study

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Background: Spinal canal invasion (SCI) occurs in 10-15% of children with peripheral neuroblastic tumors (PNTs). These patients have favorable clinical and biologic features at diagnosis and better survival probability, but are prone to develop late neurologic and orthopedic sequelae. The aim of this study was to evaluate the prevalence and type of late effects according to therapeutic approach and initial presentation in infants with neuroblastoma with SCI treated in the framework of INES-SIOPEN protocol.

Methods: This is a multicentric European cohort follow-up study including infants with PNTs treated according to INES protocol between 1999 and 2004. One hundred patients with SCI were included initially and a follow-up form based on the last examination of the patients had to be returned by institutions from 2014.

Results: A total of 63 patients were enrolled in INES-FU-SCI. Main initial symptoms at diagnosis included motor deficit (52%), neurovegetative dysfunctions (27%), bladder (21%) and bowel dysfunctions (14%) and pain (17%). The median interval between occurrence of first symptoms and diagnosis was 14 days (0-290 days). Initial treatment was chemotherapy in 51 cases and neurosurgery ± chemotherapy in 12 cases. After a median follow-up of 11.9 years (2.7-14.7 years), 31 patients (51%) had one or more sequelae, including motor deficit (34%), sphincter dysfunction (30%) and spinal deformities (33%). No significant difference between first-line two treatments on long-term outcome was found. The severity of motor deficit and the presence of sphincter dysfunction at diagnosis were correlated with the occurrence of late effects, except for spine deformities. The estimate of overall survival at 5 and 10 years was 96.8% (CI95, 87.4%-99.2%) and the event free survival was 95.0% (CI95, 85.3%-98.4%).

Conclusion: Fifty-one percent of infants with neuroblastoma with SCI developed late effects, without significant difference between first-line neurosurgery and chemotherapy. A prospective study is necessary and has recently developed to clarify the incidence and severity of sequelae and facilitate treatment data collection.

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Endogenous and Haploidentical Natural Killer Cell Reconstitution in Children Treated with Combined Chemoimmunotherapy For Neuroblastoma

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Background: Natural killer (NK) cells are effector cells of antibody-mediated cytotoxicity (ADCC). We studied NK cell reconstitution in children with newly diagnosed high-risk neuroblastoma treated with chemotherapy and the anti-disialoganglioside (GD2) monoclonal antibody hu14.18K322A, and after haploidentical NK cell infusion.

Patients and Methods: Forty-four patients (0.5 to 15.2 years) with newly-diagnosed high-risk neuroblastoma received induction chemotherapy identical to ANBL0532, hu14.18K322A (daily x4 starting on day 2 of each course), low-dose interleukin (IL-2; 1 million U/m2), and daily subcutaneous (SQ) granulocyte-macrophage colony-stimulating factor. Consolidation included melphalan/busulfan and hematopoietic stem cell rescue. Consenting patients received an additional 4-day course of hu14.18K322A and when available, purified haploidentical NK cells on day +4 followed by SQ IL-2 x4, and then maintenance therapy with retinoic acid/immunotherapy. NK cell chimerism and phenotyping studies were performed on day 0, 7, and 21 of induction course 1, 3, and 4, during consolidation, and prior to course 1, 3, and 6 of maintenance therapy.

Results: During induction, the average number of NK cells decreased significantly from 696 (577–816) cells/µL at baseline to 137 (20–256) cells/µL ($P<0.001$) on day 7 with complete recovery on day 21 (630 [513–746] cells/µL; $P>0.05$). NK cell numbers were less repressed on day 1 of maintenance therapy course 1, 3, and 6 (432 [308–555] cells/µL; $P=0.031$) and highest on day 21 after haploidentical NK cell infusion (1095 [838–1353] cells/µL). The percentage of CD16–expressing NK cells followed this trend. Twenty-eight patients received haploidentical NK cell infusions (median, 24.7x106/kg NK cells) without identifiable toxicities. Median donor chimerisms were 5% (0–81%) on day +7 and 0% (0–35%) on day +21 and higher in patients without killer immunoglobulin-like receptor (KIR)–ligand mismatches (14/28 [50%]; $P=0.029$).

Conclusion: Reconstitution of CD16–expressing NK cells changes during induction therapy but fully recovers in between cycles, suggesting there should be sufficient numbers for effective ADCC. Haploidentical NK cells can be safely given, resulting in transiently higher cell numbers than at baseline. Further studies of cytotoxic function of NK cells during therapy are underway to better understand their role in antibody therapy in neuroblastoma.

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Targeting INCENP, a Key Regulatory Component of Chromosomal Passenger Complex Inhibits Tumor Progression in Neuroblastoma

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Growing evidence indicates that epigenetic regulators play important roles in the tumorigenesis of multiple types of cancer, including neuroblastoma (NB). We performed an epigenetic-focused RNAi screen to identify critical regulators that control NB proliferation and differentiation. High-content Opera imaging identified 16 genes whose silencing led to a decrease in NB cell proliferation and also induced neuronal differentiation. Gene ontology (GO) and pathway analysis showed that these 16 priority hits were mainly involved in four important biological process including DNA damage (HMGN1, ACTR5, RAG2), cell division/mitotic event (INCENP, CENPE), histone modification (SETD8, SUV4-20h1, KDM4B, CHAF1A, PRDM2 and BRD4) and the P53 pathway (SETD8, KDM4B). Among these, NB cells were particularly vulnerable to loss of INCENP, a gene encoding a key regulatory component of the chromosomal passenger complex (CPC). CPC functions to ensure proper chromosomal segregation during mitosis and consists of Aurora B kinase, INCENP, Survivin, and Borealin. Notably, two of CPC components (Aurora B and Survivin) have been demonstrated to be important and druggable in NB. In CPC, INCENP acts as a protein scaffold and its interaction with Aurora B is essential for the activation of this kinase as well as CPC function in mitosis. INCENP is highly expressed in NB cells and its expression level decreased during Retinoic Acid induced differentiation of NB cells. We found genetic silencing reduces growth in both MYCN-wt and MYCN-amp NB cell lines tested in vitro. Silencing of INCENP using Doxycycline inducible INCENP shRNA led to significant decreases in growth of SY5Y and BE2C xenografts in vivo and increases in murine survival ($p=0.0003$ and $p=0.0004$ respectively). In primary NB tumors elevated levels of INCENP are significantly associated with poor prognosis in high-risk neuroblastoma patients. However, what leads to the elevated levels of INCENP and whether INCENP functions in NB are CPC dependent are incompletely understood and currently under investigation. Further mechanistic studies will provide insight into the role of INCENP and CPC in the pathogenesis of NB. Targeting the scaffold, INCENP, would provide an additional and novel way to disrupt the activity of CPC in NB.

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MYCN Cooperates with HAND2 to Control Neuroblastoma Growth and Differentiation

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Neuroblastoma (NB) is a malignancy of the developing sympathetic nervous system. Amplification of MYCN is found in 25% of cases and correlates with high-risk disease. How MYCN alters the transcriptional landscape of sympathoadrenal progenitors to cause NB remains ill-defined. We hypothesize that by defining the MYCN interactome we can identify critical nodes regulating MYCN's diverse effects on growth and differentiation. In this study, we define the MYCN interactome using two different antibodies to MYCN to immunoprecipitate proteins interacting with MYCN in IMR32 cells. Protein mass spectrometric analysis identified that MYCN interacts with the transcription factors Phox2A, Phox2B, Hand1 and Hand2 that are known to control sympathetic neuron specification and differentiation. Western blot analysis confirmed the interactions identified by mass spectrometry and extended findings to additional NB cell lines. To understand whether these sympathoadrenal neural transcription factors contribute to the transcriptome regulated by MYCN we individually silenced MYCN, Phox2A, Phox2B, Hand1 and Hand2 expression using siRNAs. We found a subgroup of MYCN target genes, ODC1, MTAP, PRMT1 and DKC1 were decreased only in siHAND2 cells. ChIP seq data (R2 database) indicate MYCN and HAND2 co-localize to promotors of ODC1, MTAP, PRMT1 and DKC1. Cell confluence assays showed cell proliferation decreased 45% in siMYCN, 80% in siPhox2B and 85% in siHAND2 treated NB cells, but not in siPhox2A and siHAND1 treated cells compare to sicontrol cells. This suggests that MYCN and Hand2 work together to regulate a subgroup of MYCN target genes that impact NB cell biology. We then silenced both MYCN and HAND2 and found that while growth was not significantly when compared to silencing of either gene alone. However, the combined decrease of HAND2 and MYCN significantly an elaborate network of neurites and increases in expression of neural differentiation genes GAP43 and DPYSL3 compared to controls or silencing of either MYCN or HAND2 alone. Kaplan-Meier survival curves indicate high Hand2 expression is associated with a worse prognosis in MYCN-Amp patients (P=0.025). These results suggest a mechanism for NB tumorigenesis in which the cooperation of MYCN with Hand2 raises the threshold which sympathoadrenal progenitors must overcome to implement a differentiation program.

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Activating Transcription Factor 5 (ATF5) Promotes Neuroblastoma Metastasis by Inducing Anoikis Resistance

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Purpose: We have previously demonstrated that ATF5 is highly expressed in Stage 4 MYCN-amplified tumors and silencing of ATF5 inhibits proliferation. In our current study we investigated the role of ATF5 in neuroblastoma metastasis.

Methods: Established SK-N-Be(2)C xenograft tumors, expressing a tet-controlled shRNA, were treated with Dox (2mg/ml) and monitored by bioluminescence imaging. Xenograft tumors were also treated with a novel cell penetrating dominant negative peptide agonist CP-dnATF5. Circulating tumor cells (CTC) and bone marrow metastatic burden were assessed by measuring luciferase activity in the blood and bone marrow homogenates, respectively. SK-N-Be(2)C and SK-N-DZ cells expressing anti-ATF5 shRNAs were subjected to anoikis assay in vitro by plating cells on polyHEMA coated plates and measuring dell death by TUNEL assay. Immunoblots were performed on lysates from cells, grown under anoikis condition, for pro and anti-apoptotic factors. In vivo anoikis assay was performed by injecting cells intracardically into nude mice pretreated with Dox and isolating CTC after 12hr followed by TUNEL assay. CP-dnATF5 was also evaluated in vitro and in vivo anoikis assays.

Results: In vivo, ATF5 inhibition by shRNA or CP-dnATF5, significantly inhibited SK-N-Be(2)C xenograft tumor growth in nude mice ($P=0.0083$, shRNA ; $P=0.0013$, CP-dnATF5). Both treatment modalities reduced SK-N-Be(2)C metastatic burden in the liver ($P<0.05$) and bone marrow ($P<0.01$) and decreased CTC ($P<0.05$). In vitro, anoikis induced SK-N-Be(2)C and SK-N-DZ cell death were increased 40-60% after ATF5 knock down. Immunoblots on cell lysates showed an increase in pro-apoptotic factor BMF expression upon ATF5 downregulation. In vivo, isolated circulating SK-N-Be(2)C and SK-N-DZ cells from Dox treated mice were found to be associated with increased death (SK-N-Be(2)C $P<0.01$, SK-N-DZ $P<0.05$) indicating a significant anoikis induced cell death due to ATF5 inhibition. Increased expression of BMF at mRNA level was also observed in isolated SK-N-Be(2)C CTCs from Dox treated mice. Consistent with ATF5 knock down, CP-dnATF5 also increased SK-N-Be(2)C and SK-N-DZ cell death to 50-60% in vitro and in blood circulation in vivo.

Conclusion: Our data, using a shRNA or novel inhibitor, shows that, ATF5 suppresses pro-apoptotic factor BMF inducing resistance of circulating tumor cells to anoikis and leading to increased metastasis.

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Active Inhibition of mPGES-1 Suppresses Neuroblastoma Tumor Growth and Enhances the Effect of Conventional Cancer Therapies

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Bakground: Cancer related inflammation is a complex tumor-promoting interaction between malignant cancer cells and benign stromal cells. We recently reported high-risk neuroblastomas having an immunosuppressive microenvironment compared to low-risk tumors. This microenvironment contains infiltrating cancer associated fibroblasts (CAFs) expressing microsomal prostaglandin E synthase-1 (mPGES-1), responsible for prostaglandin E2 (PGE2) synthesis. PGE2 promotes immunosuppression, angiogenesis, tumor progression and therapy resistance. We propose that targeting the PGE2 production by CAFs will reduce tumor progression.

Methods: To elucidate if mPGES-1 and PGE2 contribute to tumor progression two neuroblastoma mouse models were utilized, transgenic MYCN-driven mice and xenografts using 11q-deleted cells. The mPGES-1 inhibitor, Compound III (CIII), was administered daily i.p. In the xenograft model, treatment started at tumor cell inoculation or at tumor take. To evaluate mPGES-1 inhibition in combination with conventional cancer therapies in vitro we established a multicellular tumor spheroid (MCTS) model, simulating the neuroblastoma microenvironment by co-culturing neuroblastoma cells and fibroblasts with PBMCs.

Results: By targeting mPGES-1 activity with the small molecule CIII we effectively blocked CAF-derived PGE2 production in tumors. This non-toxic selective inhibition of PGE2 resulted in significantly reduced growth of established tumors in both xenograft and transgenic models compared to controls. In the immune-competent transgenic mice, pharmacological mPGES-1 inhibition resulted in reduced angiogenesis, induced a favorable shift in the M1/M2 macrophage ratio supporting host immune response and reduced infiltration of PDGFRβ expressing CAFs. The MCTS model successfully recapitulated the neuroblastoma microenvironment where fibroblasts expressed mPGES-1 and combination treatment studies with CIII enhanced the cytotoxic effect of chemotherapeutic drugs doxorubicin and vincristine. Furthermore, mPGES-1 inhibition significantly improved the suppressive effect of immune cell activation.

Conclusion: In this study we show that selective inhibition of prostaglandin E2 biosynthesis and its role in the crosstalk between cells of the microenvironment reduce tumor progression and that mPGES-1 inhibition enhances the effect of conventional cancer therapies, targeting both malignant cells and stromal cells. By specific targeting mPGES-1 dose-inhibiting side-effects of NSAIDs and COXibs were avoided. We therefore propose mPGES-1 inhibition for future clinical applications as an adjuvant treatment or long-term maintenance treatment for high-risk neuroblastoma.

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Clinical Characteristics of Neuroblastoma Harboring ATRX Aberrations

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Background: Loss-of-function somatic ATRX mutations or deletions in neuroblastoma were associated with older age at diagnosis (Cheung et al, JAMA 307:1062, 2012). Since that seminal publication, there have been few reports on the clinical characteristics of neuroblastoma patients harboring ATRX aberrations. We now provide further clinical details on an expanded cohort of patients: all seen at MSKCC.

Methods: With IRB approval, information on patients with somatic ATRX aberrations was retrospectively analyzed. ATRX sequencing was performed either by whole genome sequencing (~35x depth) or targeted exome sequencing (>500x depth using MSK-IMPACT or Foundation One platforms). Progression-free (PFS) and overall-survival (OS) were estimated using Kaplan-Meier analyses.

Results: Forty-nine patients (27 male and 22 female), diagnosed at 2.3-to-67.8 (median 9.4) years of age were identified. Twenty-nine (59%) tumors were analyzed before relapse and 20 (41%) at relapse. Eleven patients had ATRX sequenced prior to and at relapse; 10/11 had identical aberrations. Eight (16%) and 41 (84%) patients had locoregional and stage 4 neuroblastoma at diagnosis, respectively. Of the 8 locoregional cases, 7 relapsed as stage 4 and the remaining adult patient has short follow-up. All (47/47) evaluable tumors had unfavorable histology. ATRX was deleted in 20 (41%) tumors and mutated in 29 (59%). Coexisting ALK mutations were noted in 11 (20%) tumors but MYCN-amplification was not detected in any. High-dose chemotherapy was administered to 39 (95%) stage 4 patients but only 13 (33%) achieved CR/VGPR. At a median follow-up of 61months, 24 (49%) patients are alive, though only 7 progression-free. Five-year PFS and OS were 14±5% and 51±9%, respectively. Younger age at diagnosis (≤5years; n=20) and presence of concurrent ALK mutation were not prognostic (p>0.1) for PFS or OS. Patients with locoregional disease at diagnosis had an equally poor survival compared to those with stage 4 (p>0.1 for both PFS and OS). Patients with ATRX deletions had worse OS (p=0.05) compared to those with mutations.

Conclusions: ATRX aberrations were associated with chemorefractory neuroblastoma and poor survival outcomes regardless of stage at diagnosis. ATRX deletions were associated with a worse prognosis than mutations.

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Identification of a Novel 3q13.31 Chromosomal Rearrangement Involving the LSAMP Gene in Neuroblastoma

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Background: Despite major progress in treatment of pediatric cancer, aggressive neuroblastoma (NB) still constitutes a major clinical problem. The study of DNA copy number variations (CNVs) provides relevant information regarding patient specific alterations, as abnormal expression of oncogenes and tumor suppressor genes that might be used in targeted therapy.

Material and Methods: Whole genome sequencing (WGS) was performed for tumor DNA from 32 patients. The bioinformatic analysis of the data was done using CLC Genomic Workbench for mapping and variant calling while Ingenuity Variant Analysis software was used for the filtering. Specific breakpoints for structural variants were detected through manual inspection using IGV. To corroborate and further explore the 3q13.31 chromosomal region, SNP-microarrays from 150 NB patients were analyzed, as well as 10 different NB cell lines. Silencing and overexpression of LSAMP in NB cell lines were performed for functional evaluation.

Results: The analysis of the structural variants detected by WGS showed recurrent focal chromosomal rearrangement at chromosomal region 3q13.31 in 5 of the 32 patients. These rearrangements are affecting the limbic system-associated membrane protein gene (LSAMP). In addition, SNP-microarrays from additional 150 patients were also included, getting a positive result on a total of 6 patients for structural rearrangements in LSAMP. Regarding the SNP-microarrays from NB cell lines, 6 cell lines were positive for rearrangement in LSAMP while 4 of them were negative. Early results from in vivo experiments indicates that LSAMP has tumor suppressing capacity in neuroblastoma.

Conclusions: The analysis of genes encoded in breakpoint regions as well as the relationship between junction sites could provide information about oncogenes and tumor suppressor genes dysregulation. In total, chromosomal rearrangement involving the LSAMP gene in the 3q13.31 locus was detected in 8 different patients as well as in 6 different NB cell lines in our neuroblastoma cohort. LSAMP has been described as a tumor suppressor gene in other types of cancer such as osteosarcoma, renal and ovarian cancer; and has high potential to be a novel tumor suppressor gene in NB. Functional evaluation is ongoing and early results seem to confirm this hypothesis.

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Phase II Trial of Irinotecan/Temozolomide with Dinutuximab/GMCSF (I/T/DIN/GMCSF) in Relapsed/Refractory Neuroblastoma: A Report from The Children's Oncology Group (COG)

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Background: COG ANBL1221 was a randomized Phase II selection design trial for patients with relapsed/refractory neuroblastoma, however randomization was stopped early when I/T/DIN/GMCSF was shown be the optimal combination for further study. In the small cohort randomly assigned to I/T/DIN/GMCSF, the objective response rate was 53%. An expanded cohort was evaluated in order to more accurately assess response rates and to better define the toxicity profile of this combination.

Methods: Patients were eligible at first relapse/progression or first designation of refractory disease. Cycles were administered every 21 days. Objective responses were confirmed by central review. Toxicities were graded according to NCI CTCAE v4.0.

Results: A total of 53 eligible patients were enrolled on ANBL1221 and assigned to I/T/DIN/GMCSF; 17 during the randomized portion and 36 during study expansion. Median age at enrollment was 5.1 years (range 1.3-15.9), 39 patients (74%) had measurable disease. Fourteen (26%) had MYCN amplified tumors, 20 (38%) had previously undergone high-dose chemotherapy with stem cell rescue, and 14 (26%) had received prior anti-GD2 antibody. Twenty-two (42%) had relapsed disease while 31 (58%) had refractory/progressive disease (PD). Subjects received 378 total courses (median 6). Of 53 subjects assigned to I/T/DIN/GMCSF, 21 experienced objective responses [40%; 95% CI (26, 53)], including 10 PR and 11 CR. Seven experienced PD on therapy, 23 patients had stable disease. Two patients did not receive protocol therapy and did not undergo disease evaluations but were included in the intention-to-treat analysis. Among responders, 4 (19%) had MYCN amplified tumors and 9 (43%) had previously received an anti-GD2 antibody. Of the 51 patients evaluable for toxicity, thirteen (25%) had Grade 3 pain, 8 (16%) had Grade 3 diarrhea, and 4 (8%) had Grade 3 vomiting. Neutropenia (Grade 3) was observed in 14 (27%), Grade 3 thrombocytopenia in 5 (10%), and Grade 3 fever/infection in 11 (22%).

Conclusion: I/T/DIN/GM-CSF showed significant anti-tumor activity in patients with relapsed/progressive or refractory neuroblastoma. This combination was well-tolerated in a cohort of >50 patients. Studies of biological markers that may identify patients most likely to respond to this active chemo-immunotherapeutic regimen are in progress.

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CHAF1A Blocks Neuroblastoma Differentiation and Promotes Tumorigenesis Via Polyamine Accumulation

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Neuroblastoma (NB) arises from embryonal neural crest secondary to a block in differentiation. Efforts to define this block have led to the incorporation of differentiation strategies into NB treatment. However, retinoic acid (RA)-based therapy has had limited therapeutic success. Recently, we have demonstrated a novel function of the histone chaperone Chromatin Assembling Factor 1A (CHAF1A) in blocking NB tumor differentiation.

Through gain-of-function studies, we evaluated the contribution of CHAF1A-mediated blockage of differentiation to NB tumorigenesis and treatment resistance. We found that CHAF1A loss-of-function drives neuronal differentiation even in NB cells that fail to respond to RA. Conversely, conditional overexpression of CHAF1A in RA-sensitive cells blocks RA-induced differentiation, enhances cell proliferation and migration, and increases RAS-mediated colony formation. Moreover, by using an in vitro model system in which human embryonic stem cells (hESC) differentiate into neural crest cells (NCCs) upon Wnt activation, we found that CHAF1A remarkably suppresses the differentiation of hESC into NCCs (determined by SOX9 fluorescence immunostaining), supporting a role of CHAF1A in NC development. Importantly, using a non-tumorigenic orthotopic xenograft NB model (conditional CHAF1A overexpression in SHEP cells), we demonstrated that turning on CHAF1A expression is sufficient to promote tumor initiation (CHAF1A OFF engraftment rate: 2 out of 9, 22%; CHAF1A ON engraftment rate: 9 out of 12, 75%, p=0.02). Furthermore, by combining gene expression and global metabolomic profiling of NB cells upon CHAF1A overexpression, with patient data analysis, we have captured a novel feature of CHAF1A in metabolic reprogramming. CHAF1A significantly enhances cellular levels of polyamines (by GS-MS) and ODC1 activity (by fluorescence-based assay) to support cell growth. Blocking polyamine metabolism potently enhances the cytotoxic effects of RA and restores RA sensitivity.

Our data suggest that CHAF1A contributes to NB tumorigenesis through restriction of cell differentiation and disruption of cell metabolism. They also indicate that polyamine accumulation is key for CHAF1A-mediated de-differentiation. Further understanding of CHAF1A-induced genetic and metabolic alterations will guide the development of novel differentiation therapies for high-risk NB.

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Epigenetic Profiling to Explore Cell Identity in A Cohort of Neuroblastoma Patients

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Background: Neuroblastoma (NB) show significant intra-tumor heterogeneity, with two distinct cell states, a noradrenergic and a mesenchymal neural crest cell-like identity, identified by different core regulatory circuits defined by super-enhancers (SE). We performed epigenetic profiling to explore cell identities defined by histone modifications in 43 NB tumors.

Methods: We profiled two histone marks (H3K27ac; H3K27me3) using chromatin immunoprecipitation and sequencing (ChIP-seq). We analyzed transcriptomic profiles by Affymetrix microarrays. Patient characteristics: median age at diagnosis=27.9 months (range 0-131.2), INRG stage; L1/L2=18, M=22, MS=1), MYCN status: amplified=9; non-amplified=32. Tumor samples were either frozen (n=33) or formalin fixed-paraffin embedded (FFPE, n=10). Frozen samples were sequenced by Illumina® (TrueSeq protocol). Peak calling in ChIP-seq data was performed using HMCan. Copy number, ploidy and tumor purity were assessed using Control-FREEC; super-enhancers (SE) and large repressed regions were called with LILY. Seven samples were excluded from the analysis due to low signal-to-noise ratio in ChIP-seq profiles, and 9 due to purity level <65%. ChIP-seq profiles for the remaining samples were renormalized together using the assumption of similar binding intensity for the strongest 1000 peaks.

Results: SE were detected in PHOX2B, GATA3, HAND2, ISL1, TBX2 and other genes with previously described NB SEs. We then located large Polycomb-repressed regions using a similar procedure. Extremely strong H3K27me3 signal was observed in several large domains including important cancer-related genes: CAV1, CAV2, TES and MET were simultaneously silenced on chromosome 7 (rank 3 and 1 in MYCN-amplified and non-amplified tumors, respectively). The second strongest Polycomb-repression domain included IRX2/IRX4 (ranks 1 and 2). A region on chromosome 1p36 including a strong epigenetic repression signal (ranks 16 and 7) covering putative tumor suppressor TP73, ARHGEF16, and PRDM16 was also observed. However, for these large H3K27me3 domains, no significant difference between MYCN-amplified versus non-amplified NB, nor between stage M versus non-stage-M NB, was observed.

Conclusion: Using the analysis of histone marks, strong NB SEs previously reported in NB cell lines could be confirmed in primary NB. Interestingly, we identified large Polycomb-repression domains including important cancer-related genes, which might possibly be targeted by Ezh2-inhitors, currently undergoing clinical trials for several cancer types.

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Germline BARD1 Mutations Predispose to Neuroblastoma Through Defective DNA Double-Strand Break (DSB) Repair

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Background: Sequencing of neuroblastoma patients' germline DNA has revealed significant enrichment for potentially pathogenic BARD1 variants. The clinical relevance of these germline mutations was unknown.

Methods: Five BARD1 germline variants discovered in our neuroblastoma sequencing efforts (p.R112*, p.R150*, p.E287fs, p.Q564*, and p.R641*) were prioritized for study here. BARD1-BRCA1 heterodimerization was assessed by co-immunoprecipitation after co-transfection with BRCA1 cDNA into HEK293T. BARD1 and BRCA1 stability were assessed via cycloheximide chase, and localization was assessed via immunofluorescence (IF) and immunoblotting after nuclear fractionation. In parallel, the E287fs variant was introduced into IMR-5 cells as a monoallelic knock-in via CRISPR/Cas9 (IMR-5 BARD1 WT/E287fs). BARD1 isogenic cell line proficiency in homology-directed repair (HDR) was assessed by quantification of RAD51 foci after UV irradiation and by measuring cytotoxicity with the PARP inhibitor olaparib.

Results: Potentially damaging germline BARD1 variants were identified in 7/766 (0.9%, $p<0.001$) neuroblastoma patients; no tumors showed loss of heterozygosity. Of the five variants studied, all but one truncated BARD1 protein (p.R112*) maintained the ability to bind BRCA1, though only p.E287fs stabilized BRCA1 comparably to wild type (WT) BARD1. More than half of the variants (3/5) increased the proportion of BRCA1 localized to the cytoplasm relative to WT. BARD1 WT/E287fs IMR-5 cells assembled fewer RAD51 foci after UV irradiation than WT IMR-5 cells (38% vs. 12% of nuclei with ≥3 foci; $p<0.001$), suggesting reduced capacity for DNA DSB repair. Moreover, IMR-5 BARD1 WT/E287fs cells were sixfold more sensitive to PARP inhibition with olaparib relative to WT IMR-5 cells (IC50 of 860 vs. 5400 nM).

Conclusions: These data suggest that BARD1 germline mutations predispose to neuroblastoma by disrupting the stability and localization of BRCA1. The resultant dysregulation of DNA DSB repair and increased sensitivity to PARP inhibition may provide a therapeutic opportunity. Efforts are ongoing to engineer additional heterozygous BARD1 variant knock-ins, and to further characterize these variants' effects on BARD1-BRCA1 heterodimerization and DNA DSB repair.

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Bcl2-family Inhibitors for Neuroblastoma: Mechanisms of Activity and Biomarker Development

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Background: Bcl2-family proteins govern mitochondrial commitment to apoptosis downstream of therapeutic stress. Bcl2-family inhibitors are approved for cancer (Venetoclax; Bcl2 inhibitor) and under development (S63845; Mcl1 inhibitor; others) and a mechanistic understanding of their activities will enhance predictive biomarker development for clinical use.

Methods and Results: We measure apoptotic responses (cytochrome c release) from tumor-mitochondria exposed to synthetic BH3 death-domains to define Bcl2-family dependencies. Such mitochondrial profiles are reproducible, concordant from tissue culture and xenograft growth, and invariant when assessed serially, supporting them as stable and cancer cell-intrinsic. Tumors functionally profiled as Bcl2-dependent have Bim sequestered to Bcl2 (by coIP), and are sensitive to Bcl2 inhibitors (IC50<100nM) that displace Bim, induce death, and extend survival in vivo. Mcl1-dependent neuroblastomas have Bim sequestered by Mcl1 and resist Bcl2 inhibition (IC50>2,000nM). Mcl1 knockdown (siRNA) re-sensitizes these cells to Bcl2 inhibition (IC50<200nM). We studied neuroblastoma cell lines with the Mcl1 inhibitor, S63845. Neither Blc2- nor Mcl1-dependent cells were sensitive to S63845 monotherapy (IC50>5,000nM). Yet S63845 synergized with Bcl2 inhibition, presumably by removing this adaptive survival pathway. S63845 synergy far more potent in Mcl1-dependent tumors (shifting IC50>1 log in all tested) and ongoing mechanistic work tests a hierarchical model in which Bcl2 can sequester Bim displaced from Mcl1, but not the reverse. As neither mitochondrial profiling nor coIP are expedient for clinical biomarker use, we are developing proximity ligation assays (PLA) for FFPE-tumor slides. Assays that define cells with Bim:Bcl2 and Bim:Mcl1 binding, respectively, are being optimized using neuroblastoma xenografts of known survival dependency. Their accurate detection of Bim binding will be tested for predictive accuracy in upcoming pediatric trials of the Bcl2 inhibitor, Venetoclax (Abbvie).

Conclusion: Neuroblastoma maintains selective survival dependencies that define their vulnerabilities to Bcl2 inhibition not otherwise predictable by molecular data. We propose a responder hypothesis in which only tumors with Bcl2-dependence defined by Bim:Bcl2 binding are sensitive. While Bim:Mcl1 binding similarly predicts Mcl1-dependency, the S63845 compound does not show single agent activity in our models. Development of clinically-relevant biomarker assays to define Bim binding should prove useful for the clinical development of Bcl2-family inhibitors like Venetoclax.

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Loss of GAS7 Expression Promotes Metastasis in Neuroblastoma with High Levels of MYCN Expression

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About half of all patients with neuroblastoma, especially those over 18 months of age with MYCN amplification, present with widespread metastasis and have a high risk of treatment failure and death. However, the mechanisms underlying neuroblastoma metastasis remain poorly understood. In earlier work, we found that overexpression of LMO1 synergizes with MYCN to promote neuroblastoma metastasis. Here, we show that GAS7, the growth arrest-specific 7 gene located on chromosome 17p, acts as a brake on tumor cell dissemination in MYCN-amplified neuroblastoma, with loss of this regulator increasing metastatic potency. Computational analysis of the publicly available R2 human neuroblastoma dataset indicated that GAS7 is significantly downregulated in most neuroblastoma cases, and that low levels of GAS7 expression are associated with a poor prognosis. Interestingly, GAS7 expression is significantly lower in MYCN-amplified cases. In addition, deletion of chromosome 17p, which includes the GAS7 locus, identifies a discrete subset of high-risk patients with MYCN amplification. Together, these findings suggest loss of function of GAS7 might enhance the ability of MYCN to affect neuroblastoma pathogenesis. To test this hypothesis, we developed a gas7 knockout mutant zebrafish line using TALENs and crossed these mutant fish with transgenic fish overexpressing MYCN in the peripheral sympathetic nervous system. Strikingly, in this system, EGFP+ neuroblastoma cells showed much earlier and broader dissemination in the compound fish with overexpression of MYCN and loss of function of gas7 than in fish with overexpression of both MYCN and LMO1. With knockdown of GAS7 in MYCN-amplified human neuroblastoma cells, we consistently detected enhanced invasion and migration, in contrast to a reduction in these end points when GAS7 was overexpressed in human neuroblastoma cells with low endogenous expression of GAS7. Furthermore, our RNA-sequencing analysis of tumors from fish with MYCN-only or MYCN;gas7 loss showed that genes affecting tumor cell-cell or cell-extracellular matrix interactions were downregulated by gas7 loss of function. Together, our results provide the first in vivo evidence suggesting that loss of gas7 function in a subset of high-risk neuroblastomas promotes widespread metastasis in the context of overexpressed MYCN, apparently through deregulation of tumor cell-cell and cell-matrix interactions.

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Targeting p53 and Rb Pathway Disturbances for Neuroblastoma Treatment

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Background: Clinical complete remission is achieved in many stage 4 neuroblastoma patients, but the high risk of relapse and the accompanying treatment-resistant nature of these tumors is still a challenge. We have previously identified higher frequencies of mutations that affect both the p53 and the Rb pathway, like the homozygous loss of CDKN2A or amplification of MDM2 and CDK4. In addition, both Cyclin D1 and MDM2 overexpression is a common characteristic of primary neuroblastoma. These events affect both Rb and P53 pathways, but it is yet unknown which of the two affected pathways is the most potent driver in these cases.

Results: To study this, we first performed compound exposure assays with the most promising CDK4 (Ribociclib, Palbociclib and Abemaciclib) and MDM2 (Idasanutlin and SAR405838) inhibitors in a series of 11 neuroblastoma cell lines. These lines have well defined mutational properties as CDKN2A deletion, CDK4/MDM2 amplification, p53 mutation and MYCN amplification. Sensitivity for the MDM2 inhibitor inversely correlated to the p53 mutations status but did not correlate to MDM2 amplification or CDKN2A deletion status. Similarly, there was no clear correlation between the mutations status of CDK4/CDKN2A and the sensitivity for CDK4 inhibitors. This could relate to the overall high expression of Cyclin D1. To further explore this, we generated CDK4 and MDM2 inducible cell lines. The overexpression of CDK4 did not induce sensitivity for CDK4 or MDM2 inhibitors.

Conclusion: As was previously suggested in the first clinical trials with CDK4 inhibitors, the CDK4 nor the CDKN2A status are clear selection biomarkers for sensitivity for CDK4 inhibitors. Further testing is necessary to identify (other) biomarkers for CDK4 and MDM2 inhibitors, e.g. the recently described loss of FBXO31. We are currently also testing if combining CDK4 and MDM2 inhibition might result in synergistic effects in cell lines with genomic aberrations in these pathways.

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Alterations in Lipid Metabolism in Neuroblastoma Cells After MYCN Downregulation

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Neuroblastoma is the second more deadly solid tumor in children. The survival rate of high-risk patients remains around 50%, despite intensive multimodal therapy. In addition, long-term effects derived from treatment reduce quality of life of survivors. For these reasons, the discovery of new therapeutic targets for neuroblastoma is a high priority.

High levels of the MYCN protein is strongly correlated to poor prognosis and treatment failure in high-risk neuroblastoma. In fact, the MYCN oncogene is amplified in the 25% of the neuroblastoma cases, and it is used as a genetic marker for poor outcome. Our findings show that a small chemical molecule, 10058-F4, previously identified as a c-MYC inhibitor, also targets the MYCN/MAX complex resulting in induction of apoptosis and neuronal differentiation in MYCN-amplified neuroblastoma cells. Importantly, we demonstrated that inhibition of MYCN results in metabolic changes including mitochondrial dysfunction leading to accumulation of lipid droplets (Zirath PNAS 2013). Recently we found that ectopic expression of estrogen receptor (ER-alpha) also induced lipid accumulation in neuroblastoma cells (Dzieran PNAS 2018).

Here we have analyzed the effects of small-molecule inhibitors including 10058-F4, 10075-G5, JQ1 and I-BET762 in a panel of MYCN-amplified and non-amplified neuroblastoma cell lines, and observed accumulation of cytoplasmic lipid droplets in all tested cell lines except in SH-EP, in which neither MYCN nor c-MYC proteins can be detected.

To gain insight in the control exerted by MYCN on neuroblastoma cell biology, we performed high-resolution proteomic analysis in MYCN-amplified cells before and after downregulation of MYCN with specific shRNA. We identified around 7000 proteins, of which 6500 have been used for identification of novel pathways involved in neuroblastoma pathogenesis and for investigation of potential MYC related biomarkers. Our preliminary analysis shows that most of the altered proteins are involved in metabolism, including lipid metabolic processes. We are now comparing these data with the proteomics of ER-alpha overexpressing neuroblastoma cells to gain further insight into lipid metabolism and neural differentiation. Taken together, our results highlight the link between MYCN signaling and lipid metabolism in neuroblastoma biology, and provide information about important metabolic pathways which may be the basis for future therapies.

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Sequential Expression of Lineage Specific Transcription Factors Drives Differentiation in High-Risk Neuroblastoma (HR-NB)

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Temporal regulation of lineage specific transcription factors(TFs), guided by epigenetic changes involving super enhancers(SEs), underlie normal developmental programs. SE dysregulation is implicated in tumorigenesis. Clinical and experimental evidence indicates that HR-NB arises from an inability of sympathoadrenal progenitors to exit a self-renewal program and terminally differentiate. We utilized retinoid(RA)-induced growth arrest and differentiation of NB cells to identify critical SE regulators of NB self-renewal and differentiation. We identified 1199 SEs with H3K27ac ChIP-Seq in matched control (self-renewal) and RA treated KCNR cells [2,4,8 days(D)]. K-means clustering identified 4 temporal patterns in the dynamically RA-regulated SEs(n=940). Proximity analysis was used to link SEs to downstream genes. The 1st SE cluster(n=254) decreases with RA with GREAT analysis identifying genes regulating stem cell development and differentiation processes such as MYCN, ID2, GATA3, SOX11. Three other SE clusters showed sequential waves of activation at 2D(n=174), 4D(n=355) or 8D(n=157) with genes involved in regulation of neuron-projection and axonogenesis including genes like GATA2, SOX4, TWIST1. Under self-renewal conditions, SEs were maximally enriched for MYCN and SOX11 motifs, whereas after RA treatment SEs were enriched for SOX4 motifs. The MYCN SEs disappeared by 2Ds while by 8Ds, the SOX11 SE was lost coincident with decreasing RNA (50%) and protein (60%) levels. High SOX11 levels are associated with poor prognosis(p=2.3e-06) in NB tumors with SOX11 being over-expressed in NB vs normal cell lines (p=0.0006). siRNA mediated inhibition of SOX11 in HR-NB cell lines led to decreased cell growth (~30%) without evidence of differentiation indicating SOX11 may be a key regulator of self-renewal. In RA activated SE clusters, 2.5-fold increase in H3K27ac SE activity driving SOX4 was observed after RA treatment coincident with a 2-fold increase in SOX4 expression. High SOX4 expression is associated with good prognosis(p=1.7e-15) and more differentiated NB tumors, implicating SOX4 as a regulator of NB differentiation. To date, little is known about the involvement of the SOX TFs in NB's self-renewal process but sequential expression of SOX11 and SOX4 is required for neuronal programming of sympathoadrenal progenitors. Our SE analysis has identified candidate oncogenic lineage drivers of NB self-renewal and SEs critical for implementing a differentiation program.

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Epigenetic Modifiers MLL1 and JMJD3 Regulate Neuroblastoma Tumorigenicity By Maintaining A Cancer Stem Cell-Like Subpopulation

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High-risk neuroblastoma (NB) represents a major clinical challenge in pediatric oncology. Despite significant dose escalation of intense therapies, long-term survival for NB patients remains poor (<45%), and accounts for almost 15% of all pediatric cancer deaths. Relapse of metastatic, drug-resistant disease and treatment related toxicities mandates the development of new therapeutic strategies. Recently, we discovered a highly tumorigenic, chemo-resistant and self-renewing sub-population in NB with features similar to cancer stem cells (CSCs). This G-CSF receptor (CD114) expressing sub-population, can escape initial therapy and cause aggressively invasive relapsed disease. Therefore, developing direct targeting strategies and understanding for the molecular mechanisms maintaining this NB CSC-like sub-population is essential to delineate effective therapeutics for NB patients. By using siRNA screening approach and low-density pathway arrays, we found that the epigenetic regulators mixed-lineage leukemia-1 (MLL1; KMT2A; an H3K4me3 methyltransferase) and Jumonji D3 (JMJD3; KDM6B; an H3K27me3 demethylase) are important epigenetic regulators for NB proliferation and growth. Interestingly, we found that epigenetic regulators MLL1 and JMJD3 are aberrantly expressed in CD114+ sub-population and regulate the expression of the G-CSF receptor gene (CSF3R) itself, by maintaining active histone modifications at the promoter locus. Inhibition of MLL1 and JMJD3 with specific small molecule inhibitors reverses the histone patterns at CSF3R promoter and block gene expression, induces apoptosis selectively in CD114+ cells, and inhibits overall NB proliferation in vitro. Furthermore, inhibiting MLL1 and JMJD3 leads to dramatic tumor regression ($p<0.001$) and reduction in incidences of metastasis ($p<0.001$) in vivo. Most interestingly, both of these inhibitors increase the overall survival of the treated mice in vivo. As expected, reduction in tumor size was significantly correlated with the reduction in tumor CD114+ cells. Taken together, these data highlight that: a) neuroblastoma is maintained by complex interplay of epigenetic modifiers, and b) direct targeting of these epigenetic modifiers and combining this approach with current therapy, is a novel therapeutic approach to high-risk neuroblastoma.

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Validation of the Revised International Neuroblastoma Response Criteria (INRC)

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Background: A key requirement for assessing the efficacy of treatment regimens is a standardized method of interpreting disease response. Recognizing the importance of developing consensus criteria for evaluating response, in 1993 the international neuroblastoma community established the International Neuroblastoma Response Criteria (INRC). During the past 2 decades, new functional imaging studies and additional methods for quantifying bone marrow disease have been developed. In 2017, a task force of 52 international investigators from 13 countries with expertise in diverse fields of medicine and statistics was established to develop a revised INRC incorporating these advanced technologies for imaging and bone marrow assessments. The revised INRC was recently published by Park et al (JCO, 35 (2017)). We hypothesized that the revised INRC will lead to a more accurate assessment of response to treatment and also be more highly associated with overall survival (OS) and event free survival (EFS) than the 1993 INRC.

Methods: We analyzed high-risk (HR) neuroblastoma patients treated between 2006-2016 at the University of Chicago and Lurie Children's Hospital with known outcome data. Disease status was assessed at diagnosis and response was evaluated following induction therapy, prior to autologous stem cell transplant using both the original and revised INRC. Overall response was analyzed as an ordinal continuous variable.

Results: Preliminary analysis of 33 HR patients showed that both the original and revised INRC are good predictors of EFS (p =0.009, C-statistic = 0.68 and p=0.001, C-statistic =0.71 respectively). Neither the original INRC nor the revised INRC predicted OS (p =0.17, C-statistic = 0.58 and p=0.13, C-statistic =0.60 respectively). To determine if limiting response assessment to metastatic sites was associated with outcome, we also evaluated stage 4 HR patients with or without a complete response (CR) at metastatic sites. However, this metastatic site response assessment was not statistically associated with EFS (p=0.19, C-statistic 0.61) or OS (p=0.32, C-statistic= 0.56).

Conclusions: Our preliminary results suggest that response after induction therapy in HR neuroblastoma patients is associated with EFS but not OS. Analysis of a larger cohort and longer follow-up is needed to confirm these findings.

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Characterizing Treatment for Relapsed and Refractory High-Risk Neuroblastoma and the Resultant Impact on Survival

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Background: Most patients with relapsed/refractory high-risk neuroblastoma (HRNBL) die of disease. Treatments include chemotherapy, I-131 MIBG therapy, Phase 1 trials, immunotherapy, and no further tumor-directed therapy. While the response rate of each regimen is known, it is unknown how the totality of one's individual journey effects survival. Here we describe treatment of relapsed and refractory high-risk neuroblastoma patients from time of first relapse or progression to time of death, no evidence of disease for 24 months or stable disease defined as no change in imaging studies for 24 months.

Methods: Data from electronic medical records were abstracted on patients with HRNBL treated at Texas Children's Hospital from 2002-2013. Kaplan-Meier survival analysis was used to explore time to death and time to relapse, and the Log-Rank (Mantel-Cox) p-value was reported. Analysis was performed with SPSS vs 24.

Results: Thirty-six patients with relapsed/refractory neuroblastoma who had records available for their entire treatment course were identified. 15.4% of patients are alive with no disease or stable disease for at least 24 months. Phase 1 therapy was the most frequently utilized regimen at 31%. The mean number of days from time of diagnosis to 1st relapse was 347.1 days and 357.2 days between 1st relapse and death. The mean number of progressions and/or relapses was 3.

Those who participated in Phase 1 trials had significantly higher mean number of days from relapse to death than those not on Phase 1 trials (606.25 d vs 207.80 d; p=0.004). There was no significant difference in time to death between patients who took a break from treatment (defined as >21 days without therapy) and those who did not (p=0.52). However, those who took a break reported 439.88 mean days from relapse to death compared to 327.14 for those not taking a break.

Conclusions: Patients who participated in Phase 1 trials had a greater number of days between relapse and death, which is likely confounded by the fact that those patients are able to meet stringent enrollment requirements. Taking a break from therapy does not appear to impact time to death.

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Combination of High-Dose Radiation Therapy and Immunotherapy Inhibits Neuroblastoma Tumor Growth in A Syngeneic Mouse Model

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Introduction: Enhancement of the anti-tumor immune response with cancer immunotherapy agents has emerged as an effective complementary approach to the therapeutic mainstream of surgery, chemotherapy, and radiation in neuroblastoma. However, the overall outcome for high-risk neuroblastoma remains poor. High-dose radiotherapy (HDRT) has been shown to induce an efficient immune response and eliminate tumors outside of the radiation field. Here, we studied the effect of combining HDRT and anti-PD1 immunotherapy on tumor growth and immune response in neuroblastoma.

Method: We developed a syngeneic neuroblastoma tumor-graft model, using the murine neuroblastoma cell line 9464D. A cohort of 80 tumor-graft mice were prepared by transplanting 1 mm3 portions of 9464D flank tumors within the left renal capsule. Mice were randomized to receive either three 8Gy fractions of CT-guided HDRT or sham HDRT, and concurrent dosing of either PD-1 antibody (αPD1) (200 □g) or isotype control. Half of the mice from each group were euthanized 48 hours after the last HDRT treatment and the other half were euthanized when tumor size reached 1.5cm3. Tumor tissue from each group were stained simultaneously for DAPI, CD3, CD8, CD4, FoxP3 and Endomucin. Cell phenotypes within representative fields were visualized using Vectra multispectral imaging platform, and tumor-infiltrating lymphocytes were quantified using inForm image analysis software (Perkin Elmer).

Result: Combination of HDRT and anti-PD-1 treatment significantly increased median survival by 48 days (10 days vs 58 days, control vs combination, $p<0.0001$). Treatment of anti-PD-1 alone did not affect survival and treatment of HDRT alone increased the medium survival by 24 days (10 days vs. 34 days, control vs HDRT, $p<0.001$). 48 hours after the last HDRT treatment, tumors of mice which received combination treatment had significantly greater CD3+, CD3+CD4+ and CD3+CD8+ lymphocyte infiltration than those of control mice, or mice which received either anti PD-1 or HDRT. Specifically, tumors of mice who received combination treatment have 7-fold increase of CD3+ ($p<0.0001$), 7-fold increase of CD3+CD8+ ($p<0.001$), 4-fold increase of CD3+CD4+ ($p<0.001$) and 2.7-fold increase of CD3+CD4+FOXP3+ ($p<0.01$), comparing with tumors of control mice.

Conclusion: Combination of HDRT and PD-1 blockade significantly inhibits neuroblastoma tumor growth and promotes intra-tumor lymphocyte infiltration

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A High-Content Screening of Anti-Cancer Compounds Suggests the Multiple Tyrosine Kinase Inhibitor Ponatinib for Repurposing in Neuroblastoma Therapy

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Background: Survival of children with high-risk neuroblastoma (NB) remains a major open challenge, prompting for a search of novel therapeutic options. The repositioning of FDA-approved molecules is a strategy that has already been successfully employed to discover new applications for existing drugs in other cancers. Therefore, we performed a high-content screening of a library of anti-cancer compounds, consisting of 349 small molecules either FDA-approved or under clinical trial, to repurpose commercially available drugs for NB therapy.

Results: The anti-cancer library was tested in 3D multicellular spheroids, recognized as a reliable preclinical model to recapitulate drug responses in solid tumors. In the primary screening, three NB cell lines (CHP-134, IMR-32, and SK-N-BE(2)) were treated for 72 hours with 10 µM of the library compounds. The viability of spheroids was evaluated using a high-content imaging approach, and the data were analyzed by the Strictly Standardized Mean Difference (SSMD) metric. Compounds were classified as primary hits if SSMD was < -3 in at least two cell lines. This resulted in a primary hit list of 193 compounds, from which we selected 60 FDA-approved molecules, and then prioritized drugs with multi-target activity, discarding those already in use for NB treatment or enrolled in NB clinical trials. Hence, 20 drugs were further tested in the concentration between 0.625 and 80 µM for their efficacy to inhibit NB cell viability, both in 2D and 3D models. Dose-response curves were next supplemented with available data on severe side-effects, therapeutic index, and molecular targets, indicating ponatinib and axitinib, tyrosine kinase inhibitors, as the most promising candidates for repositioning in NB. Both molecules affected the viability of NB cells by inducing cell cycle block and apoptosis, and by inhibiting colony formation. However, only ponatinib consistently affected migration/invasion capabilities of NB cells. Finally, only ponatinib proved effective inhibition of tumor growth in NB orthotopic mice models.

Conclusions: Starting from a screening of 349 anti-cancer drugs we identified the multiple tyrosine kinase inhibitor ponatinib as a promising drug for repurposing in NB therapy.

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Development of A Neuroblastoma Image Model By 3D Reconstruction of Histological Whole Serial Sections

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Background: The use of 3D technologies in tumor pathology lets a better characterization of tissue scaffolding that give clues about tumor homeostasis, dynamics and cell growth.

Aim: To get a 3D neuroblastoma (NB) representation using mathematical algorithms designed through image analysis.

Material and methods: We made 60 serial sections of a paraffin-embedded NB sample which had been previously characterized histological and genetically with two different areas of primary tumor and an adjacent lymph node. Serial sections were stained to detect different extracellular matrix elements and blood vessels. The stained tumor sections were digitized at 20x with Pannoramic MIDI (3DHistech Ltd). We carried out the morphometric image analysis using the following software tools: NuclearQuant (Pannoramic Viewer v.1.15, 3DHistech Ltd), Image Pro-Plus v.6.0 (Media Cybernetics), and AngioPath® (UV/Incliva, UCLM and SAS). The 3D reconstruction required the use of an image registration algorithm to match different structures and therefore perform a serial section alignment of the different samples. Once this process was got in the sections of the sample, a complex computation rendering for 3D visualization was carried out by computer-assisted.

Results: We found differential tissue characteristics between each of the areas and sections of the sample, showing intratumoral heterogeneity at 180-300µm of tissue depth. Parameters of extracellular matrix elements associated with poor prognosis in NB which had been described by our group as lower density of tumor capillaries and larger reticulin fibers were found in the most undifferentiated area of the sample.

Conclusions: The digital analysis of different tissue elements and 3D reconstruction in NB highlights a greater accurate in the composition and organization of the tumor tissue to define novel therapeutic targets.

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Depleting Mesenchymal Stromal and Endothelial Cells in Tumor Microenvironment with Anti-CD105 Antibodies Enhances Dinutuximab Immunotherapy of Neuroblastoma with NK Cells

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Background: We determined if mesenchymal stromal cells (MSCs), endothelial cells, and monocyte-derived macrophages in the tumor microenvironment (TME) of neuroblastomas (NB) promote tumor growth and suppress immunotherapy with activated natural killer (aNK) cells and anti-disialoganglioside (GD2) dinutuximab. Further, we determined if depleting these cells from the TME by targeting them with anti-CD105 antibodies increases the efficacy of aNK cell plus dinutuximab immunotherapy.

Methods: Growth of NB cell lines (CHLA-255, CHLA-136) cultured alone or with MSCs, monocytes, or MSCs + monocytes or with conditioned medium (CM) from co-cultures of NB cells, monocytes, and MSCs was determined. Cytokines in CMs were quantified with Luminex® or ELISA assays. Expression of CD105, GD2, and ligands for NK cell receptors on MSCs, endothelial, and NB cells was determined by flow cytometry. aNK cytotoxicity/ADCC with anti-CD105 and anti-GD2 antibodies was tested in vitro. Human NB was modelled in immune deficient NSG mice by co-injecting human NB cells (cell lines or patient-derived xenograft, PDX) with MSCs and monocytes under the renal capsule. Immunotherapy utilized NK cells activated ex vivo with K562-mbIL21 cells, dinutuximab, TRC105 and M1043 (anti-human and anti-mouse CD105 mAbs, respectively).

Results: NB cell line growth in vitro was stimulated by co-culture with MSCs and/or monocytes. ADCC against NB cells mediated by dinutuximab with aNK cells was suppressed by CM from co-cultures of NB cells, MSCs, and monocytes. Tumors formed by human NB cells in NSG mice grew faster when co-injected with MSCs and/or monocytes. Tri-cell tumors (NB, MSC, monocyte) were less sensitive to dinutuximab and aNK cells than those formed by NB cells alone. Human MSCs and endothelial cells, which express CD105, were sensitive to ADCC mediated by anti-CD105 antibody TRC105 with aNK cells in vitro. Addition of anti-human and anti-mouse CD105 antibodies to dinutuximab and aNK cell immunotherapy depleted human MSCs and murine endothelial cells and macrophages from tumors, reduced tumor growth, and increased survival.

Conclusions: MSCs and monocytes promote NB growth and suppress immunotherapy with aNK cells and dinutuximab. Anti-CD105 antibodies deplete CD105+ MSCs and endothelial cells as well as macrophages from the NB TME and improve immunotherapy with aNK cells and dinutuximab.

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Circulating microRNA Biomarkers for Metastatic Neuroblastoma

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In this study, the circulating repertoire of microRNAs in diagnostic neuroblastoma serum was assessed for identification of non-invasive biomarkers with potential in monitoring metastatic disease. Upon initial determination of the circulating neuroblastoma miRNome, 743 miRNAs were selected for further screening in two independent cohorts of 131 and 54 patients. Evaluation of serum miRNA variance in a model testing for tumor stage, MYCN status, age at diagnosis and overall survival, revealed tumor stage as the most significant factor impacting miRNA abundance in neuroblastoma serum. Differential abundance analysis between patients with metastatic and localized disease revealed 9 miRNAs strongly associated with metastatic disease in both patient cohorts. Increasing levels of these miRNAs were also observed in serum from xenografted mice bearing human neuroblastoma tumors. Murine serum miRNA levels were strongly associated with individual tumor volumes, suggesting that this novel miRNA signature may be applied to monitor disease burden.

Also on behalf of Vanderheyden K, Demuynck F, Vermeulen J, Noguera R, Berbegall A, Combaret V, Schleiermacher G, Schramm A, Schulte JH, Rahmann S, Bienertová-Vašků J, Mazánek P, Jeison M, Ash S, and Moreno-Smith M

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Longitudinal Evaluation of Murine Serum microRNAs as Biomarkers for Neuroblastoma Burden and Therapeutic TP53 Reactivation

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Accurate assessment of treatment response and residual disease is indispensible for the evaluation of cancer treatment efficacy. However, the requirement of tissue biopsies for longitudinal follow-up poses a major challenge in the management of solid tumors like neuroblastoma. In the present study, we evaluated whether circulating miRNAs, small non- coding RNAs, are suitable to monitor neuroblastoma tumor burden and whether treatment- induced changes of miRNA abundance in the tumor can be detected in the serum. We performed small RNA sequencing on longitudinally collected serum samples obtained from mice carrying orthotopic neuroblastoma xenografts that were exposed to treatment with idasanutlin (RG7388) or temsirolimus. We identified 56 serum miRNA to be differentially expressed upon xenograft tumor manifestation, out of which 20 were also found specifically expressed in the serum of human high-risk neuroblastoma patients. The murine serum levels of these 56 miRNAs correlated with both tumor tissue expression and tumor volume, suggesting potential utility for monitoring of tumor burden. In addition, we describe for the first time serum miRNAs that dynamically respond to TP53 activation, following treatment of engrafted mice with idasanutlin. We identified idasanutlin-induced serum miRNA expression changes both upon one day and 11 days of treatment. By limiting to miRNAs with a tumor-related induction, we put forward hsa-miR-34a-5p and hsa-miR-212-3p as potential biomarkers of TP53 activation in serum.

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Establishing Mouse Neuroblastoma Cell Lines Using Conditional Reprogramming Technology

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The transgenic TH-MYCN mouse strain is predisposed to neuroblastoma and shows many features of the human disease. As such it represents a valuable model for neuroblastoma research. However, establishment and propagation of useful cell cultures in vitro from these mice is very difficult due to frequent cell differentiation. In this study we describe generation of viable neuroblastoma cell lines using conditional reprogramming (CR). This newly established cell culture method (Nature Protocols 2017, doi:10.1038/nprot.2016.174) allows indefinite propagation of cells in an undifferentiated state without the use of exogenous gene expression. Induction of cell reprogramming is rapid and expands multi-lineage cell types, rather than selective outgrowth of a small subpopulation. In conditionally reprogrammed tumor cell cultures, phenotypic and genotypic features of the primary tumor are maintained.

We optimized CR growth conditions for neuroblastoma tissue and were able to grow mouse neuroblastoma cells in vitro. The cells could be passaged, frozen and biobanked for continued studies. These cell lines demonstrate a unique phenotype and are positive for neuroblastoma markers, such as Phox2B, tyrosine hydroxylase and neuropeptide Y. Further studies are required to determine the utility of this method for isolation of the human neuroblastoma cell lines. If this method is proven successful in clinical setting, potential applications of conditionally reprogrammed neuroblastoma cell lines are far-reaching, including basic research, live biobanking, as well as diagnostic and therapeutic purposes.

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Evaluation of the Multikinase Inhibitor RXDX-105 Targeting RET and BRAF as a Novel Therapeutic in Neuroblastoma

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Background: Neuroblastoma is the most common extracranial solid pediatric malignancy and patients with high risk disease have poor outcomes despite aggressive multimodal therapy. RET tyrosine kinase is highly expressed in neuroblastoma cells and has been suggested to play an important role in their progression and survival. The RAS-MAPK pathway has also been suggested to play a role in neuroblastoma relapse, as recent studies have shown that a high percentage of relapsed neuroblastomas contain activating mutations in this pathway. RXDX-105 is a multikinase inhibitor with high potency against RET and BRAF, currently in Phase I trials for adult solid tumors. Based on the potential role of the RET tyrosine kinase and the RAS-MAPK pathway in neuroblastoma, we explored the therapeutic potential of RXDX-105 in this disease.

Methods: Ten neuroblastoma cell lines were treated with increasing concentrations of RXDX-105. Cell viability was assessed using Alamar blue assays. Cell proliferation was examined by measuring occupied percent confluence over time using continuous live cell imaging. The effects of RXDX-105 on RET inhibition and inhibition of the RAS-MAPK pathway was assessed with immunoblotting. Apoptosis rates were examined using a caspase immunofluorescence assay.

Results: The calculated IC50 values for RXDX-105 were between 3.2 uM and 20.6 uM after 72 hours of drug exposure. Immunoblots showed strong suppression of RET phosphorylation as well as suppression of MEK and ERK phosphorylation downstream of BRAF. RXDX-105 also induced apoptosis in all sensitive cell lines. Conclusion: RXDX-105 causes decreased viability and proliferation in neuroblastoma cells and shows strong inhibition of RET tyrosine kinase and RAS-MAPK pathway signaling, both of which may play an important role in NB cell survival, proliferation, and metastasis.

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Multiple Source Data Integration Identifies the CB2 Receptor as a Druggable Target in MYCN Amplified Neuroblastoma

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Despite advances in the molecular characterization of neuroblastoma, we lack effective treatments for many of the high-risk patients. We propose a new computational technique providing actionable insight from data amalgamation, aiDA, which enables researchers to select, with good accuracy, compounds that are likely to induce changes in cellular pathways that are consistent with specific clinical outcomes and subgroups. aiDA prediction is based on an integration of molecular data from childhood tumor biobanks, drug expression profiling data from NIH-LINCS, and drug-protein interaction networks. Applying aiDA to the transcriptomes of high risk neuroblastoma patients, the algorithm identified both known targets, such as the PI3K/MTOR and retinoic acid pathways and made interesting new predictions. In a set of experiments, such predictions were explored in patient-derived xenograft cell lines. The most promising targeted molecule was a cannabinoid receptor 2 agonist, which selectively suppressed viability in the NB cell cultures, and reduce N-Myc protein levels at concentrations that were non-toxic in embryonic zebrafish. The in vivo potential of cannabinoid receptor 2 interference on high risk neuroblastoma is under evaluation. aiDA will further be integrated in an easy-to-use web tool, to help researchers predict the effect of drugs in clinically defined neuroblastoma subgroups using in-house and publicly available patient data sets.

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Delineating the Mechanisms Underlying Oncogenic Transcription in ALKF1174L/MYCN Neuroblastoma

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Neuroblastoma, a malignancy of the developing sympathetic nervous system, is the most common extracranial solid tumor in children and has a survival rate of <50% in high-risk patients. One of the most influential determinants of poor prognosis in children with neuroblastoma is amplification of the transcription factor MYCN, found in approximately 20% of tumors. Because small-molecule inhibition of MYCN has not been clinically achieved, recent investigations have focused on the identification of critical targets that modulate MYCN. In a disease with few recurrent somatic mutations, a gain-of-function mutation, a phenylalanine to leucine substitution at codon 1174, exists in a gene encoding the cell surface receptor tyrosine kinase ALK. Interestingly, the ALKF1174L mutant is preferentially associated with MYCN amplification and accounts for a subset of patients with a particularly poor clinical outcome. In vivo studies have shown that concomitant expression of MYCN and ALKF1174L in neural crest cells leads to development of neuroblastoma with earlier onset, higher penetrance, and enhanced lethality compared to tumors in mice with isolated MYCN amplification. Mice bearing tumors with MYCN amplification and ALKF1174L show a distinct transcriptional profile compared to those with isolated MYCN amplification, suggesting a positive cooperative effect between the two. However, we do not have a clear understanding of the contribution of ALKF1174L in altering gene expression.

The goal of this study is to understand the mechanism(s) underlying the ability of ALKF1174L to affect oncogenic transcription of MYCN. To investigate this, I performed shRNA-mediated knockdown of ALK in cell lines expressing ALKF1174L or wild-type (WT) ALK together with and without amplified MYCN. Depletion of oncogenic ALKF1174L resulted in a decrease in MYCN protein levels, while knockdown of WT ALK showed no discernible change in MYCN protein expression, supporting a role for the mutant receptor but not its WT counterpart, in regulating the expression of oncogenic MYCN. By examining the genome-wide changes caused by inducible overexpression and deletion of ALKF1174L in isogenic cell line pairs with and without MYCN amplification, we hope to better characterize this oncogenic relationship and potentially identify therapeutically targetable nodes in high-risk neuroblastoma.

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Susceptibility-Weighted MRI Predicts Response to Vascular Endothelial Growth Factor Receptor Inhibition in the Th-MYCN Transgenic Model of Neuroblastoma

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Advanced magnetic resonance imaging (MRI) can non-invasively quantify changes in tumor vascular architecture and function, and potentially afford predictive/prognostic biomarkers of response to novel targeted therapies against neuroblastoma that directly (VEGF) or indirectly (MYCN, mTOR, ALK) modulate angiogenesis. The utility of susceptibility-weighted (SW-)MRI to provide two imaging biomarkers of tumor vascular response to pan-VEGFR inhibition was evaluated in Th-MYCN transgenic mice.

SW-MRI allows quantification of i) the native transverse relaxation rate R_2*(s-1), a proxy for deoxyhaemoglobin concentration and a biomarker of haemorrhagic neuroblastoma arising in Th-MYCN mice, and ii) fractional tumor blood volume fBV(%), derived from changes in R_2* following intravenous administration of ultrasmall superparamagnetic iron oxide (USPIO) particles. Anatomical and SW-MRI was performed on tumor-bearing Th-MYCN mice prior to, 24h and 7 days after daily treatment with 6mg/kg cediranib.

Cediranib caused a rapid reduction in tumor fBV (ΔfBV_cediranib_24h=-35±3% vs ΔfBV_vehicle_24h=-7±7%, p=0.0002), suppressing overall the characteristically aggressive tumor growth (ΔVolume_cediranib_D_o_D_7=-16±5% vs ΔVolume_vehicle_D_o_D_7=142±11%, p<0.0001); a range of volumetric response, from progressive disease to partial response, was however evident. Tumor median native R_2* and fBV correlated with aggregated erythrocytes and vascular endothelial marker Cd34 fraction area extracted from MRI-registered and digitised histology images respectively (r=0.8668, p<0.0003 and r=0.85, p<0.0001). Average baseline fBV (D_o) correlated with cediranib-induced changes in fBV at day 7 (r=-0.83, p=0.0008). Both baseline R_2*_D_o and fBV_D_o correlated with cediranib-induced tumor volumetric response at day 7 (r=-0.72, p<0.0001 and r=-0.65, p=0.02).

This study validates fBV as a sensitive imaging biomarker of vascular perfusion and its therapeutic modulation and demonstrates that fBV provides a predictive biomarker of vascular response. Interestingly, it also shows that both baseline fBV and native R_2* are predictive biomarkers of tumor response to cediranib in the Th-MYCN mice. SW-MRI can be readily implemented on clinical scanners, providing an attractive steady-state alternative for the pediatric population in which dynamic contrast enhanced-MRI can be challenging. Recent clinical studies have highlighted the safe use of the USPIO preparation ferumoxytol for MRI in children, supporting incorporation and investigation of SW-MRI in early phase imaging-embedded trials of vascular–targeted/modulating therapies against neuroblastoma.

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Colony Stimulating Factor 1 Receptor (CSF-1R) Blockade Improves the Efficacy of Chemotherapy Against Human Neuroblastoma

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Tumor-associated macrophages can promote growth of cancers. In neuroblastoma, tumor-associated macrophages have greater frequency in metastatic versus loco-regional tumors, and higher expression of genes associated with macrophages helps to predict poor prognosis in the 60% of high-risk patients who have MYCN-non-amplified disease. The contribution of cytotoxic T-lymphocytes to anti-neuroblastoma immune responses may be limited by low MHC class I expression and low exonic mutation frequency. Therefore, we modelled human neuroblastoma in T-cell deficient mice to examine whether depletion of monocytes/macrophages from the neuroblastoma microenvironment by blockade of CSF-1R can improve the response to chemotherapy. In vitro, CSF-1 was released by neuroblastoma cells, and topotecan increased this release. In vivo, neuroblastomas formed by subcutaneous co-injection of human neuroblastoma cells and human monocytes into immunodeficient NOD/SCID mice had fewer human CD14+ and CD163+ cells and mouse F4/80+ cells following CSF-1R blockade. In subcutaneous or intra-renal models in immunodeficient NSG or NOD/SCID mice, CSF-1R blockade alone did not affect tumor growth or mouse survival. However, when combined with cyclophosphamide plus topotecan, the CSF-1R inhibitor BLZ945, either without or with anti-human and anti-mouse CSF-1 mAbs, inhibited neuroblastoma growth and synergistically improved mouse survival. These findings indicate that depletion of tumor-associated macrophages from neuroblastomas can be associated with increased chemotherapeutic efficacy without requiring a contribution from T-lymphocytes, suggesting that combination of CSF-1R blockade with chemotherapy might be effective in patients regardless of the status of their anti-tumor T-cell responses.

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Socio-Economic Variation in Survival from Childhood Neuroblastoma in Northern England: A Population-Based Study

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Background: Social inequalities have been identified as factors affecting survival outcomes in adult cancers and some childhood cancers. Although neuroblastoma survival has improved recently and age and stage at diagnosis are known survival predictors, the relationship of socio-economic status (SES)and survival from neuroblastic tumours (NT) has not been studied. This study investigates the association between SES and survival from NT.

Methods: All NT cases in children aged 0-14 years, diagnosed during the period 1993-2014 were identified from a population-based specialist registry in Northern England. Paternal occupational social class at the time of birth was used as a proxy for SES and classified into 3 categories: class I/II (professional/managerial), class IIIN/M (skilled non-manual/skilled manual) and class IV/V (semiskilled/unskilled). Kaplan-Meier method was used to calculate overall survival (OS) and Cox regression analysis to investigate associations between survival and SES adjusting for age at diagnosis, stage, MYCN status and site of tumour.

Results: There were 102 cases of NT, 54(52.9%) male and 48(47.1%) female, 29(28.4%) aged <1 year, 51(50.0%) aged 1-4 years and 22(21.6%) >4-14 years. Using the INRG risk group criteria, 49(48.5%) cases were low and intermediate risk while 52(51.5%) were high risk cases. Social class was category I/II for 25 (24.5%) cases, IIIN/M for 40 (39.2%), IV/V for 14 (13.7%) and missing for 23 (22.5%). Five-year OS was 93.6% (95% CI 81.5-97.9%) for the low and intermediate risk group and 45.8% (95% CI 31.9-58.7%) for high risk cases. Cox regression analysis showed no significant disparity in OS from NT according to paternal social class although when compared to the most affluent class (I/II), those in class IIIN/M had a 22% increased risk of death and those in the most deprived class (IV/V) had 36% increased risk (adjusted HR=1.22, 95% CI 0.44-3.43 and HR=1.36, 95% CI 0.40-4.63 for class IIIN/M and IV/V respectively).

Conclusion: This study did not find a socio-economic effect on survival from NT diagnosed in northern England, where there is universal free access to primary and specialist healthcare. This contrasts with other childhood cancer studies where SES inequalities may affect outcome through adherence and treatment compliance.

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Loss of Information, and Confounding with Age When Using INPC for Risk Stratification: An International Neuroblastoma Risk Groups Confirmatory Analysis

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Background: Historically, COG has included the International Neuroblastoma Pathological Classification (INPC) as a component of neuroblastoma risk stratification. INPC (Favorable versus Unfavorable histology) is a binary composite of diagnostic category, MKI, grade, and age. In 2006, London et al demonstrated the confounding of INPC with age at diagnosis (<547days, ≥547days) and prognostic value of MKI and grade (patients diagnosed 1980-2001). Herein we attempt validation of those results in patients diagnosed 2002-2015.

Patients and Methods: 5,138 patients from the INRG database (2002-2015) were randomly divided into equal Test and Validation Sets. We tested the association of INPC with age (chi-squared test), and the relative prognostic strength of INPC and age in a multivariable Cox proportional hazards regression model. Survival tree regression with univariate Cox models was performed using age/diagnosis/MKI/grade. Analyses were repeated within the Validation Set.

Results: Age at diagnosis and INPC were highly associated ($p<0.0001$). In the multivariable model, INPC (HR=5.9; $p<0.0001$) was statistically significant but not age (HR=0.8; $p=0.06$) due to confounding. A survival tree of age/diagnosis/MKI/grade discriminated patients into seven statistically distinct terminal nodes (<547days, low/int MKI; <547d, high MKI; ≥547days, differentiating grade, low/int MKI; ≥547days, differentiating grade, high MKI; ≥547days, undifferentiated/poorly differentiating grade, low/int MKI; ≥547days, undifferentiated grade, high MKI; ganglioneuroblastoma [int/mixed]/ganglioneuroma [maturing]), but an INPC tree had only two nodes (Favorable, Unfavorable). Similar results were obtained in the Validation Set.

Conclusions: Our study validates the confounding of INPC with age and prognostic strength of MKI and grade. INPC gains prognostic strength by using age (cut-offs of 18 months, 5 years) in its definition, as demonstrated by a Cox model unable to retain both INPC and age as statistically significant. Using INPC alone results in a loss of granularity/precision in our ability to risk stratify patients using histopathology. For risk stratification, INPC is an oversimplification of histologic information, as evidenced by a survival tree with seven terminal nodes using age/diagnosis/MKI/grade, versus two nodes with INPC. Testing the individual components (age, diagnostic category, MKI, grade) rather than INPC will enhance the precision of multivariable Cox models and other statistical analyses to assess risk stratification for treatment assignment.

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Generation of Patient-Derived Organoid Biobanks for Faithful Representation of Neuroblastoma Subtypes

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Background: Developing novel therapies for Neuroblastoma (NB) has been hampered by the lack of representative preclinical models that can faithfully reflect the degree of heterogeneity seen in NB patients. Recent publications have successfully generated patient-derived organoids from different tumours types and have shown to resemble the original tumour genetically and phenotypically compared to traditional 2D cell lines. Therefore, we aimed to bridge the gap the conventional 2D culture and the complex in vivo environment by generating patient-derived NB organoids as representative preclinical ex vivo models with a high predictive value that can recapitulate the genetic and phenotypic diversity of the original NB tumour.

Methods and Results: To optimise the culturing conditions of primary NB tissue, we have exploited the R2 bio-informatics platform (http://R2.amc.nl) to identify ligands that could stimulate neuroblastoma cell growth. We tested a total of 14 different growth factors preferentially expressed in the primary tumour, from which we identified EGF, EGF IGF-1, PDGFA, PDGFB and Insulin as potential ligands based on their significant effects on cell viability and proliferation. Additionally, various 3D-forming matrices were examined to determine the most suitable for our NB organoid culture system including Matrigel (3D-base), Maxgel, methyl cellulose and using 3D human blood plasma semi-solid matrix. The last system was chosen not only because of the compatibility of the 3D matrix (near-physiological setting, free of animal serum and xenogeneic ingredients), but also their positive impact on NB cell viability. In addition, we were able to show successful single cell clonogenic outgrowth in the newly established 3D culturing environment. To determine whether we maintained the intra-tumoural heterogeneity as in the original tumour, we applied single-cell RNA sequencing in both NB organoids and primary NB tissue for which results are currently being analysed.

Conclusion: Using a 3D organoid system, we report the isolation and generation of 18 NB organoids from biopsy or resection specimens with an improved success rate of tumour organoid isolation and propagation in a near-physiological setting to preserve the intra-tumoural heterogeneity of primary NB tissue. NB organoid system is expected to be an ideal tool in preclinical drug screening and precision medicine program.

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The p53-Regulating Phosphatase WIP1 Is a Prognostic Neuroblastoma 17q-Oncogene, Significant for Cancerogenesis, Tumor-Progression and Provides a Novel Neuroblastoma Therapeutic Target

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Background: In neuroblastoma gain of chromosome 17q is the most powerful genetic predictor of adverse clinical outcome. WIP1, encoded by the gene PPM1D is a serine/threonine phosphatase directly regulating TP53 and other key proteins in cell cycle control and DNA repair, and involved in immunity and inflammation with activating mutations in cancers and developmental disorders.

Methods: Comparative genomic hybridization (CGH), expression arrays, RNA-sequencing, and whole-genome/whole-exome sequencing were used to examine PPM1D/WIP1 in tumors and cell lines. PPM1D/WIP1 knockdown SK-N-BE (2) cells were tested in vivo in tumor xenografts. Pharmacological inhibition with the p53-mdm2 modulating inhibitors RITA, Nutlin-3 and the novel Wip1-inhibitor SL-176, was used to evaluate PPM1D/WIP1 as therapeutic target. Transgenic mice were established to overexpress PPM1D/WIP1 and irradiated with sublethal doses of 4.5 Gy to induce DNA stress and further activate WIP1.

Results: CGH-array analysis of a national population-based material detected PPM1D/WIP1 extra copies in all tumors and cell lines containing 17q-gain. A novel oncogenic WIP1-activating PPM1D-truncating mutation in a MYCN-amplified infant neuroblastoma was detected. PPM1D mRNA correlates with chromosome 17-status, histologic patterns, clinicobiological subsets and prognosis with highest levels in MYCN non-amplified metastatic HR-neuroblastoma. In TH-MYCN mice PPM1D/WIP1-expression was correlated with MYC-target gene signatures during tumor development ($P<0.0001$). Neuroblastoma clonogenicity and xenograft development was significantly delayed ($P<0.001$) after PPM1D knockdown. Transgenic PPM1D/WIP1-overexpressing mice show spontaneous tumor development highly enhanced after irradiation compared to wild-type mice ($P<0.0001$). Tumor development is age- and time of irradiation-dependent displaying a wide range of p53 dependent cancers in >30% of irradiated animals. Pharmacological WIP1-inhibition with SL-176 was highly potent in cytotoxic/cytostatic effect in a variety of neuroblastoma cell lines. Furthermore, this non-toxic WIP1-inhibitor significantly inhibited growth of established neuroblastoma tumors in nude mice ($P<0.01$).

Conclusions: The p53 regulating PPM1D/WIP1 phosphatase is activated by prevalent unfavorable gain of 17q in childhood neuroblastoma, alternative splicing as well as oncogenic truncating mutations. We have confirmed an important oncogenic role of PPM1D/WIP1 regulating cellular stress and DNA-repair in neuroblastoma development. Activation of transgenic PPM1D/WIP1 through irradiation induced tumors confirming the oncogenic significance for cancer development. Molecular and pharmacological data propose WIP1 as a novel, druggable target in neuroblastoma.

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Preclinical Assessment of MDM2/p53, ALK and MEK Inhibitor Combinations in Neuroblastoma

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Background: Treatment of patients with high-risk neuroblastoma remains clinically challenging. The use of novel small molecule inhibitors targeting oncogenic pathways perturbed in neuroblastoma offers a non-DNA damaging, potentially more effective and less toxic treatment strategy. A high proportion of aberrations upstream of p53, and an increased incidence of ALK and RAS/MAPK pathway abnormalities have been observed in relapsed neuroblastoma, which support the use of MDM2-p53, ALK and MEK inhibitors as potential novel therapies in high-risk neuroblastoma.

Methods and Results: Using XTT assays, GI50 values of multiple ALK (TAE-684, Crizotinib, Alectinib, Ceritinib, Lorlatinib) and MEK (Trametinib, MEK-162, Cobimetinib) inhibitors were determined in a large panel of neuroblastoma cell lines of varying ALK and MAPK pathway status. Sensitivity to MEK inhibitors correlated with the presence of a MAPK pathway aberration with cell lines with RAF/RAS aberrations being more sensitive than those with NF1 aberrations. Sensitivity to ALK inhibitors, correlated with the presence of an ALK aberration, with cell lines with ALK mutations being more sensitive than those with amplification or copy number gain. Using median-effect analysis together with Calcusyn software, selected 2-way combinations of idasanutlin (RG7388, an MDM2-p53 antagonist), ALK and MEK inhibitors were shown to be synergistic in cell lines with wild-type p53 and an ALK or MAPK aberration. Consistent with this, in most cases, combination treatments led to increased levels of apoptosis as evident by higher levels of caspase 3/7 activity, compared to either agent alone.

Conclusions: These data highlight differences between the tested ALK inhibitors and type of ALK aberration with ALK amplified cell lines being less sensitive despite the potential clinical significance of ALK amplification in high risk NB. The data also show that combinations of MDM2-p53, ALK and/or MEK inhibitors are a potential therapeutic option for neuroblastoma patients with functional p53 and aberrations in ALK and/or the MAPK pathway and should be further explored in in vivo models.

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The Landscape of Long Non-Coding RNAs in Neuroblastoma

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Background: Sequencing studies have revealed a paucity of protein coding mutations in neuroblastoma (NBL), while the role of the non-coding genome, including long non-coding RNAs (lncRNAs), remains relatively unexplored. Here, we seek to define the landscape of lncRNA expression and genetic variation in NBL to elucidate the role of lncRNAs in susceptibility, progression, and disease relapse.

Methods: We analyzed RNA-sequencing data from NBL patient tumors (n=153) and patient-derived cell lines (n=38) using StringTie to identify both known and novel expressed transcripts. Differential expression was assessed based on clinical/biological subtypes and diagnosis vs. relapsed NBL. Genome-wide association study (GWAS) results from 3817 European NBL cases and 9974 matched healthy controls were interrogated to identify NBL-associated germline variants potentially affecting lncRNAs. Public and generated chromatin immuno-precipitation sequencing (ChIP-Seq) and chromatin capture data from NBL cells is utilized to infer regulatory sequences and interactions.

Results: To date, we have identified 76,237 expressed genes across 153 NBL patient tumors and 38 cell lines, including 14,000 lncRNAs and 14,353 previously unidentified predicted non-coding genes. Differential expression analysis comparing high- versus low-risk, MYCN amplified versus MYCN non-amplified, and diagnosis versus relapsed NBL subtypes revealed 1,528 lncRNAs and 818 differentially expressed protein coding genes (log2 fold change >2, FDR<0.1). We confirmed the previously reported NBL-associated CASC15/NBAT-1 lncRNAs at chromosome 6p22 (rs4712656, p=1.64x10-16), and identified two additional lncRNA loci at NBL-associated regions reaching genome-wide significance. AC072062.1 (rs13001462, p=6.19x10-24) located at 2q35 is a lncRNA diverging from the promoter of the BARD1 gene with highly correlated gene expression (r = 0.69). Another lncRNA, lnc-LIN28B-6 (rs72990858, p=2.07x10-15) overlaps a potential enhancer region located at 6q15, downstream of HACE1 and LIN28B.

Conclusion: The landscape of lncRNA expression and genetic variation in NBL presents a rich resource to study the role of the non-coding genome in tumorigenesis and disease relapse. Chromatin interaction data analysis to determine target gene promoters and enhancers of CASC15/NBAT1, lnc-LIN28B-6 and AC072062.1 is ongoing. Elucidation of the regulatory landscape of lncRNAs in NBL has the potential to expose new avenues for the development of disease biomarkers and therapeutics.

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RAS-MAPK Pathway Activation Causes Neuroblastoma Tumor Evolution and Can Result from Various Genomic Events, Including PHOX2B and CIC Aberrations

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Background: We have previously shown that RAS-MAPK pathway mutations are present at a high frequency in relapse neuroblastoma tumors, but also occur at a lower frequency in primary tumors. These observations suggested, that this signaling pathway plays an important role in tumor progression. In this study we further explore the role of an active RAS-MAPK pathway in neuroblastoma and identify additional genomic aberrations that are involved in activation of this pathway.

Results: First, we generated a neuroblastoma RAS-MAPK pathway signature by profiling of NF1 CRISPR knockout lines, inducible RAS overexpressing lines and cells treated with a MEK inhibitor. We then used this signature to analyse a large series of primary neuroblastoma tumor samples. Activation of the RAS-MAPK pathway clearly correlates with poor survival and is associated with known activating mutations such as ALK, SOS1 and PTPN11. There are also tumors with active signaling in which such mutations were not detected, suggesting there are other mechanisms of activation. In these tumors we detect recurrent aberrations in CIC, DMD and PHOX2B. We show that mutations in PHOX2B activate the RAS-MAPK pathway in neuroblastoma model systems in vitro¸ while mutations in CIC cause activation of downstream target genes without direct pathway activation. This results from direct interaction of CIC with downstream transcription factors from the RAS-MAPK pathway. Subsequently we confirmed the involvement of the RAS-MAPK pathway in tumor progression in 'in vivo' models. We overexpressed NRAS in xenografted neuroblastoma cells, which induced tumor growth. In addition, CIC CRISPR knock out in the neuroblastoma cells caused an even more pronounced induction in 'in vivo' tumorgenicity.

Conclusion: These results reinforce the RAS-MAPK pathway as a promising target in high-risk neuroblastoma and establish CIC as a powerful tumor suppressor related to this pathway.

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Utilization of Pencil-beam Scanning Proton Radiotherapy in High-risk Neuroblastoma of Thoracic Primary

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Purpose: The standard of care treatment for high-risk neuroblastoma includes post-transplant radiotherapy (RT) to the primary site of disease. While effective for tumor control, photon-based RT results in significant low-dose radiation exposure to normal tissue. With thoracic tumors, this can cause long-term cardiac and pulmonary toxicities. Pencil-beam scanning (PBS) proton RT is a state-of-the-art technique that optimizes tumor coverage by radiation while sparring normal tissues. However, PBS RT treatment plans are susceptible to degradation due to patient setup uncertainty, interaction patient variability and intrafraction motion. We describe a clinical case highlighting the benefits and opportunities to overcome the challenges of proton RT in thoracic primary high-risk neuroblastoma.

Materials/Methods: A 4-year-old male with neuroblastoma presented with a paravertebral thoracic soft tissue primary tumor and multiple osseous metastases. He received RT to the primary thoracic site following chemotherapy and tandem transplantation. Movement was assessed utilizing 4D computerized tomography (CT) for RT planning. PBS proton and intensity-modulated photon plans were constructed for comparison, optimized for equivalent target coverage with 21.6Gy.

Results: 4DCT confirmed 1cm of diaphragmatic motion, with minimal motion of the posterior and upper mediastinal target volumes. Posterior beam arrangement mitigated uncertainties from chest wall motion. Interfraction cone-beam CT confirmed negligible change in dose distribution from soft tissue variability during the radiotherapy course. Comparison plans revealed significant dosimetric advantages to proton RT over photons, with lung V5 (50% vs. 99%) and V20 (15% vs. 26%), and mean heart dose (3.2Gy vs. 9.8Gy) and V5 (22% vs. 98%), all superior with protons vs. photons. Patient volume receiving >2Gy, a measure of integral dose and indirect correlate of secondary malignancy risk, was reduced by a factor of 2.8 with proton RT.

Conclusion: This case illustrates the superior dosimetry of PBS proton vs. photon RT for the treatment of posterior thoracic high-risk neuroblastoma and demonstrates practical methods for mitigating common uncertainties in treatment planning for thoracic tumors. Proton RT can protect the heart and lung from unnecessary radiation exposure and reduce the integral dose to patients. These clear dosimetric advantages warrant the consideration of protons when evaluating radiation treatment options for thoracic neuroblastoma.

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Meta-acetyl Benzylguanidine (MABG) Nanoparticles for High Risk Neuroblastoma

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As an alternative to 131I-MIBG, a nanoparticle (NP) delivery system that uses an MIBG analog to target the norepinephrine transporter (NET) protein on neuroblastoma (NB) has been developed. The nanoparticle device eliminates the use of radiolabeled iodine-131, with synthesis of pegylated nanoparticles loaded with chemotherapy for the treatment of NB now possible.

Methods: Meta-acetylbenzylguanidine (MABG) was synthesized and characterized using 1H-NMR, 13C-NMR and mass spectroscopy. The targeting specificity of MABG to NET proteins was measured by chemically conjugating MABG with a fluorescently labeled poly-ethylene glycol (PEG) molecule, incubating the molecule with a NB cell line (SK-N-BE (2)), and quantifying uptake with flow cytometry. Multi-compartmental nanoparticles were then generated from a biodegradable synthetic polymer poly (lactic-co-glycolic acid) (PLGA) using electrohydrodynamic (EDH) co-jetting. PLGA-Alkyne nanoparticles were PEGylated (to reduce immunogenicity) and subsequently conjugated with MABG using sequential click-chemistry reactions, resulting in MABG-NPs. The particles were characterized using dynamic light scattering and scanning electron microscopy. In vitro studies were performed to examine the specificity for MABG-NPs to target NET. Two NB cells lines (SK-N-BE (2) and SH-SY5Y) were exposed to either a NET competitive inhibitor (desipramine), a NET upregulator (vorinostat) at varying concentrations, or neither agent (control), with NET expression measured by quantitative Western blot. Confocal microscopy and flow cytometry was used to localize uptake of fluorescently tagged MABG nanoparticles within tumor cells and quantify uptake efficiencies. MABG-NPs were then loaded with irinotecan and cytotoxicity assays performed.

Results: A yield of 85% was achieved in the synthesis of MABG. It was found that MABG was active when conjugated to linear-PEG molecules and was specifically uptaken by NB cells as measured by flow cytometry. 150 nm MABG-NPs were successfully synthesized and were found to be uptaken by MYCN amplified and non-amplified cells, at a rate related to their expression of NET. Last, it was found that irinotecan loaded MABG-NPs showed higher toxicity towards NB cells, compared to their untargeted controls.

Conclusion: The ability to synthesize non-radiolabeled MABG nanoparticles has been developed, with targeting of NET proteins on tumor cell lines now shown. In vivo studies are in progress, based upon this novel technology.

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Targeted Next Generation Sequencing of High-Risk Neuroblastoma Tumours

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Background: Using a cohort of 200 DNAs from high-risk neuroblastoma tumours and cell lines, we investigated the applicability of targeted next generation sequencing (NGS) as an adjunct to our neuroblastoma reference centre genomic analyses.

Methods: A custom Neuroblastoma Illumina Truseq amplicon kit was designed. The genes selected for investigation were chosen in collaboration with the SIOPEN Biology group (London, 2015). The exonic regions of a panel of 38 genes were targeted:- NRAS, MDM4, MYCN, ALK, IDH1, PIK3CA, PDGFRA, TERT, FGFR4, CDK6, BRAF, FGFR1, MYC, CDKN2A, CDKN2B, PTCH1, TSC1, PTPRD, PTEN, HRAS, CCND1, ATM, KRAS, CDK4, MDM2, PTPN11, MAP2K1, TP53, NF1, ERBB2, MAP2K2, ATRX, ARID1A, ARID1B, PDE6G, TENM3, MAP3K13 and PHOX2B. Sequencing was performed on the Illumina NextSeq 550 with subsequent alignment of reads using the Illumina Truseq amplicon application v2.0. Tumour DNAs were drawn from our neuroblastoma national reference centre archive, and had been previously characterised by MYCN FISH and either MLPA, oligo-array CGH or SNP array. ALK mutation status was known from Sanger sequencing for 137 tumours. Twenty-two neuroblastoma cell lines with known mutations in TP53, ALK, RAS-MAPK pathways were included as further positive controls.

Results: Twenty mutations affecting 7 genes known to be present in the 22 cell lines were all confirmed by targeted NGS. 181 patient tumour DNAs have been fully analysed by targeted NGS. 40/181 (22%) mutations considered to be clinically actionable were detected. ALK was the most frequently mutated gene, 17/40 (43%) of mutations (9%) of all cases. In 15/17 cases ALK mutations identified previously by Sanger sequencing were confirmed by NGS. Other genes harbouring mutations in this cohort in ≥ 2 cases were ATRX (4 cases), BRAF (3 cases), KRAS, NF1, PHOX2B, ARID1A/B, FGFR1, ATM, FGFR4 (all 2 cases) and MAP2K1, FGFR4, TP53, CDKN2A (all 1 case). Potentially actionable mutations were confirmed by Sanger sequencing and reported to referring clinicians. In 3 cases NGS identified germ line mutations and referral to a clinical geneticist was made.

Conclusions: Our experience indicates that the targeted NGS panel could be implemented as a primary screen for ALK mutations and will reveal further biologically significant and/or actionable mutations.

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Complete Clinical Response of Metastatic High-Risk Neuroblastoma to ALK-Inhibitor Monotherapy Targeting the Novel Activating ALK-I1171T Mutation

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Background: Precision medicine requires understanding of complex molecular mechanisms. Activating Anaplastic Lymphoma Kinase (ALK)-aberrations are important genetic factors in neuroblastoma development and aggressiveness and have been suggested for novel targeted therapies. Whereas tumors with fusion rearrangements, such as non-small cell lung cancer and anaplastic large cell lymphoma, are highly sensitive to ALK tyrosine kinase inhibitors (TKIs), response to monotherapy with the ALK TKI crizotinib has been disappointing in neuroblastoma with ALK mutations. DNA repair defects such as Fanconi anemia (FA) may induce embryonal tumors, often with poor prognosis due to a lack of therapeutic options.

Design/Methods: A 15-month-old patient with 11q-deleted HR-neuroblastoma with multiple bone and intracranial metastases, was started on COJEC-induction with early severe life-threatening multi-organ toxicity due to underlying FA confirmed with chromosome-breakage analysis and germline FANCA-mutations (c.2728C>T/c.4161-2A>C). Subsequently tumor sequencing revealed a novel somatic neuroblastoma ALK(c.3215T>C; I1171T) mutation. In search for therapeutic opportunities the biological nature of this ALK-mutation was characterized.

Results: We show here that ALK-I1171T generates a potent gain-of-function mutant, as measured in PC12 cell neurite outgrowth and NIH3T3 transformation. Pharmacological inhibition profiling of ALK-I1171T in response to various ALK TKIs identified an 11-fold improved inhibition of ALK-I1171T with ceritinib when compared with crizotinib. Immunoaffinity-coupled LC-MS/MS phosphoproteomics analysis indicated a decrease in ALK signaling in response to ceritinib. Based on these analyses, ceritinib was selected for therapeutic use in this child.

Targeted treatment with ceritinib/LDK378 (450mg/m2-540mg/m2) was started as compassionate use. Elevated urinary catecholamine-markers were normalized after the first 4-week-treatment cycle. Hemoglobin normalized, and platelets improved to stable levels (>100). Ceritinib was well tolerated and resulted in significant tumor shrinkage. After 7.5 months of treatment, residual primary tumor was surgically removed and exhibited hallmarks of differentiation together with reduced Ki67 levels. Clinical follow-up after 21 months of ceritinib monotherapy revealed complete clinical remission (incl MRI/CT/MIBG/BoneMarrow/Catecholamines) still maintained for 3 years.

Conclusions: Here we show that the novel ALK-I1171T mutation is activating and promotes neuroblastoma aggressiveness. Monotherapy with ALK-inhibitor ceritinib induced complete response in a child with metastatic high-risk neuroblastoma unable to receive conventional treatment. Therefore, ceritinib presents a viable therapeutic option for ALK-positive neuroblastoma.

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Impact of Immune Cell Profiles on High-Risk Neuroblastoma Treated with A COG Phase III Randomized Immunotherapy Study

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Background: Dinutuximab mediates antibody-dependent cellular cytotoxicity (ADCC) by neutrophils and natural killer (NK) cells via interactions with Fc receptors (FcRs). We set out to determine the ADCC activity, immune cell populations and FcR genotypes, and their correlation with outcome of 223 patients randomized to isotretinoin +/- immunotherapy on COGANBL0032 and 25 patients with biopsy proven residual disease non-randomly assigned to isotretinoin + immunotherapy.

Methods: Blood samples were obtained before and during courses 1, 4 and 5 of immunotherapy, or at days -1, 150 and end of treatment for isotretinoin only cohort. Peripheral Blood Mononuclear cells (PBMCs) and neutrophils were isolated for ADCC, NK populations and FcR genotype determination.

Results: A total of 110 patients were randomized to isotretinoin and 113 to isotretinoin + immunotherapy. On both regimens, there was a significant increase in lymphocyte population over time, mainly due to a significant rise in the CD3+ /CD56- T cells in isotretinoin patients and increases in various subsets in immunotherapy patients, despite course-associated transient decline of most cell populations. Pretreatment levels of three NK cell subsets, NKp44+, KIR+ and KLRB1+ alone and together showed strong correlation with EFS ($p<0.01$) and a trend with OS ($p= 0.07$) for immunotherapy group, but not isotretinoin. Pretreatment ADCC levels were significantly lower than that observed in eight normal donors for both PBMCs (41.6±33.9 vs. 130.0±69.0 Lytic Units (LU20), $p=0.0004$) and neutrophils (18.2±14.0 vs. 25.3±8.5 LU20, $p=0.019$). ADCC of PBMCs decreased significantly during both course 1 and 5 of immunotherapy ($p<0.05$) but recovered to pretreatment levels before subsequent courses. Neutrophil peak ADCC was unchanged during courses 1 and 5 but increased during course 4 ($p=0.0019$). ADCC of both PBMCs and neutrophils remained unchanged in isotretinoin only patients. There was no association of ADCC with EFS or OS at any of the measured time points.

FcγR genotypes failed to show any associations with EFS and OS for patients randomized to either regimen. Similarly, no association of genotype and ADCC was observed.

Conclusion: This study characterizes the state of the immune system during immunotherapy and identifies three NK markers that may serve as potential biomarkers predictive of outcome.

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Pharmacological Blockade of MYCN In Neuroblastoma Using Orally-Bioavailable CDK Inhibitors Reveals an Approach Widely Applicable to Myc-Dependent Cancers

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Neuroblastoma (NB) is a paediatric tumor wherein amplification of the oncogenic basic helix-loop-helix (bHLH) transcription factor (TF) MYCN confers clinical and biologic features prototypical of Myc-dependent cancers. TF-dependent cancers like MYCN-amplified NB are difficult to target, but the availability of clinical-candidate transcription inhibitors makes selective blockade of oncogenic TF activity a possibility. Here we investigated whether NB could be controlled via dual targeting of the transcription elongation machinery and cell cycle progression, using an orally bioavailable and selective CDK9/2 inhibitor CYC065. CYC065 blocks nascent transcription and leads to a drastic reduction in steady-state levels of short-lived transcripts. In MYCN-amplified NB, CYC065 rapidly and completely eliminated MYCN mRNA and protein, terminating expression of a MYCN-dependent gene expression programme. This result is phenocopied by multiple clinical-candidate CDK9 chemical inhibitors and genetic manipulation of CDK9. Mechanistically, CYC065 dissociates MYCN from physical proximity of P-TEFb in cells. P-TEFb co-occupies promoters and enhancers of de-differentiating pathway genes highly-occupied by MYCN, and CDK9 inhibition terminates expression of these and other highly transcribed genes. CYC065 targeted MYCN-driven neuroblastoma in vivo, resulted in tumor regression or eradication and prolonged survival in multiple NB models, and was effective against a wide range of Myc-dependent cancer cells lines in vitro. The data establish that combined CDK9/CDK2 inhibition blocks transcriptional dependence induced by MYCN, highlighting a potential clinical strategy by which many Myc-driven cancers could be targeted.

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Cytokine Profile and Association with Toxicities and Outcome of High-Risk Neuroblastoma Treated with Dinutuximab Immunotherapy on COG ANBL0931

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Background: Immunotherapy using dinutuximab, a chimeric anti-GD2 antibody, combined with IL2 and GM-CSF has become standard maintenance therapy for high-risk neuroblastoma. This study aimed to assess the cytokines profile of patients treated with immunotherapy on COGANBL0931 to determine their association with toxicities and patient survival.

Methods: Serum samples were collected before starting immunotherapy and just before the fourth doses of ch14.18 infusion on courses 1 (days -1 & 6) and 4 (days 80 & 90) for determination of cytokines by ELISA and their association with reported toxicities and outcome.

Results: Of 105 patients enrolled, the 3-year event-free survival and overall survival were 67.6±4.8% and 79.1±4.2%, respectively. The most common Grade ≥3 toxicities for cycles 1-5, respectively, were neuropathic pain (41%, 28%, 22%, 31%, 24%), hypotension (10%, 17%, 4%, 14%, 8%), allergic reactions (3%, 10%, 5%, 7%, 2%), capillary leak syndrome (1%, 4%, 0%, 2%, 0%), and fever (21%, 59%, 6%, 32%, 5%). After correcting for multiple comparisons, the average levels of 9 of 13 cytokines on course 1, and 11 of 13 on course 4 were highly significantly elevated compared to the pre-treatment values (all $p<0.001$). IL-5 exhibited the most dramatic rise, while IL12p70 and IL-8 levels did not change significantly. 1L-1Ra (2043.6 vs 2522.3 pg/ml, $p=0.02$) and IFNγ (35.9 vs.73.8 pg/ml, $p=0.02$) were associated with allergic reaction (AR) on course 1 but not course 4. However, several non-AR patients had higher IL-1rα, and higher/lower IFNγ levels at the indicated courses.

Levels of IL-6 and other cytokines at diagnosis prior to initial chemotherapy have been associated with outcome in numerous cancers including neuroblastoma. However, among the 15 analytes measured in two courses of maintenance immunotherapy, only CXCL9 at day -1 showed an uncorrected association with EFS ($p=0.05$). No association with OS was observed nor was there any outcome association with changes in levels of any of the analytes including IL-6 during course 1 or course 4.

Conclusion: This study documented the dramatic increases in cytokine release during immunotherapy, demonstrated the association of 1L-1Ra and IFNγ with AR during course 1, and implicated the pretreatment chemokine CXCL9 as prognostic.

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Targeting Oncogenic ODC1 with Difluoromethylornithine (DFMO) Inhibits Global Protein Translation and Has Anti-Tumor Activity in Neuroblastoma

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Background: Deregulated MYC links cell cycle with biomass creation to drive oncogenesis. Augmented protein synthesis is paramount and polyamine biosynthesis, coordinately regulated by MYC, is essential for oncogenic translation. ODC1 encodes Ornithine decarboxylase, a MYC target that is rate-limiting for polyamine synthesis and itself an amplified oncogene in neuroblastoma. DFMO is an irreversible Odc inhibitor that inhibits tumor progression in diverse murine neuroblastoma models. Here we focus on protein synthesis inhibition as its predominant anti-tumor mechanism and seek predictive biomarkers and required exposures for activity.

Methods and Results: ODC1 co-amplification occurs in 13% (33/256) of MYCN-amplified neuroblastomas and is associated with exceptionally poor outcome. We studied protein translation in neuroblastomas with MYCN/ODC1 co-amplification (10-15% of high-risk tumors), MYCN amplification alone (~25%), or neither (~60%). Basal global protein translation (puromycin incorporation) was variable and did not correlate with genetic subtype. DFMO reduced protein translation 25-75%, with inhibition trending with MYCN/ODC1 signalling. We show DFMO inactivates a principal translation factor, eIF5A (measured by hypusine-specific immunoblot and isoelectric-focus), at exposures >300μM in cell lines of all genetic subtype, but has no effect on 4EBP1-phosphorylation, a regulator of eIF4E and cap-dependent translation. In contrast, the ATP-competitive mTORC inhibitor, MLN0128, does not inactivate eIF5a but inhibits 4EBP1-phosphorylation, as predicted. Dissection of protein translation effects of DFMO and alternative translation inhibitors, and their impact on clonogenicity and synergy, support this as a key anti-tumor mechanism. Lower concentrations of DFMO failed to inactivate eIF5A or inhibit protein translation. PK data from our preclinical murine trials, and the NANT N1201 human Phase 1 DFMO trial, support higher dosing of DFMO to achieve protein inhibitory effects for anti-tumor activity (>5gm/m2/day). Synergy studies with DFMO and complementary translation inhibitors are ongoing. eIF5A also has an essential role in resolving translation through polyproline motifs and bioinformatic studies to define the eIF5A-dependent translatome, and its polyamine dependency, are ongoing.

Conclusions: DFMO depletes polyamines to attenuate protein translation, an oncogenic output of deregulated MYC. We present data evaluating MYC-ODC-protein signalling in neuroblastoma, with evidence that higher DFMO exposures are required to inactivate eIF5A, which correlates with anti-tumor activity.

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Single Cell RNA Sequencing of Neuroblastoma

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Background: Neuroblastoma (NB) displays important clinical and genetic heterogeneity, at tumor progression and differentiation. Single cell RNA-sequencing (scRNA-seq) can allow simultaneous measurement of global transcriptional profile from single cells.

Methods: To study heterogeneity of NB cell differentiation during ATRA treatment, an innovative method based on scRNA-seq by next generation sequencing (NGS) was performed in NB cell lines. And this method was also performed in primary culture cells derived from 5 NB tumor specimens (1L1, 3M, and 1MS).

Results: The scRNA-seq of SMS-KCNR and SMS-SAN in time-course analysis (1-5 days) under treatment of ATRA revealed the clonal movement after treatment. Those of SK-N-SH and NH-12 showed the same movement but had a small clone remained during treatment. The genes upregulated in the clone moved by the treatment showed up-regulation of retinol metabolism pathway genes including DHRS4 and CYP26A1, and other correlating genes such as NROB1 and SPP1. However, the cells in the remaining clone showed the low expression of these genes. The scRNA-seq in the cell samples derived from clinical sample revealed that L1 and MS cases consisted of the cells with the up-regulation of retinoic acid pathway genes but 3 of M cases included the cells similar to the remaining clone cells of SK-N-SH and NH-12.

Conclusion: Single cell analysis with transcriptome show the existence of undifferentiated small clone containing in the whole cells. This undifferentiated cell might be correlated with chemo-resistance or risk of recurrence, consequently correlated with unfavorable outcome. Single cell analysis might be useful to detect small subset of the undifferentiated or refractory cells in neuroblastoma.

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Efficacy of Targeted Radiotherapy with Meta-[211At]astatobenzylguanidine ([211At]MABG) in Preclinical Models of High-Risk Neuroblastoma

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Background: The alpha particle emitting radiotherapeutic [^{211}At]MABG theoretically has superior radiobiological properties for anti-tumor efficacy compared to the currently used agent ([^{131}I]MIBG). Specifically, [^{131}I]MIBG does not target microscopic disseminated disease due to the long path length of the beta energy as opposed to alpha particles which cause dense ionizations leading to double strand breaks and enhanced lethality. Here we sought to define the toxicity and anti-tumor activity of [^{211}At]MABG in cellular and murine models.

Methods: ^{211}At was synthesized using a bismuth target via the 209Bi(α,2n)^{211}At reaction and used for solid-phase radiosynthesis of [^{211}At]MABG. We performed uptake, cytotoxicity and biodistribution studies using [^{131}I]MIBG and [^{211}At]MABG in NET transfected models and extrapolated human dosimetry. Additionally, dose escalation studies with [^{211}At]MABG (n=10 at each dose, range 10-100 μCi) were performed to determine toxicity in SCID mice with periodic laboratory parameters evaluation and histopathological assessment at 6 months post injection. Therapeutic in vivo trials were conducted with NET transfected xenografts and patient derived xenograft (PDX) models injected intravenously with escalating doses of [^{211}At]MABG, [^{131}I]MIBG or vehicle (n=10 respectively).

Results: ^{211}At was produced with optimized distillation yields of 70% (99.9% radionuclide purity) followed by synthesis of up to 3 mCi of [^{211}At]MABG. Intravenous [^{211}At]MABG was well tolerated in murine SCID models at a dose of 10 and 25 μCi except for transient thrombocytopenia (nadir at 6 weeks; p=0.001 and p=0.0005 respectively), while doses higher than 50 μCi caused significant weight loss. [^{211}At]MABG was potently cytotoxic in six NET expressing neuroblastoma cell line models (EC50's ranged from 0.0006-0.1 μCi/ml compared to 0.25-46 μCi/ml with [^{131}I]MIBG). Single dose therapeutic trials showed significant regression of established NET overexpressing SKNSH xenograft and COG-N-453x PDX models (p<0.0001) comparable to single dose [^{131}I]MIBG therapy.

Conclusions: The uptake and biodistribution of [^{211}At]MABG is similar to [^{131}I]MIBG and there was no unanticipated toxicity. [^{211}At]MABG is more potent than [^{131}I]MIBG in vitro and [^{211}At]MABG shows significant activity in bulky xenograft models, remarkable due to the several log difference in half-life (7.2 hr for [^{211}At]MABG, 8.04 days for [^{131}I]MIBG). Additional toxicity and efficacy studies focused on fractionated dosing will be presented.

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GD2 Specific Murine CAR T-Cells Show Efficacy in Immunocompetent TH-MYCN Neuroblastoma Murine Model

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Background: Immunotherapy with chimeric antigen receptor (CAR) T-cells has shown promising results in the setting of leukemia, but results of CAR T-cell clinical trials in solid tumors including neuroblastoma (NBL) have been disappointing. This failure is despite the preclinical success of CAR T-cells against xenotransplanted solid tumors in immunodeficient animals and suggests utilization of immunocompetent animal models are needed to understand the biologic complexity better.

Methods: Second generation GD2-28Z CAR T-cells were generated using 14G2a ScFv linked to murine CD28 followed by CD3 Zeta. GD2-BBZ (2nd generation) and GD2-28BBZ (3rd generation) utilizing 4-1BB alone or in addition to CD28 were also constructed. In vitro cytotoxicity was analyzed against TH-MYCN driven GD2+ NBL cell lines using chromium-51 release assay and CD107a mobilization by flow cytometry. Homozygous TH-MYCN mice received 1x107 CAR T-cells following low dose lymphodepletive chemotherapy.

Results: Naïve mouse T-cells were successfully expanded with CD3/28 beads in the presence of IL2+IL7 and were efficiently transduced (range: 50-70%) with a retroviral carrier. There was no difference in transduction efficiency among the three CAR constructs. CD107a mobilization assay demonstrated GD2-CAR-T cell activity with all the constructs against TH-MYCN cell line. The three CAR T-cells also showed significant cytotoxicity against human and mouse neuroblastoma cells lines, with slightly better cytotoxicity observed with GD2-28Z CAR T-cells. TH-MYCN homozygous mice treated with 2nd and 3rd generation GD2 CAR-T cells showed an equal and statistically significant increase in survival in comparison to mock-transduced T-cells. However, the anti-tumor effect of the CAR T-cells was transient, and tumors eventually regrew. Analyses of harvested tumors revealed the increased presence of T-cells within the tumor periphery, the perivascular region, and foci of necrosis suggestive of partial anti-tumor effect.

Conclusions: We have successfully generated and optimized production of fully murine GD2-28Z, GD2-BBZ, and GD2-28BBZ CARs. Functional studies validated the efficacy of these CARs in the transgenic TH-MYCN model and demonstrated a partial anti-tumor effect. Our CAR T-cells provide the opportunity to investigate their properties in an immunocompetent model and assess the impact of NBL tumor microenvironment on their function.

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Selective High Affinity MYC-Binding Compound Inhibits MYC: MAX Interaction and MYC-Dependent Tumor Cell Growth

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The MYC family oncoproteins/transcription factors MYC and MYCN are key players in childhood tumor development, including neuroblastoma, but no specific MYC-targeting drugs are clinically available at present. Development of effective and selective MYC inhibitors is therefore much warranted. MYC and MYCN are strictly dependent on heterodimerization with MAX for activation of target genes. Targeting MYC:MAX interactions is therefore a conceivable approach to target MYC. In a cell-based Bimolecular Fluorescence Complementation protein interaction screen for small inhibitory molecules we identified MYCMI-6, which exhibits strong selective inhibition of MYC:MAX and MYCN:MAX interaction in cells and in vitro as validated by split Gaussia luciferase, in situ proximity ligation, microscale thermophoresis (MST) and surface plasmon resonance (SPR) assays. Further, it blocks MYC-driven transcription and binds selectively to the MYC bHLHZip domain with a KD of 1.6 µM as demonstrated by MST and SPR. MYCMI-6 inhibits tumor cell growth with high efficacy in a MYC/MYCN-dependent manner with IC50 concentrations as low as 0.5 µM. MYCMI-6 cell growth inhibition correlates with MYC/MYCN expression based on data from 70 human tumor cell lines, including neuroblastoma, where it discriminates well between MYCN-amplified and non-amplified cell lines. Further, it is not cytotoxic to normal cells. Importantly, in a mouse model of MYCN-amplified neuroblastoma, MYCMI-6 inhibits MYCN:MAX interactions and tumor cell proliferation as well as induces massive apoptosis in tumor tissue without causing severe side effects in the mice. Since MYCMI-6 inhibits MYC:MAX interactions without affecting MYC expression it is a unique molecular tool to specifically target MYC:MAX pharmacologically and it has good potential for drug development.

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Elevated Minimal Residual Disease Marker Expression in Peripheral Blood of Three High−Risk Neuroblastoma Cases

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Methods: To sensitively detect 7 MRD marker (CRMP1, DBH, DDC, GAP43, ISL1, PHOX2B, TH) expression in bone marrow (BM) and peripheral blood (PB) of high−risk neuroblastoma cases, we determined these expression in BM and PB samples taken form 10 healthy adult volunteers by droplet digital PCR (ddPCR) and set the cut-off value as Mean + 3SD for each MRD marker.

Patients and Results: Three patients #1, #2, and #3 were a 6-year old girl, 17-month old girl, and 2-year old boy at the time of diagnosis, respectively. They were diagnosed with stage 4 high-risk neuroblastoma and treated according to the Japan Children's Cancer Group (JCCG) Neuroblastoma Committee (JNBSG) protocol at Kobe Children's Hospital, Japan. 7 MRD marker expression in their BM and PB samples was consecutively monitored by ddPCR as frequently as possible. All of them were initially responded to the induction therapy and reached all 7 MRD marker-negative status. However, patient #1, #2, and #3 became 1, 4, and 2 MRD marker-positive status in PB samples at 5, 3, and 3 months before clinical diagnosis of tumor relapse, respectively.

Conclusion: The present cases may highlight the possibility of 7 MRD marker expression measured by ddPCR to detect the relapse-originating MRD in high-risk neuroblastoma cases.

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Telomere Biology Including Rearrangement of TERT Promoter Lesion in Neuroblastoma

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Background: Neuroblastoma (NBL) shows remarkable biological heterogeneity resulting favorable or unfavorable prognosis. We have reported telomere biology in NBL: telomerase activation and alternative lengthening of telomere (ALT) due to ATRX/DTXX mutations are landmarks for aggressive growth in unfavorable tumors, respectively. Recently, genomic rearrangements at 5p15.33 proximal of the telomerase reverse transcriptase gene (TERT) were found in some group of unfavorable NBLs. Therefore, we analyzed rearrangement in the proximal lesion of TERT in our NBL samples whose telomere biology was already analyzed.

Methods: In 121 NBLs, including 67 cases detected by mass-screening whose telomere length, telomerase activity, ALT with ATRX/DAXX alterations, and MYCN amplification were already known, TERT rearrangements were examined using FISH and next generation sequencing (NGS). The probes of FISH were designed at up and down loci of TERT gene.

Results: The 11 ATRX/DAXX mutated ALT cases and 29 cases with high telomerase activity showed poorer prognosis than other cases. In the cases with high telomerase activity, MYCN amplification and TERT rearrangements were independently detected in 16 and 13 cases, respectively. NGS revealed the translocation of the proximal lesion of TERT. In 81 infant cases, MYCN amplification, TERT rearrangements and ATRX mutations were detected in 3, 4, and 3 cases, respectively. In these 10 cases, 6 cases showed progression or recurrences.

Conclusion: In NBLs, the rearrangement of TERT proximal region and MYC amplification might independently activate TERT expression in unfavorable cases. As TERT is a catalytic component of human telomerase, the stabilization of telomere by ALT or high telomerase activity might be directly correlated with unfavorable tumors. Thus, a better understanding of telomere biology and developing of therapeutic strategies for telomere-stable NBLs may help to improve the outcome of NBL patients.

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Distinct Calcium Signaling in Neuroblastoma Subpopulations Regulates the Response to Antineoplastic Drugs

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Neuroblastoma (NB) the most common extra-cranial pediatric solid tumor in children under 1 year of age. High-risk NB patients have ~40% survival rate, a higher likelihood of developing resistance to treatments, and increased incidence of relapse. Despite the plethora of advanced combination and multimodal treatments available, current treatment strategies are often ineffective with high-risk and aggressive NB due to resistance or lack of response to treatment. In addition, after initial response to treatment, NB relapse occurs in more than 50% of high-risk NB patients, and relapsed tumors are resistant to treatment. Finding effective treatments for drug resistant NB has been a challenge due to the myriad of complex mechanisms that promote drug resistance. In order to address this, our research focuses on elucidating calcium signaling pathways that promote NB drug resistance, and on the identifying ion channels and regulators that may be novel targets for the development of more effective anticancer agents for drug resistant and relapsed NB.

The cancer-calcium signaling nexus has been shown to regulate the response of cancer cells to antineoplastic drugs and cell death. Previous studies and our preliminary data show that calcium signaling, particularly at the ER and mitochondria, plays a critical role in regulating the response of NB cells to therapeutic stress, and excessive ER-mitochondrial calcium signaling results in mitochondrial damage and cell death. Our data shows that key components of the ER-mitochondrial calcium signaling machinery are differentially regulated and significantly altered in drug resistant NB through a mechanism involving autophagosomal degradation and removal of damaged mitochondria (mitophagy) thereby promoting NB cell survival. Interestingly, our data indicates that dose-dependent exposure of patient derived NB cells with chemotherapeutic drugs triggers elevated calcium levels within seconds to minutes. Strikingly, this response is limited to a distinct subpopulation of NB and is diminished in NB cells that have acquired drug resistance. In conclusion, the results suggest that ER-mitochondrial calcium signaling and mitochondrial damage induced-mitophagy regulates the sensitivity of NB cells to therapeutic stress. Further, these novel mechanisms could be therapeutically targeted in order to re-sensitize drug resistant NB to conventional chemotherapeutic treatment regimen.

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Preclinical Model of Gated GD2+B7H3 CAR-T Cell Therapy in Neuroblastoma

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Background: Anti-disialoganglioside (GD2) specific CAR T-cell therapy has shown limited efficacy in children with recurrent/refractory neuroblastoma (NBL). A recent pre-clinical study demonstrated significant neurotoxicity in mice treated with CAR T-cells bearing a high-affinity anti-GD2 short chain variable fragments (scFv; Richman S et al. 2017). To devise a safe and more efficacious CAR T-cells, we developed a gated CAR T-cell system utilizing the Syn-Notch technology.

Methods: GD2 and CD19 specific Syn-Notch gated systems were constructed by linking corresponding scFv to the Notch core followed by Gal4Vp64. Upon target recognition, Gal4VP64-dependent UAS element would initiate expression of the B7H3-BBZ CAR. Reporter activity of CD19 or GD2 Syn-Notch systems were monitored using flow cytometry after exposure to CD19+ transduced CHLA255 NBL cell line. In vitro cytotoxicity was assessed using DIMSCAN and xCELLigence impedance assays at varying Effector: Target ratios. NSG mice with established metastatic CHLA255(GD2+CD19+) NBL were treated with 1x107 transduced and control T cells and monitored for disease burden.

Results and discussion: Jurkat and human primary T cells were successfully transduced with the Syn-Notch system. GD2-Notch-Gal4VP64 and CD19-Notch-Gal4VP64 T-cells effectively activated downstream reporter in exposure to CHLA255(GD2+CD19+) within 6 hours of exposure but not in the presence of NBL cells lacking GD2 or CD19. The GD2 system exhibited greater leakiness than the CD19 counterpart. GD2- Syn-Notch-B7H3-BBZ or CD19- Syn-Notch-B7H3-BBZ CAR T-cells demonstrated statistically significant cytotoxicity against CHLA255(GD2+CD19+) NBL cell line. Preliminary in vivo data also show the anti-tumor effect of these gated CAR systems compared to mock-transduced T-cells.

Conclusions: We have successfully established a novel GD2-B7H3 gated system using the Syn-Notch technology and demonstrate successful specificity to both antigen targets. This system increases the specificity of CAR T-cells against GD2 and B7H3 positive neuroblastoma tumors and likely minimize GD2-induced toxicity. These gated CARs show great promise for establishing immunotherapies that maximize tumor specificity while minimizing toxicity.

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A Multi-Ethnic Genome-Wide Association Study (GWAS) Identifies Multiple Novel Loci Associated with Neuroblastoma

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Introduction: An ongoing genome-wide association study (GWAS) of sporadic neuroblastoma (NB) has identified multiple common and rare variants associated with susceptibility and tumor aggressiveness. However, additional susceptibility loci remain to be discovered and studies to date have focused on cases of European ancestry due to limited power in other ethnicities.

Methods: To identify additional susceptibility loci, we have increased the number of European American's studied (3817 cases, 9974 controls), expanded cohorts to include African American (723 cases, 2249 controls) and Hispanic (593 cases, 275 Controls) populations, and updated the imputation panel to Phase 3 of 1000 Genomes. Each cohort was analyzed independently followed by meta-analysis. Additional replication was performed using United Kingdom (424 cases, 1300 controls) and Italian cohorts (432 cases, 780 controls). An annotation tool incorporating functional genomic, transcription factor binding site, and evolutionary conservation data was applied to infer putative causal variants and data were integrated with matched RNA sequencing (n=94) to perform expression quantitative trait loci (eQTL) analysis.

Results: All previously reported susceptibility loci in European and African Americans were confirmed, and we further replicated associations in the Hispanic cohort at LMO1 (rs2168101: P=2.5e-4; OR:0.71, 95%CI:0.56-0.90) and HACE1 (rs72990858: P=2.5e-2; OR:0.74, 95%CI:0.252-1.04). Additional loci trended toward significance in the same direction. Replication in the African American cohort was also observed at LMO1 (rs2168101: P=1.5e-2; OR:0.70, 95%CI:0.50-0.98). We identified eight new susceptibility loci ($P < 5.0e-7$). The top novel locus was at 6p25.3 (rs12203592: P=3.95e-10; OR:0.823, 95%CI: 0.75-0.90) and represents a multi-tissue eQTL for IRF4 in GTeX (P=1.91e-9). Moreover, the risk allele at rs12203592 was associated with increased expression of IRF4 (P=0.0167) and worse overall survival (P=3.9e-7). Several additional loci clustered on the short arm of chromosome 1 at 1p36.22 (rs2039176: P=1.05e-7), 1p36.23 (rs2039176: P=1.05e-7) and 1p32.1 (rs12068196: P=8.52e-7), a region frequently deleted in NB tumors.

Conclusions: These data refine and extend previously reported susceptibility loci and identify eight new regions, including chromosome 6p25.3 implicating IRF4 and a set of independently associated susceptibility loci clustered at chromosome 1p36-32. Additional replication in UK and Italian cohorts and integration with tumor DNA and RNA sequencing is ongoing.

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The Effect of 131I-mIBG-Therapy on The SIOPEN And the Curie Score

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Background: 131-I-mIBG-therapy is an established treatment option in high-risk neuroblastoma. Its effect on the SIOPEN and Curie score has not been investigated so far.

Methods: 121 mIBG scans of 48 patients that have received 131-I-mIBG therapy at our center between 2004 and 2017 were scored according to SIOPEN and modified Curie score before (t1; n=48), during (t2; n=43) and 3–6 months after (t3; n=30) mIBG-therapy. The effect of mIBG-therapy on the scores was analyzed and correlated to risk factors and clinical features.

Results: At t1, SIOPEN score was 0 in 15 patients while Curie score was 0 in 6 patients, indicating 9 patients with soft tissue metastases only. Five patients had mIBG-positive primary tumor only and 1 patient received mIBG-therapy without unequivocally mIBG-positive lesions.

After exclusion of scans with a score = 0, median SIOPEN score was 13 at t1 [range 1 - 52], 16 at t2 [range 1 - 56] and 8.5 at t3 [range 1 - 54]. For the same cohort Curie scores were 5.5 [t1; range 1 - 24], 5 [t2; range 1 - 25] and 4 [t3; range 1 - 26] respectively.

Of 43 patients with available scans at both time points, mIBG score was higher at t2 than at t1 in 20 (SIOPEN; median 27 vs. 18; $p<0.001$) and 21 (Curie; median 12 vs. 8; $p<0.001$) patients respectively. However, none out of 12 patients with a pre-treatment SIOPEN score = 0 and only one out of 4 patients with a pre-treatment Curie score = 0 showed a score > 0 in the treatment scan.
Of 30 patients with available mIBG-scans at t3, reduction of the mIBG-score was seen in 10 patients (SIOPEN; median 16 vs. 4.5; $p=0.005$) and 14 patients (Curie; median 7 vs 0.5; $p=0.001$) respectively and was neither correlated with status of MYCN or 1p nor with age at initial diagnosis or at mIBG-therapy nor with mIBG-score at t1 nor with concomitant therapy.

Conclusion: Only 33 – 50% of patients show reduction of mIBG-scores 3 to 6 months after mIBG-therapy. Patients with negative diagnostic mIBG-scans are unlikely to show metastases in treatment scans.

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Rare Chromosome 16p11.2 Microdeletions Associated with Neuroblastoma Susceptibility

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Background: Although much progress has been made in understanding the contribution of common variation to neuroblastoma susceptibility, rare variants remain understudied.

Methods: To identify rare variants influencing neuroblastoma susceptibility, we conducted a genome-wide analysis of large (>500-kb), rare (<1% in controls) copy number variants (CNVs) using single nucleotide polymorphism (SNP) arrays from a multi-ethnic cohort of 3,309 neuroblastoma patients and 8,855 cancer-free controls.

Results: We identified a recurrent microdeletion on chromosome 16p11.2 enriched in neuroblastoma patients (p=8.3x10-6, Fisher's Exact Test) and replicated this association in an independent cohort of 2,276 patients and 1,437 controls (p=8.7x10-3). The deletion was extremely rare, affecting 0.4% of cases and 0.02% of controls, but carried a high odds ratio of 15.4 (95% confidence interval: 4.1–57.9). It was also more prevalent in low-risk neuroblastoma patients; in particular, tumors from all 21 clinically-annotated microdeletion patients lacked amplification of the oncogene MYCN (p=0.022, Fisher's Exact Test). For one patient, the deletion was validated by whole-genome sequencing and fine-mapped to a minimal region of 552 kb which contains 28 protein-coding genes and is flanked by segmental duplications. The microdeletion will be further validated, and heritability established using whole-genome sequencing for two parent-child triads and two single parent-child dyads.

Conclusions: Rare microdeletions at chromosome 16p11.2 associate with neuroblastoma. Copy number variation at this locus has previously been implicated in autism spectrum disorder (ASD), schizophrenia, and other neurodevelopmental disorders. In ASD, deletion and duplication exhibit reciprocal growth phenotypes: deletion associates with macrocephaly and obesity, while duplication associates with microcephaly and decreased body weight. This suggests that this region may function in the regulation of proliferative and metabolic pathways; nevertheless, this is the first report implicating the CNV in cancer. This discovery highlights the importance of rare germline variation in neuroblastoma and reveals a potential role for this unstable region on 16p11.2 in neurodevelopmental pathways common to both autism and neuroblastoma.

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PI3K/AKT/mTOR Pathway Expression in Neuroblastoma - Immunohistochemical Analysis and Correlation with Patients' Survival

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The goal of the current study was to analyze the status of PI3K/AKT/mTOR signaling pathway in the primary tumor tissue samples from 103 neuroblastic tumors.

Material and Methods: The clinical data included: patients' age, tumor stage, primary tumor localization, tumor histology and NMYC status. Patients' age ranged from 1 to 169 months (median 30 months). A tissue microarray consisted of 1-2 representative 0.5mm cores from formalin-fixed, paraffin-embedded tissue blocks from 103 neuroblastic tumors. Sections were stained with monoclonal antibodies against PTEN, PI3K85, PI3K110, p-Akt (Ser-473), p-MTOR (Ser-2481), p-70S6K (Ser 235/6) and p-4EBP1 (Cell Signaling Technology, Beverly, MA). Immunoreactivity of examined proteins was assessed using semi-quantitative scale (negative, low, high).

For univariate analysis, the Chi-square test and Mann Whitney test were used to compare categorical and continuous variables, respectively. Patients' survival was calculated with the Kaplan and Meier method and multivariate Cox proportional hazard analysis.

Results: High pAKT immunoreactivity in tumor tissue significantly associated with worse patients' survival ($p<0.001$), while lack of pAKT expression was associated with the best prognosis (10-year survival 0% vs. 95%, respectively). Similarly, high expression of the mTOR target, p70S6K, significantly correlated with poor clinical outcome (45% vs. 58% 10-year survival for patients with high and low levels, respectively). A trend toward decreased survival was also observed in patients with high expression of another mTOR effector, p4EBP-1, although this difference did not achieve statistical significance ($p=0.09$). No statistically significant effects of changes in other tested protein expression on patients' survival was observed. Multivariate Cox analysis revealed statistically significant differences ($p<0.001$). However, among analyzed clinical correlates, NMYC status had significant independent, and p70S6K, stage and age showed a tendency for significant effect on patients' survival.

Conclusions: Our results indicate frequent activation of the PI3K/AKT/mTOR pathway in neuroblastic tumors. High pAKT and p70S6K immunoreactivity was associated with adverse prognosis. However, in multivariant analysis only p70S6K expression exhibited a trend toward independent correlation with patients' survival.

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Increased m6A mRNA Methylation Promotes Neuroblastoma Aggressiveness

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Neuroblastoma (NB) is a tumor of the developing neural crest and a devastating cause of children's' death. The elusive nature of the disease and the embryonic derivation of NB progenitor cells may account for a marked involvement of post-transcriptional control of gene expression to maintain the undifferentiated state and promote rapid progression to oncogenesis.

In this context, we are focused on understanding the importance of N6-methyladenosine (m6A), the most abundant covalent modification in mRNAs, in NB onset and progression.

From data collected from NB patients, we found that the expression of METTL14, a component of the methyltransferase complex, is specifically higher in NB cells compared to other solid-tumor cell types. Furthermore, it correlates with MYCN (the most potent positive determinant of NB aggressiveness) expression and its levels directly associate with worse clinical features and poor prognosis of NB patients. Coherently, expression of the m6A demethylase ALKBH5 inversely correlates with clinical features of aggressiveness and it is virtually absent in NB cell lines.

When overexpressed in NB cells, METTL14 increases cellular proliferation, invasivity, and clonogenicity, while its depletion induces a reversal of these phenotypes. ALKBH5 rescue behaves as METTL14 depletion. When xenografted into athymic nude mice, tumors generated by METTL14 overexpressing cells have a significantly higher growth rate and dimension than tumors produced by control cells, while tumors made by ALKBH5 rescuing cells have a significant reduction in the growth rate and size.

We finally performed a comprehensive analysis of mRNA dynamics in the engineered cell lines, including steady-state levels by RNAseq, mRNA stability by SLAMseq, translation efficiency by ribosome profiling, and we compared these features with a base-resolution m6A map obtained by methylation-iCLIP. We were able in this way to identify and validate downstream mRNA targets of METTL14 and ALKBH5 and how increased methylation affect their dynamics, promoting tumor progression.

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Early Use of Rituximab and Plasmapheresis for the Treatment of Opsoclonus-Myoclonus Syndrome

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Background: Traditional treatment approaches for pediatric opsoclonus-myoclonus syndrome (OMS) with long-term corticosteroids and IVIgG are associated with a high rate of motor and neurocognitive sequelae. Benefit from plasmapheresis has been demonstrated in the treatment of various neuroinflammatory disorders, but evidence for use in OMS is limited. Emerging evidence also suggests rituximab may improve clinical outcomes in OMS. The goal of this study was to evaluate clinical outcomes in a series of children with OMS with and without neuroblastoma treated using a protocol of acute intensive immunotherapy including plasmapheresis followed by rituximab.

Design/Methods: A retrospective chart review was performed of consecutive patients diagnosed with OMS from 2006-2017 at a tertiary pediatric hospital. Patients treated within 3 months of presentation using a standardized treatment protocol involving pulse methylprednisolone (3-5 days, followed by an oral steroid taper), IVIgG and/or plasmapheresis, and rituximab ("protocol group", n=6) were compared to a historical cohort treated primarily with prednisone and IVIG ("historic group", n=6).

Results: All 10 patients with neuroblastoma were treated with surgical resection, but only one also received chemotherapy. In the protocol group 4 patients received IVIgG, and 4 underwent plasmapheresis prior to rituximab induction. Median follow-up post-treatment was 14.5 (range 2-120) months. Duration of corticosteroid treatment was a median 3.5 (range 3-5.5) months in the protocol group compared to 21.5 (range 6-54) months in the historic group (p=0.01). Only a single OMS relapse occurred following rituximab therapy in the protocol group, while 5/6 patients in the historic group had at least one relapse (p=0.08). OMS rating scale at last follow-up was similar in the protocol group (median 1.5, range 0-2) compared to the historical cohort (median 0.5, range 0-6; p=0.89).

Conclusions: A treatment approach involving intensive immunotherapy and rituximab significantly reduces the duration of corticosteroid therapy in pediatric OMS and is associated with a low OMS relapse rate. Rituximab and plasmapheresis, used in 4/6 protocol patients, may have contributed to these outcomes. Future studies will determine whether motor, psychosocial, and cognitive outcomes are improved by this approach.

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Prospective Germline Next-Generation Sequencing Identifies Novel Variants and Frequent DNA-Repair Pathway Alterations in Patients with Neuroblastoma

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Background: ALK and PHOX2b are the most commonly mutated genes in patients with familial neuroblastoma (NB). However, advances in next-generation sequencing (NGS) technologies are leading to the identification of novel germline variants with potential roles in NB predisposition in patients with and without suspected cancer predisposition syndromes.

Methods: Patients with newly diagnosed and relapsed malignancies, including 29 NB patients, were enrolled on an institutional study to sequence germline DNA and tumor DNA and RNA. Germline DNA was sequenced using a custom pediatric cancer panel (Agilent Sure Select capture kit technology) targeting 15,000 exons across 886 genes (1000x coverage). Rare germline non-synonymous substitutions and indels were called using GATK. Population allele frequencies were compared to ExAC and internal databases and cross-referenced to known polymorphisms (dbSNP). Variant pathogenicity was classified according to ACMG (American College of Medical Genetics and Genomics) criteria and evaluated at multi-disciplinary molecular tumor boards. Rare variants of uncertain significance (VUS) with supportive corresponding somatic data, patient phenotype and/or literature or in-silico functional analyses were termed “Variants of uncertain significance with limited evidence for pathogenicity’ (VUS-LEP)”.

Results: To date, analyses have been completed on 17/29 NB patients. Patient history was retrospectively categorized as “high genetic risk” (HGR) in 5/17 patients based on ≥1 of the following criteria: (1) family history of NB and/or significant family history of other cancer(s); (2) patient with NB and another primary tumor; or (3) NB plus congenital anomalies. Pathogenic variants in known cancer-predisposition genes involved in DNA-repair, including BRCA1, CHEK2, and PALB2, were identified in 2/5 HGR patients. For the 12 non-HGR patients, ≥ 1 rare likely pathogenic variant or VUS-LEP in DNA-repair genes, including BAP1, BRCA2, ERCC2, RAD51, and BLM, were identified in 7 patients (58%). Notably, an additional 3/12 non-HGR and 2/5 HGR patients harboured VUS-LEPs in other cancer-predisposition genes including EZH2, SOS1 and DICER1.

Conclusion: Our preliminary results using prospective NGS identified frequent DNA-repair pathway alterations and variants in genes not commonly associated with NB predisposition syndromes, even in the absence of family history. These findings may have implications for therapies (e.g. PARP inhibitors) and genetic counselling for patients and families.

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Development of a Syngeneic Mouse Model for T-Cell Engaging Bispecific (huCD3 x GD2) Antibodies

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Background: T-cell engaging bispecific antibodies (BsAb) can redirect polyclonal T-cells against tumors, but to date most preclinical models utilize transplanted human cell xenografts in immunodeficient mice, with limited ability to identify relevant immune cell subsets, or the vaccination effects of the therapy. GEMM disease models are powerful ways to interrogate biology but limited to targets with identity or strong high human homology. GD2, one of the proven neuroblastoma antigens for immunotherapy, is natively expressed in most animal species, allowing for direct assessment of GD2-targeted therapies.

Methods: We utilized a transgenic mouse line, which expresses the human CD3-ε gene, to allow testing of BsAbs specific for huCD3ε and GD2. Using this model, we tested a panel of 10 recombinant anti-GD2 BsAb using the sequences of 5 different anti-GD2 antibodies in two different bsAb formats (monovalent heterodimers and bivalent IgG(L)-scFv). Antibodies were synthesized in expi293 cells and purified by affinity chromatography. Affinity was measured using SPR (biacore, GE) and potency was measured in vitro, and in vivo using both the huCD3ε transgenic and xenograft models.

Results: Hu3F8-BsAb showed strong GD2-specific anti-tumor activity against both melanomas and lymphomas in the huCD3ε transgenic immunocompetent mice. Additionally, potency of BsAb strongly correlated with GD2 binding affinity that ranged from ~0.5μM to 1nM. Monovalent binding (heterodimer) substantially reduced the overall binding avidities and anti-tumor potency when compared to bivalent IgG(L)-scFv formats. However, both the transgenic and the xenograft models suggested that an upper limit of affinity (~10nM) beyond which affinity improvements could no longer improve potency.

Conclusion: The transgenic mouse model confirms the high potency of T-cell engaging BsAb directed against huCD3 in an immunocompetent environment, without being confounded by graft versus host reactions, or graft versus graft reactions, as well as other cross-species artifacts inherent in the humanized SCID mice models. We concluded that in vitro and in vivo potency improved with affinity until it reached ~10nM. Monovalency towards the target antigen significantly degraded BsAb potency. This mouse model has the potential to increase the understanding and to optimize the design of BsAb as an emerging immunotherapeutic platform for GD2 and other tumor targets.

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Patterns of Structural Variation, Chromothripsis and Mutational Burden Differ Across Neuroblastoma Subtypes

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Background: Neuroblastoma (NB) exhibits few recurrent somatic single nucleotide variants (SNVs); however, chromosomal instability (CIN) including copy number (CN) and structural variation (SV) is extensive. Our understanding of the mechanisms driving these mutational processes and how they differ across NB subtypes remains limited.

Methods: To characterize the mutational burden across different NB subgroups and further refine the landscape of SVs (duplication, deletion, inversion and translocation), we sequenced the genomes of 128 primary diagnostic tumors and matched normal DNA. Cases were divided into three groups: high-risk with MYCN-amplification (MNa; n=29), high-risk without MYCN amplification (S4na, n=76) and low/intermediate-risk stage 4S tumors (S4s, n=23). A comprehensive analysis of SNVs, CN and SV was performed.

Results: We first obtained per mega-base frequency of somatic alterations for SNV, SV, and CN breakpoints (ΔCN > 0.2) and discarded outlier regions (kataegis or chromothripsis). S4na tumors harbored higher CIN (SV and CN) burden than MNa (Wilcox. Test: PSV=1.2x10-3, PCN3.3x10-3), with specific enrichment for tandem duplications when excluding the MYCN locus (P=7.8x10-6). However, no difference in SNV burden was observed (P=0.94). S4s displayed significantly lower SV, CN and SNV burden than high-risk tumors (P<1.0e-5). Chromothripsis was observed in 23/105 (22%) high-risk tumors and included 25 distinct regions. Eleven cases involved chromosome 2p in MNa tumors while nine cases involved chromosome 5p in S4na, concurrent with SVs near the TERT gene. The most frequently mutated genes included MYCN(34%), TERT(24%), PTPRD(19%), ALK(16%), ATRX(14%), SHANK2(12%) and DLG2(12%). Among them, TERT, ATRX, SHANK2 and DLG2 were almost exclusively altered in S4na tumors; indeed, SHANK2 and DLG2 occupy frequent breakpoint regions associated with 11q deletion. Functional enrichment analysis of 67 genes harboring recurrent SVs (n>3 samples) showed enrichment in neurodevelopmental genes and autism susceptibility (P<0.0001; FDR<0.01).

Conclusions: Differences in CIN (CN and SV) but not SNV burden across high-risk subtypes suggests different underlying mechanisms drive these mutational types. The co-occurrence of MYCN-amplification and TERT rearrangements with chromothripsis suggests that catastrophic events followed by selection may represent an underlying cause of those alterations. Finally, recurrently altered genes in neuroblastoma drive telomere maintenance and interfere with normal neurodevelopmental pathways in high-risk NB.

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New Role of MYCN In the Mitochondria: Anti-Cancer Effects of Diphenyleneiodonium Chloride (DPI) In MYCN-Amplified Neuroblastoma

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Despite extensive research, the five-year survival rate for children with high-risk neuroblastoma (NB) is still as low as 40-50%; thus, better therapies are desperately needed. Amplification of MYCN drives about half of the aggressive forms. Therefore, our aim was to identify therapeutically targetable nodes that function as critical effectors or regulators of MYCN in NB by using a systematic discovery approach.

We mapped the MYCN interactome across a panel of NB cell lines with differential MYCN status and paediatric tumour samples by using label-free quantitative interaction proteomics (CoIP-MS). Interestingly, we found that MYCN is physically interacting with a mitochondrial transcription factor. We confirmed that MYCN is present in the mitochondria of NB cells by Western blot analysis of purified mitochondrial fractions and by confocal microscopy. Then we performed transcriptomics with NB cells rendered MYCN deficient by siRNAs; and demonstrated that MYCN may repress or activate mitochondrial gene expression. Moreover, we found that certain mitochondrial genes are downregulated in patients with MYCN-amplified NB, and they correlate with poor patient survival (Kaplan-Meier analysis of 709 patients).

In addition, we performed high-throughput drug screening (~4000 compounds) with NB cells, and we found that Diphenyleneiodonium Chloride (DPI) inhibits the proliferation of MYCN-expressing NB cells. Interestingly, DPI significantly downregulated MYCN expression in NB cells, and in turn, modulated mitochondrial gene expression dependent on the MYCN status. Moreover, DPI treatment resulted in an increase in mitochondrial oxidative stress and induced the differentiation of MYCN-amplified NB cells. In addition, DPI treatment reduced transformation of MYCN-amplified NB cells in soft agar assays.

Furthermore, DPI reduced tumour size in the zebrafish model of MYCN-driven neuroblastoma (Tg[dβh:EGFP-MYCN] zebrafish; a generous gift from A. T. Look, Dana-Farber Cancer Institute).

In summary, we found a new role for MYCN; we demonstrated that MYCN may reside in the mitochondria and directly regulate mitochondrial gene expression. In addition, we showed that DPI affects mitochondrial functions dependent on the MYCN status. DPI induced mitochondrial superoxide-mediated apoptosis and differentiation of MYCN-amplified NB cells; and inhibited NB growth both in vitro and in vivo. Therefore, DPI might serve as a potential novel drug to treat MYCN-amplified NB.

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In Vivo Identification of Active Drugs for Refractory Neuroblastoma Using Zebrafish Models

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Neuroblastoma is a high-risk pediatric solid tumor that accounts for 15% of childhood cancer deaths. Most children with neuroblastoma respond initially to chemotherapy, but a large proportion will experience therapy-resistant relapse. A recent international study found that in 80% of cases of relapsed neuroblastoma, the tumors have somatic mutations that activate RAS-MAPK signaling, including loss of the NF1 tumor suppressor or activating mutations of the ALK, NRAS, HRAS, KRAS, BRAF or PTPN11 oncogenes.

Thus, upregulation of RAS-MAPK signaling through multiple mechanisms poses a major barrier to the cure of disseminated neuroblastoma. To identify novel effective therapies for these devastating diseases, we developed zebrafish models of refractory neuroblastoma, in which nf1-loss or transgenic expression of mutant ALK (ALKF1174L) makes MYCN-driven neuroblastomas resistant to isotretinoin. These zebrafish develop aggressive neuroblastoma with up to 80% tumor penetrance by 3 weeks of age, ideal for the rapid analysis of small-molecule drugs by adding the drugs to fish water. Using these faithful preclinical models of refractory neuroblastoma, we are analyzing libraries of FDA-approved drugs and drugs currently in clinical trials by simultaneously assessing drug toxicity to normal tissues and activity against primary neuroblastoma tumors in juvenile zebrafish with primary neuroblastoma. In our preclinical testing to date of small molecule inhibitors, the most active drug is entrectinib, a receptor tyrosine kinase inhibitor that is active against NTRK1/2/3, ALK and ROS1. We are currently testing this drug in combination with other drugs including isotretinoin and CDK4/6 inhibitors.

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A Phase I Study of Lenalidomide in Combination with ch14.18 and Isotretinoin (RA) in Patients with Refractory/Recurrent Neuroblastoma (RR-NB)

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Background: Ch14.18 (dinutuximab) increases event free and overall survival in patients with high-risk neuroblastoma when given post autologous bone marrow transplant in a regimen that includes GM-CSF and IL-2. However, this therapy has significant toxicities, and 40% of patients still relapse. Lenalidomide has shown immunomodulatory effects in pediatric solid-tumor patients and is well tolerated. The combination of lenalidomide with ch14.18, instead of GM-CSF and IL-2, was supported by preclinical data that demonstrate the activity of the combination in neuroblastoma. We are conducting a phase I trial to determine the tolerability of lenalidomide in combination with ch14.18 and istotretinoin in patients with RR-NB.

Methods: Lenalidomide dose escalation is following a 3+3 design (25, 50, 75 and 100mg /m2/day), The administration schedule is: Lenalidomide days 1-21, ch14.18 (17.5mg/m2/day) days 8-11, and isotretinoin (160mg/m2/day) days 15-28 (Dose level 2-5).

Results: 27 patients enrolled with a median age of 8 years (range: 3-20), of whom 23 were evaluable for dose escalation. The median number of courses was 4 (range 1–12). No MTD was identified after completing dose escalation. There were 7 patients with dose limiting toxicities (course occurrence): grade 3 diarrhea(C1), grade 3 diarrhea/delayed neutrophils(C1), delayed neutrophils(C2), grade 4 ALT(C8), anaphylaxis(C5), delayed platelets(C5), delayed neutrophils(C4), delayed neutrophils/grade 4 sinus bradycardia(C6). Overall regimen was tolerable with no grade 3 capillary leak, 7% grade 3 hypotension and 4% grade 3 fever. Of the 21 patients evaluable for response, there was 1 complete response, 3 partial responses, 3 minor responses, 8 stable diseases and 6 progressive diseases.

Immunomodulation was seen with statistically significant increases in frequency of circulating effector NK cells (CD56low/CD16high), an increase in the frequency of CD4+ (T-helper) effector memory T lymphocytes (CD3+CD4+CD45RA-CCR7-CD27-) and increased antibody dependent cytotoxicity (ADCC) in patients. Plasma protein concentrations demonstrated statistically significant therapy-associated increases in granzyme B, IL-15, IFNg, CXCL9, CXCL10, CXCL11, sIL-2R, and GM-CSF as well as IL-6, IL-8, IL-10, MCP-1, and M-CSF.

Conclusion: Lenalidomide 100 mg/m2 QD for 21 days per cycle is tolerable in combination with ch14.18 and RA and is associated with antitumor response and immunomodulatory effects. Further studies are warranted in studying this combination.

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Polyamine Blockade Alters NK Cell Infiltration in The Neuroblastoma Tumor Microenvironment to Augment Immunotherapy Activity

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Background: Neuroblastomas coordinately deregulate polyamine homeostasis downstream of Myc. Ornithine decarboxylase (Odc), the rate-limiting enzyme in polyamine synthesis, is a direct Myc target that can be irreversibly inhibited by difluoromethylornithine (DFMO), an FDA-approved drug with broad preclinical activity in neuroblastoma. Arginine is an evolutionarily conserved immunomodulatory metabolite, and we hypothesized that the altered arginine-ornithine-polyamine signaling induced by DFMO would impact the tumor microenvironment (TME).

Methods and Results: DFMO extends the survival of TH-MYCN+/+ transgenic neuroblastoma-prone mice, and induces distinct and reproducible alterations in the immunoenvironment, most notably a marked increase in the frequency of NK cells. Moreover, we observe an upregulation in the tumoral expression of NK cell-activating ligands and a concommitant increase in the expression of activating and a decrease in inhibitory receptors on NK cells. These effects are largely tumor-restricted and not apparent in other immune organs. Although DFMO has antimicrobial uses, 16S rRNA deep-sequencing revealed no significant changes in the intestinal or respiratory microbiota of DFMO-treated mice. The anti-GD2 mAb dinutuximab exerts its effects via NK cells suggesting relevance for immunotherapy. Further, cross-talk between NK cells and invariant natural killer T (iNKT) cells has been demonstrated for solid tumors. iNKT cells have been shown to not only exert direct cytotoxic responses against cancer cells, but to also nucleate the activity of other anti-tumor effector lymphocytes, including NK cells, via robust proinflammatory cytokine secretion, and studies have demonstrated that higher frequencies of intratumoral iNKT cells portends increased survival in neuroblastoma. Using proof-of-concept reagents, we are able to redirect iNKT cells to kill GD2-bearing neuroblastoma targets. We are now investigating whether such reagents can induce sustained intratumoral iNKT cell and NK cell activation, and whether the use of these reagents could provide additional or synergistic control of tumor growth in combination with DFMO.

Conclusions: Alterations in polyamine-arginine-ornithine signaling induce unique changes in the tumor microenvironment that may augment neuroblastoma immunotherapy and could shed light on novel mechanisms by which to improve outcomes for this malignancy.

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Stress Mediators Interfere with Sympathetic Neuron Differentiation, Providing Potential Mechanism for Increased Neuroblastoma Formation in Prenatally Stressed TH-MYCN Mice

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Neuroblastomas (NBs) are pediatric malignancies of sympathetic origin with phenotypes ranging from spontaneously regressing to aggressive tumors. Although the disease appears to have a genetic background, its etiology and heterogeneity cannot be explained solely by genetics, suggesting that non-genetic factors may affect disease development and phenotype. Indeed, using TH-MYCN mice as a model, we have previously shown that inserting slow release pellets with corticosterone or placebo to pregnant mothers, which mimics prenatal stress, resulted in increased tumor frequency in TH-MYCN offspring and more malignant NB phenotypes. The goal of the current study was to validate these findings using an established stress model and identify stress mediators that contribute to increased NB tumorigenesis. To this end, pregnant mice carrying TH-MYCN hemizygous offspring were subjected to chronic unpredictable stress (various stressors administered daily) at embryonic days 10-17, the time of sympathetic neuroblast proliferation and differentiation. The tumor frequency was significantly elevated in offspring of stressed mothers, as compared to the control group ($p=0.02$), validating our previous results with stress mimic. To determine if increase in NB formation and malignant phenotype triggered by prenatal stress results from its impact of on neurogenesis, we performed in vitro assay using PA6 cells promoting differentiation of mouse embryonic stem cells (mESCs) to sympathetic neurons. The assay was performed in the presence of stress mediators mimicking stress-induced conditions in utero. The experiments were carried out under normoxic and hypoxic conditions, as maternal stress results in vasoconstriction of uterine vessel and fetal hypoxia. Sympathetic neurotransmitters - neuropeptide Y, norepinephrine and epinephrine - in combination with intermittent hypoxia, significantly decreased the number of colonies differentiating to the sympathetic neuron phenotype, while corticosterone had no such effect. These data confirm the direct effect of stress-induced changes in the fetal environment on neurogenesis and implicate sympathetic system activation as the main trigger of these effects. Altogether, our data implicate maternal stress during pregnancy as a potential environmental factor modifying the effects of genetic aberrations and promoting NB development by direct impact on sympathetic neuron differentiation.

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Drugging MYCN transcription using BRD inhibitors in combination with TOPI inhibitors

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Background: Children with high-risk, MYCN-driven neuroblastoma (NB) continue to have poor long-term survival and significant comorbidities despite intensive multidisciplinary treatments. More effective and less toxic therapies are needed. BRD4-dependent phosphorylation of RNA Polymerase II (RNAPII) stimulates topoisomerase I (TOPI) activity. Recent studies indicate that c-myc-driven RNAPII mediated transcription requires TOPI to relieve DNA torsional stress. We hypothesize that a similar mechanism operates in N-myc-driven NB tumors and renders these tumors sensitive to inhibitors targeting BRD4 and TOPI.

Methods: We treated six NB cell lines (3 MYCN-amp and 3 MYCN-wt) with varying concentrations of BRD4 inhibitors alone or in combination with TOPI (topotecan or camptothecin) or TOPII (etoposide) inhibitors. The Bliss Independence Model was used to evaluate synergy of drug combinations. DNA damage was assessed by western blot analysis for P-γH2AX, P-ATM, and P-ATR in protein lysates from cells treated with synergistic drug combinations and single agents.

Results: Synergistic concentrations of JQ1 and topotecan, but not JQ1 and etoposide, were found to block cell growth. In NGP MYCN-amp NB cells a similar cell kill effect was achieved with a 4-fold lower dose of topotecan (7.5nM) when combined with JQ1(310nM) compared to topotecan (30nM) alone. Single drug agents did not induce detectable expression of P-γH2AX while the combination of a BRD4 inhibitor with a TOPI inhibitor produced a >3-log increase in the levels of P-γH2AX. Levels of P-ATM or P-ATR were relatively unchanged.

Conclusion: We infer that at the low dosages selected, single agents alone are not sufficient to cause significant DNA damage. Our data suggest that a synergistic drug dose that reduces cell growth and induces DNA damage can be attained, and the combination with TOPI inhibitors may be more effective than TOPII inhibitors. Understanding the operational features and pathways affected by these drugs at low dosages may enable a new strategy to selectively target transcription-driven MYCN tumors.

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Home in Time for Supper: Humanized anti-GD2 Antibody in the Outpatient Setting

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Background: Neuroblastoma is the most common extracranial solid tumor of childhood. With the addition of anti-GD2 immunotherapy, >50% of high risk patients are curable. Currently, the available anti-GD2 antibodies are dinutuximab and humanized 3F8 (hu3F8). While dinutuximab is typically administered inpatient, at MSKCC, hu3F8 is administered in the ambulatory setting.

Methods: We summarize the experience of the first 160 patients treated with hu3F8, with emphasis on major side effects and their management in clinic and at home. The safety of outpatient anti-GD2 immunotherapy has previously been established over 25 years in >800 patients at MSKCC who received murine 3F8. We modified the previously established standard operating procedure (SOP) for the administration of hu3F8. Specially trained clinical nurses administer hu3F8 with supportive care provided by nurses from the ambulatory care center. Premedication before hu3F8 includes oral antihistamines, antiemetics and IV analgesics. During the infusion, emergency medications (including Narcan, EpiPen and Levalbuterol) are available at the bedside and given when necessary. Nurses follow the SOP, outlining pain management as well as guidelines for administration and intervention during infusion. Following the infusion of hu3F8, patients are monitored until safe for discharge to home. Families receive comprehensive discharge education and emergency contact information. Patients receive three doses of hu3F8 during each cycle, 3-4 weeks between cycles.

Findings or Outcomes: The SOP was put into place when outpatient hu3F8 was first piloted. Using this SOP, coupled with integrative medicine techniques, satisfactory pain control has been achievable and administration of this antibody in an outpatient setting has proved possible for up to 10 patients per day. Of the 160 patients treated with hu3F8, there has been no incidence of transverse myelitis, motor neuropathy, capillary leak syndrome, or death.

Conclusion: With close monitoring, continual assessment and intervention following the SOP, hu3F8 can be administered safely in the outpatient setting to allow discharge early enough for supper at home.

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Genomic Characterization of High-Risk/Ultra-High-Risk Neuroblastomas Found in 610 Patients Registered in Japan Children's Cancer Group (JCCG) Neuroblastoma Committee (JNBSG)

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Purpose: Neuroblastoma (NB) is known to exhibit wide ranges of clinical behavior and is sometimes highly resistant to chemotherapy. Genome-based precision medicine has been applied recently in cancer therapy and its introduction to children with NB is urgent demand. In this study, we conducted a series of genome analysis for 610 tumors including high-risk/ultra-high risk NBs and searched for new therapeutic candidates for future clinical application.

Methods: We obtained array CGH data for 610 NBs registered to JCCG-JNBSG from 1995 to 2014 including 77 tumors from the patients enrolled in the two JCCG-JNBSG phase II clinical trials for high-risk NBs (JN-H-07, JN-H-11). Whole exome sequencing, target-resequencing of 409 cancer-related genes and CpG island methylator phenotype (CIMP) analyses were also performed for 146, 67 and 91 tumors, respectively.

Results:We collected follow-up data for 610 patients according to the criteria of the iINRGdb. CIMP marker (high:≥40% of methylation in PCDHB) showed a good power to predict favorable prognosis of the patients (outcome at 2y or 5y: $p<0.05$). CGH profiles (P:partial/segmental; S:silent; W:whole/numerical) with MYCN amplification (a;MYCN-amp; s; MYCN-non-amp), 1p-loss, 11q-loss and 17q-gain were used to classify tumors. As we previously reported, genome group Pa and Ps with both 1p/11q losses showed most aggressive phenotypes (OS: 21% n=13 and 29% n=34; EFS:0% n=9 and 24% n=27, respectively). These subgroups were preferentially involved in the high-risk/ultra-high-risk subset of tumors [died <5y (n=160) / <2y (n=90) after diagnosis, respectively]. Pa tumors occasionally accompanies ALK mutations which was extremely aggressive. On the other hand, there was a tendency that Ps and Ws tumors with aggressive characters had amplifications of 12q (CDK4 locus, in 4 Ps and one Ws), chromothripsis-like unstable genomic regions in chromosomes 2,5,6 and 11(8 tumors in Ps or Ws) and mutations in 6q (cf. FOXO3, IGF2R), whose losses have been recently reported as a potential indicator of poor outcome of NBs in older age (>5 y).

Conclusions: We are currently planning to make NGS-based oncopanel which covers NB- and other pediatric cancer-related "actionable" genes. These omics data will be shared with domestic and international databases and contribute to precision medicine for NB.

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Integrative Mass Spectrometry and RNA-Sequencing Identifies Candidate Immunotherapeutic Targets in Neuroblastoma

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Background: The cell surface landscape of neuroblastoma is currently unknown. An unbiased survey of these proteins would greatly facilitate the identification of candidate immunotherapeutic targets for preclinical validation.

Methods: To identify proteins on the cell surface of neuroblastoma, we are performing plasma membrane protein extraction utilizing an optimized sucrose density gradient methodology followed by nano-liquid chromatography coupled to mass spectrometry (nLC-MS/MS) in NB cell lines (n=10), patient derived xenografts (PDX; n=10) and patient tumors (n=10). We utilize our generated and existing normal MS data to identify highly expressed proteins in neuroblastoma compared to normal tissue not predicted by RNA-sequencing. We further integrated MS data with RNA-sequencing (NB=2242; Normal=7859) data to evaluate proteins differentially expressed in neuroblastoma, those that correlate with MYCN amplification, and those with an annotated extracellular domain. Proteins are validated by tissue microarray (TMA) and in-vitro functional studies are performed following genetic manipulation of candidate targets to assess cell proliferation, differentiation and viability.

Results: To date, we have applied our proteomic approach to nine neuroblastoma cell lines and six PDXs. We have identified 4826 membrane proteins and yielded on average 66% (range:60-68%) membrane protein enrichment with high reproducibility between biological replicates (80%; range:78-84%). We detected 1010 proteins with an extracellular domain and filtered based on abundance and reproducibility to 343 NB-specific plasma membrane proteins. Our analyses confirmed known cell surface proteins in development as immunotherapeutic targets in neuroblastoma (ALK, GPC2 and NCAM1) and prioritized DLL3, DLK1 and CNTN1 for further evaluation as candidate targets. These proteins have all been validated using TMAs. Silencing of DLL3 with two shRNAs (range:48-78% knockdown) resulted in reduced viability (P=3.8X10-3) and proliferation (P=1.1X10-3) compared to control in two neuroblastoma cell lines with high endogenous expression. Lastly, we created GiaPronto (Mol. Cell Proteomics, 2017), an efficient tool for MS data analysis and visualization.

Conclusion: We have developed a robust methodology for cell surface protein isolation and quantification, produced GiaPronto for MS data analysis, and identified several candidate immunotherapy targets that are undergoing functional characterization. Proteomic and transcriptomic studies in additional PDXs and patient tumors are ongoing to further define the neuroblastoma cell surface landscape.

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Premature Epiphyseal Growth Plate Arrest After Isotretinoin Therapy in Preadolescent High-Risk Neuroblastoma (HR-NBL) Patients

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Background: Isotretinoin is a Vitamin A derivative used as standard therapy for high-risk neuroblastoma given with immunotherapy. The use of Vitamin A derived synthetic retinoids have been reported to cause skeletal abnormalities ranging from hypercalcemia, diffuse hyperostosis, osteopenia and premature epiphyseal closure. Premature epiphyseal growth plate arrest has severe lifelong sequelae that can greatly impact the length of long bones, the angulation of joints and the overall growth of the child.

Methods: We identified patients at Children's Hospital Los Angeles (CHLA) with neuroblastoma who experienced premature epiphyseal growth plate arrest. We then performed a review of the literature of this complication. Data collection included: diagnosis age, presentation age, agent of exposure, dose, exposure range and skeletal deformity.

Result: 3 patients were identified in the CHLA neuroblastoma program all of whom had isotretinoin exposure at 7 years of age. Review of the literature identified 7 additional neuroblastoma patients who presented with isotretinoin associated skeletal abnormalities. The median age of diagnosis for these 10 patients was 6 years old ranging from 1-8 years old. The median range of isotretinoin exposure was between 6.5-7.625 years old (range 2-14). 8/10 showed premature growth arrest, with 6/8 experiencing asymmetric growth arrest resulting in a valgus or varum deformity (median age of deformity presentation was 9 years old). 2/10 patients had isotretinoin and fenretinide therapy. No case reports were found of patients whose full exposure to isotretinoin therapy occurred before the age of five.

Conclusion: Bone toxicity associated with isotretinoin and retinoid exposure is a concern in patients treated with this agent. Growth plate arrest is a serious adverse effect that is rare in the neuroblastoma population but is attributable to isotretinoin therapy. Since all 10 patients with neuroblastoma and skeletal abnormalities had a similar age of exposure to isotretinoin that is later than the typical age of exposure to isotretinoin for neuroblastoma (more commonly in patients younger than 5 years of age), the prepubescent growth plate may be most at risk for this toxicity. We recommend special attention to this population and consideration for better awareness monitoring to help prevent long-term consequences.

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PEDS-PLAN – Pediatric Precision Laboratory Advanced Neuroblastoma Therapy: Molecular Guided Therapy for High Risk Neuroblastoma at Diagnosis

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Background: While the poor prognosis for high risk neuroblastoma (HRNB) underscores the need for new treatment strategies, the elucidation of specific biologic subsets of neuroblastoma suggests a way to improve disease management. The identification of agents that target specific molecular pathways associated with the development or progression of diseases holds promise. The hypotheses for this study were that: 1) the incorporation of a targeted therapy, selected based upon upfront genomic interrogation of the tumor, into standardized induction chemotherapy for HRNB is feasible and may increase the PR/CR/VGPR response rate at the end of Induction therapy; and 2) that the addition of DFMO as maintenance therapy during and for 2 years after the completion of immunotherapy is safe and may decrease the relapse rate.

Methods: A prospective, multicenter feasibility pilot clinical trial in subjects with newly diagnosed HRNB within the Beat Childhood Cancer Consortium. At diagnosis, patients' tumors underwent DNA exome and RNA sequencing which were analyzed within a molecular tumor board to identify the single best drug out of 6 targeted agents to be added to cycles 3-6 of induction chemotherapy. After consolidation with ASCT and radiation, the patients received DFMO along with standard dinutuximab and retinoic acid and DFMO for 2 years after immunotherapy. Patients were evaluated for additional toxicities with the addition of targeted agents and DFMO in addition to induction response.

Results: The pilot study of 20 eligible patients has shown this process to be feasible. To date, 20 patients have completed induction through immunotherapy portions of the study. The combination of targeted agent with chemotherapy was shown to be safe without any unexpected toxicities. Delays experienced between induction cycles were less than 2 weeks and related to surgery, infection, or thrombocytopenia. The induction response demonstrated an 88% CR/VGPR/PR rate, which suggests improvement over historical 80%. In addition, the combination of DFMO with dinutuximab and retinoic acid was well tolerated and safe without additional toxicities.

Conclusion: The pilot study of 20 patients has demonstrated that the process of sequencing and the addition of a targeted agent to upfront chemotherapy is feasible and safe without any unexpected toxicities.

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Combination Treatment with Selinexor and Bortezomib Strongly Inhibits Neuroblastoma Growth More Than Single Agent Therapy

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Background: Exportin-1 (XPO1), a nuclear transport protein with over 200 recognized cargo, is overexpressed in patients with fatal cases of high-risk neuroblastoma. Targeting XPO1 with selinexor (Karyopharm Therapeutics, USA), a selective inhibitor of nuclear export, has demonstrated pre-clinical efficacy and is in phase II clinical trials for refractory pediatric solid tumors. The use of proteasome inhibitors, which block superfluous degradation of proteins such as IkB, in combination with conventional agents improves chemosensitivity in neuroblastoma. In neuroblastoma cell lines, XPO1 protein overexpression correlates with decreased abundance of the recognized cargo protein IkB, the inhibitor of NF-kB. We hypothesize that treatment with a combination of selinexor and the proteasome inhibitor bortezomib will work synergistically to decrease NF-kB proliferation signaling by halting excessive exportation and proteasome degradation of IkB.

Methods: Neuroblastoma cell lines (NLF, IMR5, KELLY, and SKNSH) were used for all experiments. Drug sensitivity and cellular effects were assessed with MTT assays and flow cytometry. On-target effects were measured by comparing downstream effects of selinexor to siRNA against XPO1. Western analysis was used to quantify protein expression and NF-kB activity was further measured using ELISA.

Results: Sequential combination treatment of bortezomib followed by selinexor showed greater than additive inhibitory effects on cellular proliferation compared to either single agent alone. Active and inactive forms of NF-kB protein decreased in response to treatment. Flow cytometry and ELISA experiments are still in progress to assess apoptotic markers, cell cycle, and NF-kB transcriptional activity.

Conclusions: Our work provides biological insight into highly aggressive neuroblastoma through the assessment of changes in NF-kB proliferative signaling through selective inhibition of nuclear export with selinexor and knockdown of XPO1. Regulation of IkB protein expression through nuclear localization and decreased proteasome degradation can also halt NF-kB signaling. This ongoing work will illuminate the mechanism of action behind combination treatment of selinexor and bortezomib and provide a rationale for further in vivo evaluation and ultimate clinical development of novel therapy for patients with highly aggressive neuroblastoma.

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A Case of Relapsed Neuroblastoma Analyzed with Whole Exome Sequencing

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Background: Relapsed neuroblastomas often acquire chemoresistance and are refractory. The genomic character of the relapsed tumor may be distinct from the original tumor and may contribute to the aggressive behavior. We herein report the result of the whole exome sequence of the primary and relapsed tumor in a case metastatic neuroblastoma.

Material and Method: The case was a 3-year-old patient diagnosed of left adrenal neuroblastoma stage M (unfavorable histology, MYCN non-amplified, diploid). One year after being treated with multimodal treatment for high-risk neuroblastoma, the patient experienced relapse at the retroperitoneal lymph node, which was resected. The patient eventually died of disease six years after diagnosis. DNA samples were extracted from the peripheral blood, primary and relapsed tumor and were subjected to exome sequencing. Nonsynonymous mutations that were not observed in normal tissue were analyzed as somatic mutations. Candidate genes with a coverage number≧10, read number≧4, frequency≧10% for tumorigenesis or progression were further selected through protein function prediction.

Results: A total of 26 genes from the primary tumor and 78 genes from the relapsed tumor were screened as somatic mutations. Somatic mutations in PTPN14, SV2C and KMT2C genes were detected in the primary tumor. PTPN14, SV2C and 24 other relapse-specific genes were mutated in the relapsed tumor. DISCUSSION Mutations in the Ras signaling pathway molecules have been reported to correlate to relapse and chemoresistance of neuroblastoma. However, such mutations were not observed in our case. Relapse-specific mutation of PTPN14 has previously been reported in other cancers and might have contributed to the acquirement of chemoresistance in our case.

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Reevaluation of PDGFRα Signal in Neuroblastoma: High Expression and a New Nonsynonymous Mutation

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Background: PDGFRα (platelet-derived growth factor receptor alpha), a receptor tyrosine kinase encoded by PDGFRA gene, has essential roles for embryonic development of nervous systems and mesenchymal stem cells. In oncology, missense point mutations, gene fusion and amplification have been identified in several types of cancers, such as glioblastoma and gastrointestinal stromal tumor. According to previous studies in neuroblastoma (NB), the stimulation of the receptor promoted NB cell growth and a multi-kinase inhibitor, imatinib, attenuated tumor growth in vitro and in vivo. One of the ligand, PDGFA, was abundant at mRNA level in advanced stage tumors. On the other hand, PDGFRα signal was also implied to involve neurite outgrowth in NB cells. Therefore, the function of PDGFRα has been still controversial in NB, which motivates us to investigate its biological roles and druggability.

Results: In several cohorts, the high expression of PDGFRA indicated poor overall and event-free survival rates of NB patients. Additionally, PDGFRA was highly expressed in MYCN-amplified cases. Although no genetic alteration was previously reported, our target DNA sequence analysis identified nonsynonymous single nucleotide variants (SNVs) of PDGFRA in 5 out of 45 high-risk NBs. Notably, 3 SNVs were identical and positioned at tyrosine kinase (TK) domain. To validate the biological effects of PDGFRα wild-type (WT) and the TK mutant, we exogenously expressed either of them in NB cell lines. Intriguingly, exogenous PDGFRα WT enhanced AKT phosphorylation, and the TK mutant further elevated phosphorylation of the receptor itself and AKT. Consistent with the signal upregulation, PDGFRα WT and the TK mutant accelerated NB cell growth without apparent morphological change. Inversely, the knockdown of endogenous PDGFRα decreased NB cell proliferation.

Summary: Herein, we newly identified a nonsynonymous mutation of PDGFRA in high-risk NB, the product of which elevated the down-stream signal and cancer cell proliferation. Furthermore, the high expression of PDGFRA associated to poor prognosis in NB patients, and exogenous PDGFRα WT increased the down-stream signal. Therefore, PDGFRα signal is to be a potential therapeutic target in a part of high-risk NB.

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Chromosome 19 Encoded Genes Are Deregulated in Relapse Disseminated Tumor Cells (DTC): RNA-seq and First Steps Towards DTC-Derived Zebrafish Xenografts

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More than 90% of stage M neuroblastomas present with disseminated tumor cells (DTCs) in the bone marrow. These cells are frequently refractory to therapeutic interventions and/or cause disease relapse. In order to learn about the transcriptomic makeup and identify genes that may be causative for relapse and/or are targetable, we performed the first RNA-seq of DTCs at diagnosis and relapse. For functional validation of these findings and to enable therapeutic targeting of DTCs, we are establishing a DTC-derived xenograft model in zebrafish larvae.

DTCs of stage M neuroblastomas were enriched by magnetic bead-based cell sorting. mRNA of diagnostic (n=22) and relapse (n=20) DTCs was used for RNA-seq. Furthermore, four DTC-derived cell lines were established and characterized by RNA-seq. As zebrafish larvae cannot develop properly at temperatures above 34°C, we performed in vitro testing on the impact of the low temperature on apoptosis and proliferation in the cell lines. A GFP-transduced cell line was injected into the perivitelline space of 48-hours post-fertilization zebrafish embryos and monitored by fluorescence microscopy.

The gene expression signature of relapse DTCs largely resembled those of diagnostic DTCs with only 113 differentially expressed genes. Down-regulated genes in relapse samples were enriched on chromosome 19, with PUMA and CADM4 (tumor suppressor genes) down-regulation being associated with a worse event-free survival (R2, Oberthuer dataset). In order to establish DTC zebrafish xenografts, we next tested four DTC-derived cell lines for proliferation and apoptosis at 34°C. Fewer STA-NB-15 and STA-NB-19 cells were found in S phase at 34°C as compared to 37°C (18.6% vs 38% and 24% vs 45.1%, respectively). However, STA-NB-8 and STA-NB-12 cells were not affected. Additionally, more STA-NB-15 cells underwent apoptosis at 34°C (50%) as compared to 37°C (34%). After injection into the perivitelline space, STA-NB-12 cells survived for eight days in the developing zebrafish.

RNA-seq analysis of DTCs revealed a positional enrichment of down-regulated genes encoded by chromosome 19 at relapse. We have successfully piloted DTC-derived cell line xenografts in zebrafish embryos and demonstrated tolerance to low temperature as critical determinant. These models will enable in vivo functional validation and therapeutic testing of genes deregulated in DTCs.

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Chromatin Reorganization Underlies Drug Resistance in Cancer

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Molecularly targeted agents are rapidly becoming standards of care in the treatment of cancer, yet resistance invariably limits their efficacy. We identified mechanisms of acquired resistance to the ALK inhibitor (ALKi) TAE684 in neuroblastoma (NB) cells driven by amplified MYCN and mutated ALK(F1174L). ALKi-resistant cells generated by long-term exposure to escalating concentrations of TAE684 lost their dependence on functional ALK signaling and the transcriptional amplifier effect of deregulated MYCN. Instead, there was a striking downregulation of MYCN expression and a concomitant genome-wide increase in the repressive mark H3K27me3. Integrative analysis of open chromatin marks H3K27ac and BRD4, together with SMC1-based ChIA-PET data, demonstrated extensive chromatin reorganization in the resistant cells, leading to the generation of short-range functional loops. These new enhancer-promoter interactions were associated with an altered super-enhancer landscape, that together were essential for the induction of a pro-neural transcription factor (TF) network that included genes with critical roles in early neural development. These TFs were enriched at both promoter and enhancer sites of the novel functional loops themselves, thereby establishing a feed-forward mechanism. Finally, by integrating previous results with droplet-based single-cell RNA sequencing of ALKi-resistant cells, we were able to partially reconstruct the intricate dynamics of the TF network during the evolution of resistance, establishing a central role for tumor cell plasticity as opposed to the selection of a subset of cells. A prominent feature of this altered cell state was the increased dependency on BRD4, creating a therapeutic window for the BET inhibitor JQ1, which showed a 10-fold augmented efficacy in ALKi-resistant cells. Our results suggest that tumor cell plasticity leading to structural chromatin reorganization can drive the selection of alternative gene regulatory programs that affect cellular identity.

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Selective Covalent CDK7 Inhibitor and its Synthetic Lethality in Combination with BET Inhibitors in Neuroblastoma

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The covalent transcriptional CDK inhibitor, THZ1 that inhibits CDK7 and to a lesser extent, CDK12, has shown impressive cytotoxicity and selectivity in cancers driven by aberrant transcription, including neuroblastoma. CDK7 has essential roles in transcription and cell cycle progression. As part of the TFIIH transcription initiation complex, it phosphorylates the C-terminal domain (CTD) of RNA polymerase II (RNAPII), and as a CDK-activating kinase (CAK) it activates not only CDKs involved in transcription, such as CDK9, but also those with roles in the regulation of the cell cycle. To further delineate the effects of CDK7 inhibition in neuroblastoma, we developed a highly selective CDK7 inhibitor, YKL-5-124, and examined its effect in neuroblastoma cells. YKL-5-124 was derived from a different chemical scaffold from THZ1 and has an enzymatic activity against CDK7 that is higher than THZ1, while at the same time not targeting CDK12. In contrast to our findings withTHZ1, pharmacologically relevant doses of YKL-5-124 led to cell cycle arrest in NB cells, with no evidence of apoptosis, suggesting that the cell cycle activity, rather than transcription function of CDK7 was being targeted. Indeed, YKL only caused marginal effects on RNA polymerase II CTD phosphorylation and transcripts of known super-enhancer-associated genes. Importantly, these effects were seen in all NB cells tested, irrespective of MYCN/MYC expression status. To identify agents that in combination with YKL-5-124 would lead to a greater effect on transcription, we screened a library of representative small-molecule epigenetic inhibitors and activators. We observed that the combination of YKL-5-124 and Bromodomain and Extra-Terminal (BET) inhibitors resulted in downregulation of global transcription and caspase-mediated apoptotic cell death. Together, our study provides a preclinical rationale, for combining targeted agents against CDK7 and BET, independent of MYCN status, in high-risk neuroblastoma.

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Comparison of 131I-Metaiodobenzylguanidine (MIBG) and 18F – fluorodeoxyglucose (FDG) Semi-Quantitative Scores in risk stratification in patients with Stage 4 Neuroblastoma

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Objective: Radioiodinated metaiodobenzylguanidine (mIBG) scintigraphy is used not only for staging but also for semiquantiative assessment of the extent and severity of disease in neuroblastoma. Established semiquantitaive scores include the SIOPEN and the Modified Curie score. The present study aims to use 18F–fluorodeoxyglucose(FDG-PET/CT) for semiquantitative scoring and compare with conventional mIBG scores thereby establishing its clinical utility.

Method: This is a retrospective study of 97 children with high risk neuroblastoma who underwent paired mIBG and FDG-PET/CT scans at initial diagnosis. mIBG scans were analysed using modified Curie and SIOPEN scoring methods. Similarly, FDG-PET/CT scans were assessed using these semi-quantitative scoring systems. These were compared using the 'paired t' test. A modified Curie score of ≤2 and > 2 and a SIOPEN score of ≤4 and >4 (best cutoff) was used to classify these scans into low and high risk. This was subsequently correlated with the clinical course of the disease.

Results: At baseline staging there was a statistically significant difference between the Curie-FDG scores (Mean:10.94 ± 10.0) as compared to Curie-mIBG scores (Mean 7.99 ± 9.00) across the study group with a mean increase of 2.94 (p=0.001). Additionally, the SIOPEN-FDG scores was also significantly higher compared to the SIOPEN-mIBG scores (Mean:20.00 ± 26.00 vs 11.72 ± 20.00, p=0.012) suggesting that FDG PET/CT picked up more lesions than mIBG. Additionally, all mIBG avid lesions were detected by FDG PET/CT. Post induction, there was no significant difference (p >0.01) between the Curie-FDG and Curie-MIBG scores since there was a significant response to treatment noted at majority of metastatic sites. Using the 'cut off' values for Curie and SIOPEN scores, 20 patients at initial staging show discordance with higher FDG scores, amongst whom 8 patients (40%) showed disease progression or death.

Conclusion: 18F FDG-Modified CURIE and SIOPEN scores show an incremental value over conventional mIBG scores. This could be attributed to the better spatial resolution and associated CT correlation. Semi quantitative score using 18 F FDG PET/CT could potentially replace conventional mIBG scoring in risk stratification of neuroblastoma.

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Human Pluripotent Stem Cell Models of High-Risk and RAS/MAPK Pathway-Mutated Neuroblastoma

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Nearly half of patients with neuroblastoma present with high-risk disease, which often has poor outcome. High-risk disease remains a relative clinical enigma, and the lack of actionable targets has prevented sufficient advances in improving survival for this large subset of NB. The proto-oncogene MYCN is known to be amplified in ~45% of high-risk cases, and recent sequencing studies of matched relapsed and primary tumors demonstrate an abundance of mitogen activated protein kinase (MAPK) pathway mutations in relapsed neuroblastoma, including mutations in anaplastic lymphoma kinase (ALK) and NF1. Mouse and zebrafish studies have also indicated that ALK and NF1 mutations can accelerate NB tumorigenesis with MYCN misexpression.

To better understand the genetic requirements of high-risk neuroblastoma, we generated a human induced pluripotent stem cell (iPSC) model of neuroblastoma. Human induced pluripotent stem cell (iPSC) models are superior to GEMMs at representing the chromosomal landscape and telomere biology of human neuroblastoma.

Normal human iPSCs were differentiated toward trunk neural crest, the cell of origin for neuroblastoma. We subsequently introduced candidate and established drivers of neuroblastoma and orthotopically implanted the resulting trunk neural crest cells (NCCs) into the renal capsules of immunocompromised mice. We transduced NCCs with MYCN, hyperactive mutant ALK(F1174L), and transfected NF1 CRISPR sgRNAs with Cas9. Within 3 months, tumors developed in 80% of MYCN/NF1 modified cells, 60% of MYCN/ALK cells, and 10% of MYCN-misexpressed mice, with no tumors arising in mice injected with empty vector or ALK(F1174L)-modified NCCs alone. Tumors expressed markers typically found in neuroblastoma on histological and RT-PCR analyses, while lacking markers of other tumors such as rhabdomyosarcoma, Ewing sarcoma, and lymphoma.

Our data suggest that RAS/MAPK pathway mutations may be involved in high-risk neuroblastoma tumor maintenance. Not coupled with other driving mutations, ALK(F1174L) and NF1 loss alone are capable of causing cell proliferation and then regression in vivo, perhaps suggesting that these alterations may result in senescence or apoptosis when altered individually and that additional dysregulation, including MYCN and TERT misexpression, may be required in order to bypass a cell cycle checkpoint. Continuing studies in human iPSCs promise to clarify the genetics of high-risk neuroblastoma.

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Disrupting the Aurora Kinase A Interactome in Neuroblastoma

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Neuroblastoma is the most common extra-cranial solid tumor of childhood. Amplification of the MYCN proto-oncogene occurs in 50% of the high-risk neuroblastoma and indicates an aggressive and lethal form of the disease. Recent analysis has established a second subset of high-risk neuroblastoma with oncogenic c-MYC (MYC) activation that occurs in ~10% of patients and is associated with poor prognosis. As a transcription factor with no apparent small-molecule binding surfaces, direct inhibition of the MYC proteins has proven challenging, however MYC protein stability is tightly regulated in neuroblastoma in part by an activity-independent scaffold function of Aurora Kinase A (AURKA). AURKA is also directly implicated in neuroblastoma tumorigenesis downstream of LIN28B and RAN/TPX2, and misexpression of AURKA is associated with defective microtubule assembly and aneuploidy. More recently AURKA has been shown to stabilize MYC in liver cancer, but the role of AURKA stabilization in MYC-driven neuroblastoma and other pediatric cancers such as leukemia/lymphoma, is still not well defined. We hypothesize that the dynamic expression and activation of AURKA allows for precise cell cycle regulation of critical oncogenic partners. Utilizing co-immunopreciptation, flow cytometry, and subcellular fractionation, we aim to delineate a novel mechanism of targeting the AURKA interactome by conformation disruption of AURKA for MYC degradation. We have recently described a neoteric class of confirmation disrupting “amphosteric” inhibitors of AURKA (CD-AURKAi) that orthosterically inhibit the ATP-binding pocket to dramatically disrupt the active conformation of AURKA to dissociate and degrade MYCN, a critical oncogenic driver of the pediatric cancer neuroblastoma. We and others have shown that lead compound CD532 also disrupts AURKA interaction with MYCN and MYC in other cancer models. Our preliminary results indicate CD532 will also dissociate MYC and allosteric regulators in neuroblastoma. We hypothesize that conformation disruption of AURKA and subsequent blockade of an array of protein-protein interactions will delineate the roles of AURKA, MYC, and MYCN in the cell cycle and will reveal new potential therapeutic targets in MYC-driven pediatric cancers.

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Curing Human Neuroblastoma Using T Cells Armed with Zeptomole (10-21) Scale Recombinant anti-GD2 Bispecific Antibodies (BsAb)

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Background: Ex vivo, polyclonal T cells can be armed using chimeric antigen receptor or CD3xGD2 BsAb for infusional cytotherapy. Humanized 3F8-BsAb using the IgG(L)-scFv format, where the anti-CD3 huOKT3 scFv is linked to the carboxyl end of the hu3F8 light chain has shown promising anti-tumor properties against GD2(+) tumors in vitro and in vivo (Xu et al., Can Immunol Res 2015). The Phase I study (NCT02173093) of T cells armed with chemical conjugate of hu3F8xOKT3 (BiAb) was safe at $>10^9$ cell doses with suggestions of clinical response. We now report the safety and efficacy of T cells armed with recombinant hu3F8-BsAb in preclinical models.

Method: T cells from normal volunteers were affinity purified, expanded and activated by 37°C culture in the presence of CD3/CD28 beads to form ATCs. Between day 8 and day 9 when CD3(+) T cells reached >95%, ATCs were harvested and incubated at room temperature for 20 minutes with a GMP grade hu3F8-BsAb. Density of arming was confirmed using Quantum Beads. These armed ATCs were tested for cytotoxicity in vitro and in vivo against human GD2(+) cell lines and PDXs xenografted in BALB-Rag2-/-IL-2R-c-KO [DKO] mice.

Results: Hu3F8-BsAb armed ATCs showed robust antigen-specific tumor cytotoxicity against neuroblastoma and other GD2(+) tumors such as melanoma in vitro over a range of antibody dose (0.5, 0.05, 0.005 ug/10^6 cells). Optimal arming occurred at 9-90 zeptomoles (10-21 moles), 4500 to 45000 molecules of BsAb per cell without activation induced cell death when confronted by antigen or tumor target. In vivo hu3F8-BsAb armed ATCs showed potent anti-tumor effect against both neuroblastoma and melanoma tumors over a range of cell doses ($10x10^6$, $20x10^6$ and $40x10^6$ per injection, twice a week for 3 weeks), without significant side effects in DKO mice. By immunohistochemistry, the frequency of tumor infiltrating CD3(+) T cells strongly correlated with tumor response.

Conclusion: Hu3F8-BsAb armed ATCs may provide a viable immunotherapy platform against neuroblastoma and other GD2(+) tumors in both adult and pediatrics. In contrast to virus-based gene modification, BsAb uses standard manufacture methods and distribution networks, and has the potential for much higher cell doses without cytokine storm.

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Genomic Determinants of Tumor Microenvironment Predict Survival in High-Risk Neuroblastoma

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Background: A T cell-rich tumor microenvironment is a prognostic marker for improved clinical outcome in multiple adult cancers. Although the immunobiology of neuroblastoma is not completely understood, this pediatric neoplasm is an immunotherapy-responsive disease. We hypothesized that neuroblastoma tumors with a T cell-inflamed microenvironment would be associated with improved response to immunotherapy compared to non-T cell-inflamed tumors. Understanding the molecular mechanisms that drive the presence or absence of T cell infiltration may provide insights for the development of new therapies. To test this hypothesis, we analyzed high-risk neuroblastoma genomic data from the Therapeutically Applicable Research to Generate Effective Treatments (TARGET) database.

Methods: RNAseq gene expression, somatic mutations, and associated clinical data were obtained from 118 high-risk neuroblastoma tumors (diagnosed after year 2000) in TARGET harmonized by The NCI's Genomic Data Commons. Using a defined T cell-inflamed immune signature, we categorized patients as either non-T cell-inflamed, T cell-inflamed, or intermediate. Differentially expressed genes, activated pathways, and predicted neoantigens were compared in the non-T cell-inflamed and T cell-inflamed tumors. Cox proportional-hazards model was used to test association between the molecular variations and survival outcome.

Results: 39% of high-risk neuroblastoma tumors were classified as T cell-inflamed, and this cohort had a significantly better overall survival compared to patients lacking T cell infiltration ($P<0.05$). Integration of neoantigen load with the T cell-gene signature improved the prediction of prognosis ($P<0.0001$). The significance remained after adjusting for age, MYCN amplification status, and ploidy. MYCN amplification was significantly enriched in patients with the absence of T cell infiltration ($P<0.0001$, Fisher's exact test). We identified three additional neuroblastoma-cell-intrinsic oncogenic pathways including ASCL1, SOX11, and KMT2A significantly upregulated in non-T cell-inflamed tumors (FDR-adjusted $P<0.05$, activation z-score>1.95). Together with MYCN amplification, the four pathways present the potential major drivers for immune escape accounting for 85% of patients with non-T cell-inflamed tumors.

Conclusions: Our results indicate that tumors from children with high-risk neuroblastoma harboring a strong T cell-inflammatory signature have a more favorable clinical outcome, and that strategies to target MYCN and associated signaling pathways associated with non-T cell inflamed tumors should be pursued as potential immune-potentiating interventions.

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High Throughput Functional Genomics Reveals Novel Therapeutic Vulnerabilities in Neuroblastoma

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MicroRNAs (miRNA) are potent regulators of gene expression and cellular phenotype and are emerging as an exciting new class of therapeutic. We conducted functional genomic screens of microRNA mimics and inhibitors in cancer and 'normal' cell lines to discover cellular vulnerabilities in neuroblastoma. We mined the data using a novel web-accessible database that integrates screen data with mRNA and miRNA expression, miRNA-target predictions and transcription factor networks.

We discovered more than 136 'lethal' miRNA that reduced viability by >80%, 32 of which were not lethal to normal cells. These encompassed tumour suppressors including the Let-7 family and miR-34a. Target discovery identified enrichment of relevant neuroblastoma pathways including IGF and FOXO and neuronal differentiation. We discover HAND1, RGS16 & RBFOX2 as novel direct targets of Let-7 miRNAs that are highly expressed and required for neuroblastoma viability. We also discovered a novel role for miR-515 family miRNAs in neuroblastoma, with 14 lethal family members. The LIM homeobox factor ISL1 was identified as a direct target of miR-515 family miRNAs, specifically expressed in neuroblastoma. ISL1 knockdown reduced proliferation and increased neuronal differentiation.

Finally, to discover miRNA that synergise with chemotherapy, we conducted a screen combining miRNA perturbations with low doses (IC30) of doxorubicin or vincristine. Three miRNA, miR-380-3p, miR-99b-5p and miR-485-3p had a potent synthetic lethal interaction with doxorubicin, acting to prevent DNA damage repair. Each of these miRNA undergo recurrent copy number loss in neuroblastoma, and low expression predicts poor outcome, suggesting they are novel tumour suppressors. Using an siRNA screen with predicted targets of these miRNA, we discover many coding genes with a novel role in doxorubicin sensitivity.

Using functional genomics and novel computational approaches we have discovered many new vulnerabilities in neuroblastoma, both miRNA and protein coding. These are currently undergoing preclinical evaluation as novel therapeutics.

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Role of Neutrophils in Dinutuximab-mediated Antibody-Dependent Cellular Cytotoxicity (ADCC)

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Background: The chemo-immunotherapy arm of COG ANBL1221 trial that combined chemotherapy with dinutuximab (anti-GD2 antibody), followed by GM-CSF revealed remarkable responses in children with relapse/refractory neuroblastoma. Mechanisms of tumor clearance and resistance to this therapy are unknown. We hypothesize that neutrophils play a significant role in this response through antibody-dependent cellular cytotoxicity (ADCC) and evaluated a murine model with chemo-immunotherapy.

Methods: A panel of human neuroblastoma cell-lines (n=16) was used for neutrophil-mediated ADCC assay. Flow cytometry was used to assess expression of GD2, CD47, CD274 (PDL1), and CD276 (B7H3). A metastatic model using a chemo-resistant neuroblastoma cell line was established in immunodeficient (NOD-SCID-Gamma) mice. Mice were treated with chemo-immunotherapies containing combinations of chemotherapy (cyclophosphamide+topotecan), dinutuximab, and GMCSF.

Results: Neutrophils exhibited significant cytotoxicity against GD2 positive (13 out of 16) neuroblastoma cell lines but only in the presence of dinutuximab (mean cytotoxicity 48.5%, range 22.7-71.9%). ADCC was not observed in cell lines lacking GD2. Pre-treatment of neuroblastoma cell lines with chemotherapy, enhanced in vitro ADCC, as did addition of GM-CSF. Expression of GD2 (among GD2+ cell lines), CD47, CD276, and CD274 did not correlate with neutrophil-mediated ADCC. Chemo-immunotherapy of NSG mice with large metastatic disease burden showed significant improvement in median survival time (tm) and progression-free survival (PFS) in mice treated with combination of chemo+dinutuximab (n=13) or chemo+dinutuximab+GMCSF (n=14) vs. chemotherapy alone (n=6) [tm=69days, PFS=15% and tm=74days, PFS=14% vs. tm=28days, PFS=0%, $p<0.0001$ and $p<0.0001$, respectively]. Attempt at eliminating neutrophils, the most likely source of ADCC in NSG mice led to unacceptable level of death likely due to infection.

Conclusion: Neutrophils have significant anti-tumor effects against neuroblastomas in vitro but only in presence of dinutuximab, an effect that is enhanced with the addition of GM-CSF, or pre-treatment with chemotherapy. Absence or low expression of GD2 is a mechanism of resistance to neutrophil-mediated ADCC, while expression of CD47, CD276 or CD274 did not correlate with ADCC. Chemo-immunotherapy with dinutuximab doubled median survival time and led to progression-free survival in a high-tumor burden metastatic xenogeneic neuroblastoma model.

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The Novel Histone Deacetylase Inhibitor OBP-801 Induces Apoptosis in Neuroblastoma Tumor Cells

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Background: Despite advances in therapy, the prognosis of high-risk neuroblastoma (NB) remains poor. Thus, alternate therapeutic options are required to improve survival rate and reduce side effects of existing therapies in NB patients. Histone deacetylase (HDAC) inhibitors comprise a diverse class of compounds targeting these HDAC proteins, and are being rigorously studied for their role in several types of cancers, including NB. The aim of the present study was to investigate the antitumor effects of a novel HDAC inhibitor, OBP-801, as a potential therapeutic agent for treating NB.

Methods: Human NB cell lines IMR32, GOTO (MYCN gene amplified), SK-N-AS, and SH-SY5Y (MYCN gene not amplified) were used. Cell survival rate was evaluated by the WST-8 assay and cell cycle was analyzed by flow cytometry. Apoptosis was detected by Annexin V staining, and the expression of apoptosis related proteins was investigated by western blotting. Western blotting and quantitative RT-PCR were performed to examine the expression of MYCN.

Results: In all the NB cell lines, OBP-801 almost completely inhibited cellular proliferation with the up-regulation of p21. Cell cycle arrest at the G2/M phase was mainly observed after 24-h exposure to pharmacological levels of OBP-801. OBP-801 induced apoptosis in NB cells with the induction of cleaved caspase 3. Interestingly, OBP-801 repressed the expression of MYCN only in MYCN-amplified NB cells.

Conclusion: The novel HDAC inhibitor, OBP801, effectively inhibits growth and induces apoptosis of NB cells. Therefore, OBP801 may be a potent therapeutic option for NB.

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MYCN Mediates Cysteine Addiction and Sensitizes to Ferroptosis

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Oncogenic MYC profoundly alters metabolism and mediates an antioxidant response to maintain redox balance. The purpose of the study was to analyze the interplay of oncogenic MYCN or c-MYC, referred to here as MYC(N), activity with cysteine metabolism and ferroptosis, an oxidative, non-apoptotic, and iron dependent form of regulated cell death caused by ROS mediated massive lipid peroxidation (L ROS), using MYC(N)-driven childhood neuroblastoma (NB) as a model.

The intracellular amino acid levels at MYC(N)-high and MYC(N)-low cellular states were analyzed by HPLC. Effects on cell viability upon depletion of individual amino acids from the growth medium was tested in various cancer cell lines with regulable MYC(N). An unbiased high-throughput MYCN synthetic lethal siRNA screen was used to identify genes preferentially acting in the 'MYC(N)-high' state and protecting cells from ROS accumulation and ferroptosis. The capacity of cyst(e)ine uptake, intracellular cysteine synthesis via transsulfuration and glutathione biosynthesis was assessed in various NB cell lines and tissues using metabolome, RNAseq, ChiP-seq and global proteome analyses. Ferroptosis inducers (FINs) and inhibitors of transsulfuration were used to test their activity in various NB cell lines and in vivo xenograft models.
We found that intracellular cysteine depletion in a 'MYC(N)-high' context induces cell death by ferroptosis and identified multiple points in glutathione synthesis and metabolism, particularly detoxification of L-ROS, that are vulnerable in the 'MYC(N)-high' state as compared to the 'MYC(N)-low' context. Ferroptosis was dependent on MYC(N) expression and was enhanced by iron. Both cystine import and intracellular cysteine synthesis via transulfuration achieved the intracellular state supportive of oncogenic MYC(N)-driven growth without endangering the cell to ferroptosis. MYC(N) drives increased transsulfuration activity, rather than cysteine import, in tumor cells to maintain the cellular cysteine supply for glutathione synthesis.
Our findings together with new descriptions of the ferroptotic process establish a novel functional link between oncogenic MYC(N) and ferroptosis and imply regulation by cysteine-dependent glutathione availability. In MYCN-amplified childhood NB, MYCN mediates resistance to ferroptosis by activating transsulfuration of methionine to cysteine. We identified enzymes and antiporter proteins crucial to ferroptotic escape, providing multiple previously unknown sites that may be acted on therapeutically.

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High-Dose Chemotherapy for Children with Neuroblastoma – Review of Practice, Toxicities and Outcome in a Children's Hospital

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We performed a retrospective review of all neuroblastoma patients who underwent high dose chemotherapy at the country's children's hospital from 1998 – 2017 (20 years).

A total of 140 patients with neuroblastoma were seen during this period. We excluded 39 patients who only came for opinion or surgery or initial work-up only. Out of the 101 patients available for analysis, 58 had high risk disease (47 were stage 4 and over 18 months of age at diagnosis; 5 were below 18 months at diagnosis, with stage 4 NMYC-amplified disease; 6 were stage 3 with NMYC amplification and/or unfavorable histology).

High-dose chemotherapy were given to 38 patients (36 with high risk disease, and 2 with initial low/intermediate-risk disease who relapsed). High dose chemotherapy was not given to the 20 high-risk patients because of disease progression/refractoriness (10 patients), parental refusal (5 patients), or unclear reasons (5 patients, diagnosed between 1998 and 2001). Two patients were still ongoing induction chemotherapy. Before 2011 (21 patients), the high-dose therapy regime used was the "modified VAMP regime" consisting of teniposide, melphalan, cisplatin, with/without doxorubicin, and total body irradiation (TBI). From 2011, the regime given was busulfan/melphalan ("BuMel", 16 patients), or carboplatin/etoposide/melphalan ("CEM", 1 patient). Median days to neutrophil engraftment was 13 days (range 9-28). Two patients received autologous cord blood; their neutrophil engraftment was achieved on day +25 and day +28. There were 2 deaths from toxicity – 1 patient died from sepsis on day +33 (modified VAMP regime); 1 patient died from acute pulmonary edema 13-months post-BuMel, possibly related to severe late-onset sinusoidal obstructive syndrome (SOS) with desaturations, progressive renal impairment, and platelet refractoriness. There were 2 patients with mild-moderate SOS which resolved with defibrotide (1 patient) and supportive care (including oxygen). To date, 16/38 patients have died from disease- 13 from the modified VAMP cohort and 3 from the BuMel cohort; 20 are alive without disease. The 3-year overall survival is 47%.

Toxicities from BuMel, though rare and generally manageable, can be severe and life-threatening. The new VOD/SOS diagnosis/severity criteria will likely help improve diagnosis and management of SOS in neuroblastoma patients receiving BuMel.

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Low Dose Induction-Differentiation Therapy for Neuroblastoma in Children

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Purpose: The aim of this study is to report the efficacy of low dose induction-differentiation chemotherapy in the treatment for neuroblastoma (NB).

Materials and Methods: From 1992 to 2016, 199 NB patients enrolled into this study. Patients were separated into two groups, the initial treatment group: never received the chemotherapy; treated group: relapse patients or received chemotherapy in other hospitals. All the 199 cases were treated by low dose induction-differentiation therapy and traditional Chinese medicine, or low dose chemo+1st initial surgery (mass only partially resected) +low dose chemo continuously+2nd surgery.

Results: Among the 199 NB patients, 71 patients were completely release(CR), 45 patients were partially release(PR), 25 patients were stable disease(SD), 4 patients were progressive disease(PD), 49 patients were dead, and 5 patients loss of follow-up. K-M survival analysis showed that the 5 years survival rate of the initial treatment group was higher than the treated group (68.4% VS 49.6%,p=0.035). And 13 patients showed amazing therapeutic outcome, there were 10 patients who were treated by low dose induction-differentiation chemotherapy and traditional Chinese medicine only, the tumor disappeared completely. In these 10 cases, five were Stage IVs, four were Stage IV and one was Stage III. And the three stage III NB patients was treated with low dose induction-differentiation chemotherapy + surgery (only partial resection) after surgery low dose chemotherapy continued, finally, a second surgery was performed, and the mass was completely removed, each tumor mass was transformed into benign GN confirmed by pathology.

Conclusion: The low dose induction-differentiation therapy is an efficient method for the treatment of NB in children, and it can improve prognosis of the NB children.

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Preclinical Development of a LIN28B Inhibitor of Neuroblastoma

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The two LIN28 RNA binding proteins, LIN28A and LIN28B, are well-studied oncogenes demonstrated to be drivers in several solid tumors. They seem to exert this effect mainly by preventing maturation of miRNAs of the let-7 family, which are potent inductors of cell differentiation. Therefore, when LIN28 proteins are highly expressed, they lock cells in a stemness program. LIN28A and LIN28B expression is usually mutually exclusive in tissues.

Lin28A/B and let-7 miRNAs are essential for sympathetic neuroblast proliferation during normal development. The first published genome-wide association study for familiar neuroblastoma (NB) unveiled a polymorphism closely associate with disease and patient survival, promoting LIN28B transcription. LIN28B is highly represented in NB samples compared to other tumor types, and its enforced expression in the sympathoadrenergic lineage of mice reproduces the human disease.

Therefore, LIN28B is a validated target for NB therapy. We aimed at identifying small molecules able to interfere with the binding of LIN28B to the pre-let-7g miRNA, exploiting the amplified luminescent proximity homogeneous assay technology on recombinant human LIN28B produced in human cells with a library of 5000 molecules. By validation of the hits with orthogonal approaches, including RNA electrophoretic mobility shift assay, we identified a family of candidate molecules with common scaffold binding to the LIN28B cold shock domain and displacing the let-7 interaction with high affinity. We produced by medicinal chemistry some derivatives of the original scaffold and found by structure-action relationship studies the pharmacophore, which we validated by soaking experiments in LIN28B crystals and X-ray diffraction. We finally performed on these candidates some in vitro ADME tests.

We then run an extensive molecular and phenotypic characterization of the action of two selected leads in NB cell lines, spheroids and neurospheres from primary tumors. The same molecules were tested, with or without the use of carriers, in xenotransplanted nude mice and two genetic mouse models of neuroblastoma, driven by LIN28B and by MYCN.

We, therefore, report the first complete preclinical characterization of two inhibitors of LIN28B in human cells and mouse models of neuroblastoma, which could pave the way for clinical development.

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Label-free Isolation of Circulating Tumor Cells Using Spiral Microfluidic Chip for Prognostication and Disease Monitoring in Neuroblastoma

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Background: Minimal residual disease (MRD) is an important cause of relapse in neuroblastoma but is not considered in current disease and treatment response evaluation. Historical MRD detection methods using RT-PCR and immunocytology on bone marrow aspirates (BMAs) have low sensitivity and provide limited quantitative and biological information. While single-cell capture systems allow isolation of peripheral blood circulating tumor cells (CTCs), current affinity-binding methods may be biased. We sought to study the utility of a label-free size-based cell separation system, and MRD characteristics of CTCs isolated with it.

Methods: We obtained blood and BMAs at 6 key disease evaluation points from neuroblastoma patients undergoing current treatment protocols and processed them through a spiral microfluidic chip (Clearbridge Biomedics) to isolate CTCs in an ultra-fast, label-free manner. Isolated CTCs were enumerated and characterized by immunocytochemistry and RT-qPCR using established MRD markers TH, PHOX2B and GD2 synthase. Expression profiling of CTCs was performed using NanoString platform. Results were correlated with clinical, pathological and outcome data.

Results: Samples from 10 neuroblastoma patients of varying stage and histological grade were recruited from KK Women's and Children's Hospital. Identity of CTCs was confirmed by expression and morphological analysis of established MRD markers. PHOX2B appeared to be the most reliable marker and sufficient for confirming the presence of CTCs. From CTC fractions of blood samples taken at diagnosis, PHOX2B expression was significantly associated with liver, marrow and bony metastases ($P<0.05$), and number of isolated CTCs – characterized by cellular atypia – were significantly associated with metastases to marrow and bone ($P<0.05$). Interestingly, PHOX2B expression was present in the CTC fractions of peripheral blood and BMA of a patient with metastasis to multiple sites. Conversely, PHOX2B expression was present only in the CTC fraction of BMA but not peripheral blood of another patient with only marrow involvement. This demonstrates the potential for the method to assess metastatic risk.

Conclusion: Quantification, characterization and profiling of isolated CTCs from peripheral blood of neuroblastoma patients using spiral microfluidic chip at various disease evaluation points could be a less invasive, less costly, more upfront and more predictive option in prognostication and disease monitoring.

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Gain of Telomerase Reverse Transcriptase (TERT) Expression Together with Loss of MicroRNA-128A Expression is Associated with Poor Survival in Neuroblastoma

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Background: Clinical heterogeneity is a hallmark of neuroblastoma (NB). Rearrangements of TERT gene with correspondent gain of expression were identified as adverse prognostic markers (M. Peifer,2015), while significance of microRNA expression profile in NB is undiscovered.

Aim: Investigation of prognostic significance of miR128A and TERT expression in primary NB.

Methods: RNA samples from 103 fresh-frozen NB tissues were reverse transcribed and used in quantitative PCR for normalized miR128A and TERT expression evaluation. Copy number variations (CNVs) relevant for NB were determined by MLPA and FISH. Correspondence of miR128A and TERT expression levels to event-free survival (EFS) was evaluated by ROC-analysis and established threshold levels (TL) were applied for group separation in subsequent survival analysis. Median of follow-up time achieved 5.8 years.

Results: Both abundant TERT expression and downregulation of miR128A were associated with superior rate of adverse events (ROC-analysis p=0.027, TL=4,7E-3 and p=0.004, TL=4.6E-2 respectively). Based on the presence of expressional abnormalities patients were separated into four groups. EFS in group of patients with TERT expression above 4,7E-3 (group TERT) was 0.66SE0.07, in patients with miR128A expression below 4.6E-2 (group miR128A) was 0.64SE0.15, patients harboring both TERT overexpression and lack of miR128A expression (group miR128/TERT) had dismal outcome: EFS 0.29SE0.11, comparing to patients without these abnormalities (group neither): EFS 0.92SE0.06, p<0.001. Analogously cumulative incidence of progression in the group TERT was 0.32SE0.07, miR128A – 0.36SE0.15, miR128A/TERT – 0.71SE0,11, neither – 0.08SE0.06, p<0.001.

MYCN amplified (MNA) cases tended to accumulate in the groups TERT and miR128/TERT (p=0.061), while gain of chromosome 17, 9p and 14q deletions prevailed in the group neither (p=0.014, 0.043 and 0.017 accordingly). MNA and 14q deletion had prognostic significance in the correspondent groups (p<0.001, p=0.022). The combination of expressional biomarkers and CNVs allowed distinguishing of patients with unequal outcome: MNA EFS=0.25SE0.11, MYCN single copy patients divided into groups miR128A/TERT (0.40SE0.15), miR128A (0.60SE0.15) and TERT (0.74SE0.08). Group neither was separated basing on presence (0.71SE0.17) or absence (EFS=1.00) of 14q deletion, p<0.001.

Conclusions: Levels of miR128A and TERT expression together with CNVs could be useful prognostic biomarkers for risk defining in neuroblastoma patients.

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Early-Phase Patient-Derived Cell Cultures of Neuroblastoma as a Personalized Preclinical Model

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Introduction: Personalized preclinical models can serve as avatars to facilitate individualized treatment strategies. However, patient-derived xenografts (PDXs) are limited by cost and efficiency; cell cultures, while inexpensive and more scalable, traditionally have limited recapitulation of original tumor cell populations, and no established methods exist for their derivation from embryonal tumors. We developed a system to generate early-phase multi-lineage patient-derived cell cultures (PDCs), evaluated its efficiency and utility, and compared the generated PDCs with parallel PDX and original patient tumors.

Methodology: PDCs were derived from pre-treatment, post-treatment and relapse neuroblastoma tumors from KK Women's and Children's Hospital. Four media conditions were compared sequentially in two phases to select for an optimal media condition. In-vitro growth dynamics, morphology, immunoreactivity, gene expression and functional chemoresponse of PDCs were characterized, and compared with PDX and patient tumors.

Results: From 28 neuroblastoma specimens, 24 PDC and 4 PDX lines were established. Engraftment in-vitro was associated with INPC classification and histology ($P<0.05$), metastatic disease status ($P<0.001$), and MYCN amplification ($P=0.085$). Achievement of log-phase growth was associated with 11q deletion and metastatic disease status. Median time to first passage was faster in-vitro compared with PDXs (23.6 and 79.3 days, respectively)–ahead of median time patient relapse and death events (292.5 and 657.5 days, respectively).

The optimal media yielded engraftment in 84.6% (11/13 lines), and log-phase growth in 81.8% (9/11 lines) which sustained for mean 47.8 days; total cell numbers expanded exponentially from median $1x10^5$ to $6.8x10^8$. The ratio of cell sub-populations was maintained over 10 passages (neuroblastic (N-type, 40.9±1.4%), stromal (S-type, 35.1±1.2%) and stem-like/intermediate (I-type, 24.0±1.7%)) in optimal media, whereas other media did not sustain I-type cells beyond 5 passages, with correspondingly shorter times to senescence. Immunoreactivity to PHOX2B, TH and GD2 verified the tumor characteristics of the PDCs, which were further characterized by gene expression and functional chemo-response assays.

Conclusion: We developed an optimized in-vitro system of early-phase patient-derived neuroblastoma cultures that efficiently expanded and recapitulated original tumor cell subpopulations in a consistent manner from pre-treatment, post-treatment and relapse tumors. This platform could be potentially utilized for individualized real-time ex-vivo characterization and drug screening of patient tumors.

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MES and ADRN Type Neuroblastoma Cells Reveal Principles of Epigenetic Gene Regulation in Neuroblastoma

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During development, gene expression is controlled by integrated action of transcription factors and epigenetic programming. This system progressively creates the divergent cell lineages and their developmental stages. Major principles of this system are being resolved and it has become clear that aberrant epigenetic programming is among the key drivers of cancer. Elucidation of the epigenetic control mechanisms defining neuroblastoma growth and behavior is therefore of pivotal importance.

We recently described that most neuroblastoma tumors consist of two cell types. A majority of cells has an adrenergic lineage differentiation phenotype (ADRN type) and a minority of cells shows an undifferentiated mesenchymal character (MES-type). MES cells are clinically relevant as they are relatively resistant to chemotherapeutics used for neuroblastoma treatment. Both cell types spontaneously transdifferentiate into one another, giving high plasticity to neuroblastoma. MES- and ADRN-type cell lines derived from the same tumor and analyzed for mRNA expression, chromatin modifications and super enhancers allowed the identification of their Core Regulatory Circuitries (CRC). In each cell type, about 20 transcription factors form a feed-forward network that imposes the lineage- and stage-specific transcription profiles.

We have used the isogenic pairs of MES and ADRN cell lines to establish the genome-wide DNA binding patterns of a series of the core transcription factors. This allowed us to establish the wiring of CRC transcription factors with their hundreds of downstream target genes. In addition, we analyzed the isogenic pairs of MES and ADRN cell lines for various active and repressive chromatin modifications. Both cell types appear to have strong differences in chromatin modification, both at the level of individual genes and at the level of entire chromosome bands. The chromatin patterns associated with gene silencing and gene activation are not symmetric in both cell types and testify that MES and ADRN cell types reflect subsequent developmental stages. The identified core regulatory circuitries and chromatin modification systems give a first insight in the principles of epigenetic control of mRNA transcription in the two key neuroblastoma cell types.

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Long Term Follow Up of Children>18months with Stage 4 Neuroblastoma Treated with Immunotherapy, Chemotherapy and Local Radiation Therapy

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Background: Children >or=18 months with INSS stage 4 neuroblastoma had poor outcome despite surgery, chemotherapy, local radiation therapy (RT) and auto-hematopoietic stem cell transplant (auto-HSCT). In recent years, the addition of anti-GD2 monoclonal antibodies (3F8/Ch14.18/Ch14,18-CHO) had shown additional benefit in this group of patients but the long-term outcome remains unknown.

Methods: Children >or=18 months with INSS stage 4 neuroblastoma were treated with N7 based (MSKCC) chemotherapy regimen. The recruitment period was from 1999 to 2011. Attempted surgical excision was performed after 4th or 5th course of chemotherapy. Focal RT was given to the primary site before or after the auto-HSCT. 3F8 was started at the 5th course before the stem cells harvesting. A total of 5 courses of 3F8 were given and each course consisted of 5 days of infusion. There was no IL-2 or GM-CSF given with the 3F8.

Results: 38 children were recruited, and their median age was 3.4yrs (1.5yrs-6.9yrs). M:F=23:15. 31% had MYCN amplified. 92% had positive bone marrow infiltration and 74% had positive bone involvement by bone scan. The 3 patients with no bone marrow infiltration had positive bone scan involvement. 47% achieved CR after 2 courses of induction chemotherapy and another 35% had stable disease. All survivors have at least >5 years of follow up. The median follow-up time was 13.4yrs. The 5yrs EFS was 42.1±7% and OS was 50±8%. However, we noticed there were late relapse up to 8.5yrs after treatment. The 10yrs EFS was dropped to 30% and OS was 32%. The relapse could occur in the bone, brain, lung, lymph nodes and we have no local relapse in this cohort. When compared to historical control using similar chemotherapy without immunotherapy, the 5yrs & 10yrs OS were significantly better 50% vs 31% (p=0.02) and 30% vs 20% respectively.

Conclusion: Immunotherapy using a short 5 days course without addition of growth factors or cytokines could improve long term outcome of children with stage 4 neuroblastoma. However, late relapse still could happen, and additional strategy may be needed to prevent such occurrence.

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Isotretinoin Bone Toxicity – A Case Illustration of a Known but Rare Complication

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Retinoid bone toxicity is a known side effect of retinoic acid therapy. However, it is not commonly encountered in clinical practice.

The patient is a female who was diagnosed at 9 months of age to have NMYC-amplified neuroblastoma involving both adrenals, with multiple liver metastases and also bone marrow metastases (<10%). She was risk-stratified as high-risk and treated with intensive chemotherapy, surgery, high-dose chemotherapy with busulfan-melphalan and autologous stem cell rescue, radiation to abdominal areas, as well as maintenance with 12 cycles of 13-cis-retinoic acid (isotretinoin), as per institution preference. Two years post-completion of therapy, she started to complain of intermittent pain in her wrists and ankles, with occasional mild limping. Disease evaluations did not show evidence of neuroblastoma recurrence. She gradually developed increasing prominence of bilateral lateral malleoli and flaring of her left wrist. Plain X-rays and magnetic resonance imaging (MRI) scans demonstrated premature physeal fusion affecting her left distal radius and bilateral distal tibia. There was no X-ray evidence of ossification of the anterior longitudinal ligament of the spine to suggest the presence of diffuse idiopathic skeletal hyperostosis. Her serum biochemistry profile was normal. The child has not had any significant physical discomfort or functional impairment. However, with continued growth of the unaffected long bones, the premature physeal fusion will likely lead to increasing deformities. The child therefore underwent arthroscopic assisted physeal bar resection and autograft fat interposition. She is continuing close surveillance to monitor her bone growth and to see if the bone toxicity process will stabilize or continue to evolve.

Premature physeal fusion is a rare but potentially deforming and debilitating toxicity of retinoic acid therapy. The effect of retinoic acid therapy on bone health in young children is not well understood. Extended period of retinoic acid therapy beyond the usual 6 months should probably be avoided.

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Evaluation of Protein Kinase C Iota (PRKCI) as a Target for the Treatment of Recurrent Neuroblastoma

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Although prognosis of high-risk neuroblastoma (NB) has improved recently, recurrent tumors still pose an important clinical problem. We previously observed an increased expression of Protein Kinase C Iota (PRKCI) in recurrent neuroblastoma compared to the paired primary tumors. Therefore, PRKCI might play a yet unknown role in the development of difficult to treat metastases and /or recurrent tumors. We used CRISPR / Cas9 gene editing approaches to knockout and overexpress PRKCI in NB cells. Both, knockout and overexpression were confirmed by qPCR and Western Blot analysis, and resulting cells were analyzed with regard to proliferation, clonal outgrowth, migratory capacity and invasiveness. PRKCI knock out (KO) surprisingly increased proliferation of NB cells compared to parental cells and cells with overexpression of PRKCI. However, migratory capacity was reduced for PRKCI KO cells compared to the controls. Further investigation of migratory and invasive capacity by Boyden chamber assays showed that overexpression of PRKCI results in increased invasiveness of SHEP cells, however, this did not reach statistical significance. Although PRKCI is found to be elevated in aggressive and recurrent NB tumors, both CRISPR-mediated PRKCI knock-down or overexpression resulted in formation of viable subclones in SHEP cells. While PRKCI knock-down reduced migratory capacity, it surprisingly increased clonal survival and proliferation. In vivo experiments will shed light on the role, if any, for PRKCI in modulating neuroblastoma aggressiveness.

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Possibility of Standardization of Laparoscopic Surgery for Abdominal Neuroblastoma: Results from a Nationwide Survey in Japan

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Background: Recent advances in minimally invasive surgery (MIS) brought many advantages including less operative scarring, an earlier recovery and cosmetic appearance, not only in adult patients, but also pediatric patients. Neuroblastoma (NB) is suitable for MIS approach, but indication and technical issue are unclear. The aims of this study were to clarify the present status of MIS for NB in Japan, and to discuss the possibility of standardization of MIS approach for NB.

Methods: The preliminary questionnaires requesting the numbers of NB cases who underwent MIS approach from 2004 to 2016 were sent to 159 Japanese major institutes of pediatric surgery. The secondary questionnaires were sent the institutions which had the NB cases underwent MIS approach to collect the detail data.

Results: We received replies from 133 institutes (83.6%). Of 136 institute, 82(61.6%) institute had the operative cases of NB. Total number of operative cases were 1435. MIS approach was applied for 174 cases (12.1%). Of 174 cases, the completed forms of 137 (78.7%) patients were sent back, and 39 cases had mediastinal NB and 98 cases had abdominal NB. As regarding abdominal NB, detail analysis was performed. Male/Female ratio is 50/48. Clinical stage was as follows: 1:30(30.6%), 2:11(11.2%), 3:11(11.2%), 4:42(42.9%), 4S:3(3.1%), unknown: 1(1.0%). Forty-seven cases (48.0%, tumor size: 8.0±3.1 cm) underwent laparoscopic biopsy, and 2 cases (4.3%) were converted to laparotomy. Sixty cases (61.2%, tumor size: 4.1±2.3 cm, Primary resection:37) underwent MIS for radical resection and 9 (15%) cases were converted to laparotomy in radical resection. MYCN amplification was 10 cases in radical resection. The reasons for conversion were bleeding and severe adhesion, and there was no relation to tumor size and MYCN amplification in conversion cases. Postoperative complication were recognized in 7 cases (4:renal dysfunction, 2: lymphorrhea, 1:port site hernia). IDRF was confirmed in only 12 cases (56%), preoperatively

Conclusions: MIS was performed in limited case of NB. Indication was determined based on institution's experiences. Resectable tumor size was almost below 5cm but depending on the patients' height. The indication and technical standardization of MIS approach for abdominal NB would be conducted based on prospective trial.

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In Vivo Modelling of Chemoresistant Metastatic Neuroblastoma Identifies JAK-STAT Pathway as A Potential Therapeutic Target

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Neuroblastoma is a pediatric solid tumor that in high-risk form is aggressively metastatic and poorly responsive to conventional treatment, with a 3-year survival of <15%. Development of improved therapy regimens active in the relapse-refractory setting has been hampered by lack of suitable preclinical models that natively recapitulate the rapid acquisition of chemoresistance and bone-marrow metastasis characteristic of this aggressive tumor. The Th-MYCN genetically engineered mouse model develops aggressive neuroblastoma driven by tissue-specific expression of MYCN (Th-MYCN) but is chemosensitive and lacks spontaneous metastases representative of human disease. Following individualized and dose-adjusted multicycle chemotherapy attempting to mirror the genotoxic stress characteristic of clinical induction chemotherapy regimens, we achieved mice (Th-MYCNR) that are reliably cross-resistant to chemotherapy with bone-marrow metastases present in 70% of cases.

DNA exome analysis was used to monitor for genomic alteration associated with bone marrow metastatses. We identified resistant clones with unique genomic signatures that allowed us to genetically track the development of metastatic disease. Resistant tumors showed altered microenvironment; enhancement of tumor stroma and tumor associated fibroblasts. RNA sequencing analysis of resistant tumors revealed upregulation of genes that are associated with high-risk neuroblastoma; Cyclin B1 interacting protein 1 (Ccnb1ip1) a cell cycle regulator, Thymidine Kinase (Tk1), and genes found to be enriched in association with the 17q gain subgroup of patients. Furthermore, we found activation of the anti-apoptotic pro-metastatic JAK-STAT pathway in resistant tumors. Inhibition of this pathway in vitro and in vivo using the JAK1/JAK2 inhibitor, CYT387, resulted in reduced growth.

Our results highlight the benefit of in vivo modeling of resistance in order to recapitulate the tissue complexity and patient diversity seen in the clinic and suggest JAK-STAT inhibition as a potential therapeutic approach.

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The Metastatic Role of SOX9 in Neuroblastoma

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Approximately 50% of neuroblastoma patients suffer from metastatic disease at diagnosis and require intensive treatment. Despite intensive therapy, the vast majority of patients still have poor clinical outcome. Investigating the molecular basis of neuroblastoma metastasis is crucial to develop an effective therapy. The similarities between neural crest development and neuroblastoma progression have been recognized. Transcription factor SOX9 is involved in cell migration during neural crest delamination and is implicated in formation and growth of various tumors. Currently, little is known about the role of SOX9 in neuroblastoma pathogenesis, hereby we aim to investigate the function of SOX9 in neuroblastoma metastasis. Overexpression of SOX9 significantly enhanced cell migration, invasion, and colony formation in IMR-5 and SH-SY5Y cells. On the other side, knockdown of SOX9 reduced these abilities in SK-N-AS. In orthotopic model, tumor growth remarkably increased in IMR-5 SOX9 overexpressing compared to IMR-5 vector control, while it significantly decreased in SK-N-AS SOX9 knockdown compared to SK-N-AS control. Interestingly, lung metastasis was detected in 3 of 6 SK-N-AS control mice, and none of 11 SOX9 knockdown mice was found metastasis. By comparing gene expression profile of IMR-5 versus SK-N-AS and SK-N-AS control versus SOX9 knockdown, 1394 genes were identified as SOX9-activated genes and 739 genes were identified as SOX9-repressed genes. Analysis of these genes revealed that SOX9-activated genes were highly related to extracellular matrix organization, cell adhesion, and cell migration, which was compatible with our in vivo and in vitro results. On the other side, SOX9-repressed genes were mainly involved in neuron development and differentiation. Taken together, these results clearly indicate that SOX9 enhances cell motility and invasive ability of neuroblastoma cells in vitro and promotes tumorigenesis and metastasis in in vivo model. Our data point out that SOX9 is a new promising therapeutic target in neuroblastoma.

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Investigating the Role of Cancer Stem Cell Related Genes in Acquired Drug Resistance in Neuroblastoma

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Introduction: Neuroblastoma is a paediatric tumour that develops from embryonic neural crest cells that give rise to the sympathetic nervous system. Aggressive high-risk disease remains a clinical challenge and despite multimodal therapy survival rates are poor. A major obstacle in the successful treatment of this disease is the development of acquired resistance to chemotherapeutic agents. We hypothesize that aggressive neuroblastomas acquire a more immature phenotype coupled with an increase in expression of cancer stem cell related genes that contributes to drug resistance.

Aim: To investigate the role of cancer stem cell genes in neuroblastoma cell line models of acquired drug resistance.

Methods: To study the drivers of acquired drug resistance we performed RT2 profiler - Human Cancer Stem Cell Array (Qiagen, UK) on three paired parental and vincristine resistant cell lines (IMR5, IMR32 and NGP) were derived from the Resistant Cancer Cell Line (RCCL) collection (www.kent.ac.uk/stms/cmp/RCCL/RCCLabout.html). Raw expression data were log transformed and processed using quantile normalization method. Linear models were applied for the selection of significantly differentially expressed genes using criteria P.Val.Adj < 0.05 and log2FC > 1 & < -1. Functional enrichment analysis was performed on 11 genes appearing among three comparisons for their potential involvement in functional processes and pathways of drug resistance.

Results and Summary: Several genes were identified that were deregulated in 3/3 drug resistant cell lines compared to parental cell lines including Sox-2, Lin28A, Lin28B, MYC and Snai1. Many of these genes are associated with drug resistance mechanisms in other solid cancers and have been shown to be involved in pathways related to stem cell signalling, differentiation and development, as well as the Notch signalling pathway. Our data suggests a potential enrichment of cancer stem cell related genes that contribute to the development of acquired drug resistance in neuroblastoma and warrants further study on the functional effects of these genes/ pathways.

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In Vivo and In Vitro Methods to Assess and Possibly Improve Killing of Human Neuroblastoma by Natural Killer Cells

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Neuroblastoma (NB) is the most commonly occurring extra-cranial solid tumor in children and accounts for ~10% of all childhood cancers. Half of NB cases occur in children under two years of age. NB is thought to originate from neural crest precursor cells of the developing sympathetic nervous system, with primary tumors localizing in the abdomen, adrenal gland or lumbar ganglia; however, its metastatic spread includes such diverse tissues as the lymph nodes, bone, bone marrow, liver and skin. Low and intermediate risk NB have excellent prognosis, with greater than 90% survival. However, the high-risk metastatic form that accounts for ~50% of NB has survival outcomes of less than 30% with conventional therapy. Due to the heterogenous genetic landscape inherent to NB, a major focus for therapy development is aimed at harnessing the anti-tumour capabilities of the immune system, in particular that mediated by natural killer (NK) cells.

Approaches to evaluate killing of target cells in vitro include flow-based and chromium-based assays. Here we describe a simple and reproducible system that facilitates both in vitro and in vivo monitoring of NB cell killing by NK cells based on dual color click beetle red and green luciferase bioluminescence for separate labeling of target and effector cells. In vitro, the system is convenient for longitudinal monitoring and optimization of target cell killing, while in vivo there is the potential for tracking of activities within the target tissues. This approach could reveal the impact of modulation of patient NK cells on targeting tumor niches and increasing cytolytic activity. This will establish a more stringent pre-clinical testing platform for the development of immune therapies for NB.

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Organoid Patient Derived Cells Guide Personalized Neuroblastoma Treatments

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Goal: Neuroblastoma (NBL) is the second most frequent pediatric solid tumor. We have seen an increase in survival for children with NBL, however treatment still consists of many cycles of chemotherapy and radiation. We reasoned, that in vitro high throughput drug screening (HTDS) of patient derived organoids using a library of targeted therapies, would highlight novel targeted and personalized treatment options.

Methods: We optimized an in vitro 3D culture for high-risk NBL samples, initially established as patient derived xenografts (PDX). We screened a total of 10 patient samples with genomic information using two different drug libraries: Informer Set (320 drugs) compiled by the Cancer Target Discovery and Development network which is focused on cancer pathways and SEngine Library (120 drugs) focused on clinically relevant therapies. The screen was performed in a 384 well-format and cell viability was measured by Cell Titer Glow. Organoids were characterized by immunohistochemistry and selected genomic and pathway alterations from the original tumors were confirmed.

Results: Here we highlighted unique results from 3 cases. The first sample showed sensitivities to CDK inhibitors in concordance with amplification of CDK4. However, for this patient additional drug sensitivities were not directly linked to a genetic alteration, such as Talazoparib (PARP inhibitor) and MK-1775 (WEE1 inhibitor) indicating sensitivities in the DNA repair pathway. The second case had unique sensitivities to IGF1R and Insulin Receptor inhibitors (Linsitinib and BMS-754807) indicating a specific addiction to this growth-factors/survival signal. The third sample had multiple sensitivities linked to genomic alterations, such as Dabrafenib, concordant with the presence of a BRAF activating mutation. In addition, sensitivity to Alisertib was detected as previously reported for MYCN amplified NBL, but this was not true for other MYCN amplified cases. Interestingly, this third sample also showed profound sensitivities to PARP inhibitors, most likely related to a mutation in the BRP1 gene a BRCA1 interacting protein gene.

Conclusion: A newly established NBL 3D patient organoid culture system coupled with personalized HTDS and genomic analysis offers an effective platform for the discovery of novel personalized therapies and drug combinations tailored to individual patients. In this NBL cohort, we discovered both drugs predicted by the patient-specific genomic alterations, as well as novel patient-specific drug sensitivities without a known genomic marker. More in depth genomic analysis in underway, but epigenetic alterations could explain these exceptional responses. In summary, this investigational study of 10 NBL highlights the need of personalization of targeted therapies.

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IgA anti-GD2 As an Improved Immunotherapeutic for Neuroblastoma

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Neuroblastoma is the most common extracranial tumor of childhood. Recently, the antibody dinutuximab, directed against the ganglioside GD2, was FDA-approved for treatment of high-risk neuroblastoma patients. Dinutuximab improved event-free survival in comparison to standard treatment.

Strikingly, Antibody Dependent Cellular Cytotoxicity (ADCC) by dinutuximab is predominantly mediated by granulocytes, though NK cells are indicated as the main effector cells for IgG treatment of cancer. Next to ADCC, complement dependent cytotoxicity is another effector mechanism of dinutuximab. Unfortunately, complement activation is also an important cause of severe toxicity, which is predominantly expressed as neuropathic pain.

On these grounds, we converted the isotype of dinutuximab from IgG1 to IgA. Because IgA lacks the C1q binding site, it is unable to initiate activation of the classical complement pathway. In addition, IgA exclusively interacts with leukocytes via the activating Fc receptor CD89 and is therefore a potent activator of granulocytes, monocytes and macrophages. Our data show that IgA dinutuximab is superior to the currently used IgG isotype in killing of neuroblastoma cells in vitro without complement activation. Additionally, when tested in vivo, IgA displayed a strong reduction in neuropathic pain compared to IgG. Collectively, these data provide a proof of concept for IgA as an attractive alternative to treat neuroblastoma.

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Phosphoproteome and Gene Expression Profiling of ALK Inhibition in Neuroblastoma Cell Lines Reveals Important Conserved Oncogenic Pathways

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Anaplastic Lymphoma Kinase (ALK) is a tyrosine kinase receptor which is a clinical target of major interest in cancers. Mutations and rearrangements trigger the activation of this receptor and its downstream signaling pathways. ALK mutations have been identified in both familial and sporadic neuroblastoma (NB) cases and have also been reported in 30-40% of relapsed NB cases, which makes ALK a bonafide target in the therapy for neuroblastoma. ALK tyrosine kinase inhibitors (TKIs) are currently in clinical use for the treatment of ALK-positive non-small cell lung cancer (NSCLC) patients. However, monotherapy with the ALK inhibitor crizotinib did not result in important responses in neuroblastoma patients with ALK alterations, raising the question if combinatory therapy would be more effective. In this study we established both phospho-proteomic and gene expression profiles of ALK activity in neuroblastoma cells employing first and third generation ALK TKIs, to identify the underlying molecular mechanisms and identify relevant biomarkers, signaling networks and new therapeutic targets. This analysis has unveiled a number of important leads for novel combinatorial treatment strategies for neuroblastoma patients as well as an increased understanding of ALK signaling involved in this disease.