BAPTA-AM significantly reduced PKC activity following PKL inhibition. These data highlight the power of phosphoproteomic profiling for the rational design of drug combination strategies, which need to be tested in vivo prior to clinical trials for DIPG.

**DIPG-31. MOLECULAR MECHANISMS AND FUNCTIONAL IMPACT OF ABERRANT SPLICING IN DIFFUSE MIDLINE GLIOMAS**

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Fewer than 1% of children diagnosed with diffuse-midline glioma (DMG) survive for more than 5 years, because no effective therapies exist for these pediatric cancers. Here, we sought to identify and characterize mechanisms of aberrant splicing (AS) in primary DMG tumors. We observed transcriptome-wide AS (9,805 differential splicing variations in 4,734 genes), and identified a DMG-specific splicing signature, that included known cancer genes. We hypothesize that AS of cancer genes play a role in DMG tumor formation. Assessing whether splicing factor dysregulation impacted known cancer transcripts, we discovered several splicing factors, including SRRM4, SRRM3 and RBFX03 to be down-regulated in DMG. Additionally, we found that at least half of binding motifs for these proteins were within AS regions of these mis-spliced exons. We also observed recurrent significant exon inclusion in tumor suppressor SMARC4, an integral member of the SWI/SNF family of proteins involved in chromatin remodeling. Further, we identified AS of exon 7 in DPP2, creating a complete mRNA transcript switch in DMG. Since SRRM4, SRRM3 and RBFX03 are known regulators for neural-specific microexons, we focused on microexon splicing changes. We hypothesized that these data would provide insights into disease-specific microexon-rich splicing in these tumors. We identified 245 known microexons lost or gained in DMG. Moreover, a quarter of which were observed in known cancer genes, with the most frequent splicing event being gain of a clathrin-binding site in the tumor suppressor BIN1 with a concurrent loss of an out-of-frame microexon in the oncogene BAK1, presumably activating it. Altogether, our results suggest that aberrant splicing may be an alternative mechanism driving DMG tumorigenesis and we are currently molecularly validating a subset of these events with the overall goal of identifying novel therapeutic targets for DMG tumors.

**DIPG-32. AKT SIGNALING DRIVES RESISTANCE TO ONC201 IN DIFFUSE INTRINSIC PONTINE GLIOMA (DIPG)**

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Diffuse intrinsic pontine glioma (DIPG) is a highly aggressive, childhood brainstem cancer with a median overall survival of 10 months post diagnosis. Remarkably, 80–90% of patients harbor recurring point mutation in histone H3, which induces a lysine for methionine substitution at amino acid 27 (H3K27M) in either H3.1 (H3F3A~25%) or H3.3 (H3F3A ~65%) variants. Using the blood-brain barrier (BBB) permeable DRD2 antagonist, ONC201 in clinical trials for DIPG and H3K27M-mutant gliomas (~100% of WT-H3 and H3K27M mutant DIPG cell lines (~n=5), compared to 50% of H3.3K27M-mutant DIPG (n=6). Investigations to identify the mechanisms of resistance to ONC201, revealed that cell lines with decreased sensitivity upregulated the PI3K/AKT/MTOR signaling axis to drive phosphorylation of AKT and increase metabolic activity. Combined administration of ONC201 and the BBB-permeable PI3K/AKT inhibitor, paxalisib (previously DCC-0084, in clinical trials for newly diagnosed DIPG – NCT03696353), showed synergistic cytotoxicity, reduced PI3K/AKT signaling and metabolic reprogramming to drive apoptosis in all DIPG cell lines tested. This combination was used to treat a 3-year-old DIPG patient, commencing 14 weeks post disease progression, completing 40 weeks of therapy prior to her passing, December 2019. These studies highlight the potential of combined administration of two safe, BBB penetrant, oral targeted therapies and supports testing under clinical trial conditions.

**DIPG-33. CHARACTERIZING THE NEURO-VASCULAR UNIT IN DIFFUSE INTRINSIC PONTINE GLIOMA**

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Diffuse intrinsic pontine glioma (DIPG) is a childhood brainstem tumor with a median overall survival of eleven months. Lack of chemotherapy efficacy may be related to an intact blood-brain-barrier (BBB). In this study we aim to compare the neuro-vascular unit (NVU) of DIPG to healthy pons tissue. End-stage DIPG autopsy samples (n=5) and age-matched healthy pons samples (n=22), obtained from the NIH NeuroBioBank, were immunohistochemically stained for tight-junction proteins claudin-5 and zona occludens-1 (ZO-1), basement membrane component laminin, and pericyte marker PDGFRα. Claudin-5 stains were also used to determine vascular density and diameters. In DIPG, expression of claudin-5 and ZO-1 was reduced, and subcutaneous lamination was decreased compared to end-stage Pontine cell nuclei. Laminin expression at the glia limitans was reduced in both pre-existing vessels and neovascular proliferation. In contrast to healthy pons, no PDGFRα expression was detected. The number of blood vessels in DIPG was significantly compared to healthy pons (P=0.01). Distribution of larger blood vessels (~50µm) did not differ between groups (P=0.223). Mean vessel diameter was 3.1±0.9µm for DIPG versus 7.7±2.4µm in healthy pons (P=0.016). Our study demonstrates evidence of structural changes in the NVU in end-stage DIPG. Chemotherapeutic inefficacy could be a consequence of the H3K27M mutation and that this deregulated SEC signaling overcomes repressive transcriptional regulation in order to suppress differentiation and promote self-renewal. We hypothesized that aberrant SEC expression occurs as a consequence of the H3K27M mutation and that this deregulated SEC signaling overcomes repressive transcriptional regulation in order to suppress differentiation and promote self-renewal of DIPG tumor stem cells. We interrogated the role of AFF4 in DIPG using an shRNA lentiviral approach. We demonstrate a significant decrease in estrogen receptor alpha (ERα) and core binding factor beta (CBFβ) in tumor stem cell maintenance following AFF4 depletion. We employed RNA-seq-based gene set enrichment analysis to delineate differentiation programs under SEC regulatory control. Finally, we sought to determine whether AFF4 plays a role in DIPG cell viability. Our findings suggest CDK9 is a targetable kinase for DIPG and have potential for future DIPG therapies.

**DIPG-34. SUPER ELONGATION COMPLEX AS A TARGETABLE DEPENDENCY IN H3K27M+ DIFFUSE MIDLINE GLIOMA**

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Mutations in the histone 3 gene (H3K27M) are the eponymous driver in diffuse-midline pontine glioma (DIPGs) and other diffuse midline gliomas (DMGs), aggressive pediatric brain tumors for which no curative therapy currently exists. To identify specific epigenetic dependencies within the context of the H3K27M mutation, we performed an shRNA screen targeting 408 genes classified as epigenetic/chromatin-associated molecules in patient-derived DMG cultures. This identified AFF4, a component of the super elongation complex (SEC), as a necessary dependency for DIPG cells to maintain growth and self-renewal. We hypothesized that aberrant SEC expression occurs as a consequence of the H3K27M mutation and that dis-regulated SEC signaling overcomes repressive transcriptional regulation in order to suppress differentiation and promote self-renewal of DIPG tumor stem cells. We interrogated the role of AFF4 in DIPG using an shRNA lentiviral approach. We demonstrate a significant decrease in estrogen receptor alpha (ERα) and core binding factor beta (CBFβ) in tumor stem cell maintenance following AFF4 depletion. We employed RNA-seq based gene set enrichment analysis to delineate differentiation programs under SEC regulatory control. Finally, we sought to determine whether ERα plays a role in DIPG cell viability. Our findings suggest CDK9 is a targetable kinase for DIPG and have potential for future DIPG therapies.
Despite 50 years of clinical trials, no improvement of survival has been observed in DIPG, and most children die within 2 years of diagnosis. Only radiotherapy transiently controls disease progression. The study was conceived as a randomized multi-arm multi-stage program. It started with an open-label phase-II trial comparing three drugs (everolimus, dasatinib, erlotinib) combined with irradiation, allocated according to the presence of their specific targets (PTEN-loss, EGFR-overexpression) defined with a stereotactic biopsy after central confirmation of the diagnosis (presence of histone H3K27M mutation or loss of K27 trimethylation). Targeted therapies were started concomitantly with radiotherapy and were continued until disease progression. No biopsy-related death was reported and diagnostic yield was excellent, with only 5 non-informative biopsies. Biopsy excluded the diagnosis of DIPG in 8% of the cases. At the 3rd interim analysis, based on 193 randomized patients, the IDMC concluded that the study was unlikely to show a difference of OS between the 3 drugs even if 250 patients would be randomized. The median OS from the time of diagnosis was 11.9, 10.5 and 10 months for everolimus, dasatinib and erlotinib. Treatment was discontinued due to toxicity in 2%, 13%, and 15%, respectively. BORDELETT et al, reported the feasibility of biopsy treatment in DIPG on a large international scale. Based on the better toxicity profile and the slightly better efficacy, although not statistically significant, the steering committee proposed that everolimus should be used as the control arm for the next BIOMEDE 2.0 trial.

DIFFUSE INTRINSIC PONTINE GLIOMA (DIPG)

BACKGROUND: Diffuse midline gliomas (DMG) are aggressive brain tumours with 10% overall survival (OS) at 18 months. Predicting OS will help refine treatment strategy in this patient group. MRI based texture analysis (MRTA) is a novel technique that provides objective information about spatial arrangement of MRI signal intensity and has potential as an imaging biomarker. To investigate MRTA in predicting OS in childhood DMG, we used a large drug-perturbation database. Patients clustered by their biomarker. OBJECTIVES: To investigate MRTA in predicting OS in childhood DMG. METHODS: Retrospective study of patients diagnosed with DMG, based on radiological features, treated at our institution 2007–2017. MRIs were accomplished at diagnosis and 6 weeks after radiotherapy (54 Gy in 30 fractions) at MDTA, performed using a 3.0T MRI scanner. Affymetrix Genomic view and chromatin immunoprecipitation on tissue and cell lines that were used in previous studies to investigate the utility of these signatures as biomarkers for diagnosis and insight into mechanisms of tumorigenesis and therapy response. Further investigation of the utility of these signatures as biomarkers for diagnosis and monitoring treatment response are therefore underway.

DIFFUSE INTRINSIC PONTINE GLIOMA (DIPG)

INTRODUCTION: Diffuse midline glioma is a highly morbid pediatric cancer that impacts up to 80% harbor Histone H3K27M mutation in all pathologies. H3 post-translational modifications (PTMs) and genomic enrichment patterns, affecting chromatin structure and transcription. We previously identified tumorigenic patterns of histone H3K27Ac/bromodomain co-enrichment and preclinical efficacy of bromodomain inhibition (JQ1) in DMG. Here, we employ a novel proteomics approach developed at our institution to further elucidate the impact of H3K27M mutation on glioma epigenetic signatures and treatment response. METHODS: Epitope specific analysis was performed on Histone H3 (H3K27M, H3K27Ac, H3K27me3) to characterize 95 distinct Histone H3 N-terminal tail modification states. Cells were treated with JQ1 or DMSO, and collected at 0h, 24h, 48h, Histones extracted from isolated nuclei and immunopurified, then analyzed by LC-MS/MS. Results were integrated with RNA-Seq and ChIP Seq (H3,3K27M, H3,3K27Ac, H3K27me3, H3K4me1, H3K4me3) from the same cell lines. Pediatric glioma tissues (H3K27MT WT n=3, H3K27MT ns =9) were similarly analyzed to validate cell line results. RESULTS: Cell PTM profiles cluster by H3 mutation status on unsupervised analysis, significant differential PTM abundance and genomic enrichment of H3K27M, H3K27Ac and H3K27me3 were observed between mutant and wild type cell lines with epigenetic-targeted therapy, correlating with cell transcriptomes. CONCLUSIONS: Histone H3 tail analysis reveals the effects of H3K27M mutation and Histone H3 N-terminal domain in pediatric glioma biology, providing insight into mechanisms of tumorigenesis and therapy response. Further investigation of the utility of these signatures as biomarkers for diagnosis and monitoring treatment response are therefore underway.

DIFFUSE INTRINSIC PONTINE GLIOMA (DIPG)

INTRODUCTION: Diffuse midline glioma (DIPG) remains a fatal disease with no effective drugs to date. Mutation-based precision oncology approaches are limited by lack of targetable mutations and genetic heterogeneity. We leveraged systems biology methodologies to discover common targetable disease driver, master regulator pathways (MRPs) or DIPG, to define therapeutically for DIPG. The metaVIFER algorithm, we interrogated an integrated low grade glioma and GBM gene regulatory network with 31 DIPG-gene expression signatures to identify tumor-specific MRs by differential expression of their transcriptional targets. Unsupervised clustering identified MR signatures of upregulated activity in RRM2/TOPA2 in 13 patients, CD3D in 5 patients, and MMP7, TACSTD2, RAC2 and SLCL1A1/LC3A2 in individual patients, all of which can be targeted. Notably, intratumoral ad-ministration of cisplatin to upregulate the pathway, and addition of muprine monomeric aurora in which TOP2 was identified as a MR while RRM2—targetable by drugs such as cladribine—has been shown to be a positive regulator of glioma progression whose knock-down inhibits tumor growth. We also prioritized drugs by their ability to reverse MR-signature disturbances using a large drug-perturbation database. Patients clustered by molecular biology diagnostics. Immune therapy is a powerful and promising approach for improving the overall survival (OS). A retrospective analysis for feasibility, immune responsiveness and OS was performed on 41 children treated with Newswanger, CCG, patch, denileukin diftitox, and autologous dendritic cell vaccines as part of an individualized combined treatment approach for DIPG patients at diagnosis (n=28), or at time of progression (n=13). All except one patient had reduced values of at least one immune test before starting immunotherapy. In all patients, at least one PanTum Detect test was outside the normal range. Ten patients had PDL1 mRNA expression in circulating tumor cells at diagnosis. Multi-modal immunotherapy was feasible as scheduled, until progression, in all patients without major toxicity. When immunotherapy was part of primary treatment, median PFS and OS were 8.4 and 14.4 months respectively. Th1 shift and rise in PanTum Detect test scores were linked with longer OS. Multi-modal immunotherapy is feasible without major toxicity, and its value as part of a combination treatment for primary diagnosed DIPG should be elaborated in clinical trials.