SCIENTIFIC ABSTRACT BOOKLET

NJ Pediatric Hematology and Oncology Research Center of Excellence (NJ PHORCE)

From Pilot to Progress in Pediatric Hematology-Oncology Research Showcase

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Hosted by:

Peter Cole, MD, Chief, Division of Pediatric Hematology/Oncology

Katie Devine, PhD, MPH, Chief, Section of Pediatric Population Science, Outcomes, and Disparities Research

Table of Contents

Mapping Microenvironment-Specific Adaptations of Leukemic Cells in Pediatric T-ALL at Single Cell Resolution

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T-cell Acute Lymphoblastic Leukemia (T-ALL) is a hematological malignancy in need of novel therapeutic approaches. Here, we identify the ATP-citrate lyase ACLY as overexpressed in human T-ALL and as a promising therapeutic target for its treatment. To test the effects of ACLY in leukemia progression, we developed an isogenic model of NOTCH1-induced *Acly* conditional knockout leukemia. Importantly, we observed intrinsic antileukemic effects upon loss of ACLY, which further synergized with NOTCH1 inhibition *in vivo.* Metabolomic profiling upon ACLY loss revealed a metabolic crisis with reduced acetyl-CoA levels, as well as a decreased oxygen consumption rate. Gene expression profiling analyses showed that the transcriptional signature of ACLY loss very significantly correlates with the signature of MYC loss *in vivo*. Mechanistically, the decrease in acetyl-CoA led to reduced H3K27ac levels in *Myc*, resulting in transcriptional downregulation of *Myc* and drastically reduced MYC protein levels. Moreover, pharmacological inhibition of ACLY led to reduced MYC levels and antileukemic effects in human T-ALL cell lines and patient-derived xenografts (PDXs). Interestingly, our analyses also revealed a reciprocal relationship whereby *ACLY* itself is a direct transcriptional target of MYC, thus establishing a feedforward loop that is important for leukemia progression. Overall, our results identified a relevant ACLY-MYC axis and unveiled ACLY as a novel promising target for T-ALL treatment.

Social Connectedness Among Adolescents and Young Adults with Cancer: Examining the Role of Social Networks

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Background: Given their age and developmental period, adolescents and young adults (AYAs; ages 15-39) coping with cancer often experience abrupt changes in their social relationships. Diminished social connectedness (connections/relations with others) is a major risk factor for poorer health and well-being. Limited work has focused on identifying the extent of changes within AYAs social networks, such as network structure (network size) and network composition (sociodemographic characteristics) that may confer risk. To understand this, we present (1) preliminary quantitative data mapping the social networks of AYAs, analyzing their changes over a 6-month period, and (2) qualitative data on the lived experiences of social connectedness.
Methods: AYAs (N=50) completed questionnaires (baseline, 3- and 6-months post baseline) capturing aspects of their social networks and psychological distress. A subset of AYAs (n=24) completed a brief qualitative at 6 months on social connectedness within their social networks. Quantitative analyses examined changes in social network metrics overtime and associations between baseline metrics and psychological distress at 6 months. Content-structuring analysis was used to identify qualitative themes.
Results: On average AYAs (N=50; M_{age}=27.1, SD=6.0, range= 16-38) social networks were statistically significantly smaller at 6 months, contracting by 1.5 people over time (Z=-2.7, p=0.01), with significant decreases in friends (Z=-2.2, p=0.03), second-degree relatives (Z=-2.7, p=0.01) and in network closeness (Z=-2.4, p=0.02). Network composition remained stable. Baseline network size was positively associated with depressive (β=0.46, p=0.01) and anxiety (β=0.41, p=0.01) symptoms at 6 months, whereas baseline number of second-degree relatives was negatively associated with depressive (β=-0.37, p=0.01) and anxiety (β=-0.35, p=0.02) symptoms at 6 months. Four main qualitative themes emerged: *relationship shifts:* changes in relationship dynamics and communication over time; *adapting to a new social lifestyle:* difficulty adjusting back to pre-cancer social life; *resource needs:* desire for felt connection to cancer peers; *cancer survivor identity:* integration of the cancer experience into social connections.
Conclusion: Social challenges are a major issue for AYAs coping with cancer. Our findings shed light on how social networks among AYAs are changing over time. Results additionally highlight areas of social connectedness that can be leveraged to better deliver targeted interventions that can enhance the long-term health and well-being among AYAs with cancer.

Dietary Supplementation to Prevent T Lymphoma

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Leukemias/lymphomas are the most common type of childhood cancer, accounting for about 30% of all cancers in children. In New Jersey, there is about 3-10% incidence (per 100,000) and although mortality rate has decreased due to improved treatments, there remains a need to devise more effective strategies since survival rates after relapse remain low. Importantly, there is also considerable toxicity with current treatment regimen that generates long-term undesirable consequences such as damage to other tissues, particularly for pediatric patients. Leukemias/lymphomas often develop when immune cells lose important "brakes" that normally keep their growth in check. One such brake is a gene called PTEN, which is often mutated in different types of tumors. Our laboratory created a mouse model in which PTEN is specifically turned off in developing T cells in the thymus. These mice reliably develop lymphoma around 15 weeks of age, roughly equivalent to early adolescence in humans, thus allowing us to closely follow how metabolic remodeling triggers malignancy. By tracking the metabolic changes in these mice, we discovered that PTEN-deficient cells deregulate mitochondrial metabolism over time. In response, they progressively activate mTORC2 and GCN2 signaling, allowing them to survive. Surprisingly, we found that the developing tumors were extremely sensitive to α-ketoglutarate, a key molecule used by the mitochondria. Dietary supplementation with α-ketoglutarate (a-KG) allowed proper thymocyte differentiation, thus significantly delaying lymphomagenesis. A-KG is already widely available as a diet supplement and touted to benefit healthy aging. Defining the mechanisms underlying its effects on rewiring tumor metabolism and mTORC2/GCN2 signaling will provide insights on how to improve its efficacy as a dietary strategy for pediatric lymphoma prevention and treatment.

Plasma Biomarker Validation of Platelet-Derived MPN Signatures

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Patients with myeloproliferative neoplasms (MPN), including polycythemia vera (PV), essential thrombocythemia (ET), and myelofibrosis (MF), present with a paradoxical and clinically challenging risk profile encompassing both thrombosis and hemorrhage, splenomegaly, and microcirculatory disturbances. While expansion of one or more myeloid cell lineages contributes to these morbidities, the qualitative abnormalities driving vascular risk remain incompletely understood, and are particularly understudied in pediatric MPN. Building on our prior work profiling the MPN platelet transcriptome (n=120) and proteome (n=140) across independent adult ET, PV, and MF cohorts contrasted with healthy donors, we identified a robust, disease-progressive platelet signature associated with clinical severity across the MPN spectrum. To translate these cell-type-specific findings into a clinically accessible format, we completed a plasma biomarker validation study in a cohort of n=45 adult MPN plasma specimens, encompassing ET, PV, and MF patients alongside healthy donors. Using a targeted Luminex-based multiplex platform, we evaluated candidate markers derived from our platelet transcriptomic and proteomic signatures in plasma, a specimen type that is readily obtainable across clinical settings, including community practices without access to high tier academic centers. In this completed adult cohort, we successfully validated a core set of platelet-derived plasma markers that associate with MPN disease subtype and clinical severity indices, demonstrating proof-of-concept for plasma-based surrogacy of the underlying platelet molecular phenotype. These findings establish a translatable biomarker framework with potential prognostic and predictive utility for MPN thrombosis and bleeding risk. Extension of this work to pediatric MPN patients, a population with even greater unmet need and for whom molecular disease characterization is nearly absent, is currently ongoing through a multi-site collaborative effort. Pediatric plasma specimen accrual and analysis are in progress, with the goal of determining whether the adult-validated platelet-derived plasma signature generalizes across the age spectrum and retains clinical relevance in younger patients. Taken together, this work advances an accessible, prognostic, and potentially actionable plasma biomarker tool that is designed to complement rather than replace existing genetic assays, and to extend the reach of precision MPN monitoring to patients who may not otherwise have access to specialized molecular profiling.

Developing Advanced Liquid Biopsy Diagnostics Targeting Acute Lymphoblastic Leukemia-Associated Extracellular Vesicle miRNAs Using the CRISPR/Cas13a System

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This project developed a nanotechnology-enabled liquid biopsy platform for sensitive detection of disease-associated extracellular vesicle microRNAs (EV-miRNAs), with the long-term goal of enabling non-invasive monitoring of pediatric acute lymphoblastic leukemia (ALL). The platform design was built on a modular biosensing strategy, in which disease-associated EVs are selectively enriched on a functionalized gold nanoarray and directly analyzed using CRISPR/Cas13a-based miRNA detection without conventional RNA extraction or amplification. In the proposed ALL application, the platform integrates three key technological components: antibody-functionalized gold nanoarrays for selective capture of leukemia-associated EVs, liposome-mediated delivery of CRISPR/Cas13a sensing probes into intact EVs, and fluorescence signal amplification via Cas13a collateral cleavage. Methodologically, the platform is designed to address major limitations of current liquid biopsy workflows, including low EV specificity, miRNA degradation during extraction, and limited sensitivity in small-volume plasma samples. Gold nanoarrays provide a high-surface-area, reproducible substrate for the immobilization and enrichment of EVs. For ALL targeting, the nanoarray can be functionalized with B-cell leukemia-associated capture ligands such as CD19 antibodies, allowing selective isolation of ALL-derived EVs from heterogeneous plasma EV populations. After capture, CRISPR/Cas13a-loaded liposomes fuse with immobilized EVs, forming nanoscale reaction compartments that preserve EV integrity while concentrating target miRNAs and sensing reagents within confined vesicular volumes. Upon recognition of leukemia-associated miRNAs, such as miR-155 and miR-125b, the activated Cas13a complex cleaves quenched RNA reporters, generating an amplified fluorescence signal that can be quantitatively read from the nanoarray. Overall, this work establishes a flexible and clinically translatable EV-miRNA liquid biopsy platform that can be adapted across cancer types by modifying the EV capture ligand and CRISPR guide RNA design, providing a foundation for future pediatric oncology diagnostics and longitudinal treatment monitoring.

Genotoxic Effects of Synthetic Food Dye Exposure in Fanconi Anemia Patients

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Cancer predisposition and early onset malignancy remain critical challenges for Fanconi anemia (FA) patients, with cancer serving as the leading cause of mortality. FA proteins play essential roles in inter-strand crosslink repair, replication fork protection, and restart processes vital to cell survival. Despite strict dietary and lifestyle restrictions, FA patients still develop cancer over time, suggesting additional, currently unidentified genotoxic exposures may contribute to disease progression. Notably, very little research has explored dietary metabolites as a source of DNA damage in FA, which is predominantly a pediatric disease. Synthetic food dyes, particularly azo dyes, are now present in over 90% of children's candies, juices, colas, jellies, and jams in the United States. Although the FDA has established recommended daily intake limits for these dyes, these thresholds are not grounded in rigorous pre-clinical or clinical testing. Only a handful of studies over three decades have assessed their mutagenic potential, most conducted exclusively in rodent models. Due to concerns over adverse effects and potential carcinogenicity, the European Food Safety Authority and several major countries have banned these dyes in food products. In this study, we examined the cytotoxic effects of azo dye exposure in FA patient cells. Our findings demonstrate that exposure of FA protein-deficient cells to azo dyes causes extensive DNA replication fork stalling, elevated DNA damage, prominent activation of replicative checkpoints, and increased apoptosis. These phenotypes were recapitulated in primary human hematopoietic stem and progenitor cells and ex-vivo Fanconi mouse cells. Critically, azo dye exposure drives accumulation of reactive oxygen species, resulting in oxidative damage and exacerbated mitochondrial dysfunction, which may alter the reactive metabolome. Overall, our results indicate that azo dye exposure can exert cytotoxic, mutagenic, and potentially carcinogenic effects in FA patients, highlighting synthetic food dyes as an overlooked environmental risk factor warranting further investigation in this vulnerable population.

Targeting S-adenosylmethionine Metabolism and Chromatin Methylation in Synovial Sarcoma

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Tumors consume nutrients to support growth and treatment resistance. The metabolite S-adenosylmethionine (SAM), synthesized from the amino acid methionine, is an essential substrate for chromatin methylation and contributes to tumor redox defenses. Effective targeted interventions for the pediatric soft tissue cancer synovial sarcoma are lacking. Here, we define the control of cellular SAM supply on regulation of oncogenic gene expression in synovial sarcoma. Stable isotope tracing reveals SAM levels determine the rates of global DNA methylation. Unlike nearly universal loss of DNA methylation at genomic loci induced by small molecule inhibition of DNA methyltransferases, depletion of SAM causes a genome wide redistribution of methylation at distinct CpG islands. Consequently, pharmacological targeting of SAM synthesis disrupts unique gene expression programs supporting oncogenic growth. This work provides insights into how metabolic supply of SAM tune chromatin methylation dynamics and suggests an actionable vulnerability for developing a novel therapy for synovial sarcoma.

AAV-Mediated Superoxide Dismutase Gene Therapy for Preventing Cisplatin-Induced Hearing Loss in Pediatric Cancer Survivors

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Cisplatin is a cornerstone platinum-based chemotherapy used to treat a wide range of pediatric and adult solid tumors. Although cisplatin has substantially improved cancer survival, its clinical benefit is limited by permanent treatment-related toxicities that can persist throughout survivorship. Among the most common and debilitating of these is cisplatin-induced hearing loss (CIHL), which can significantly reduce quality of life. This is especially consequential for young children, in whom hearing loss can disrupt speech, language, cognitive development, academic performance, and social communication. CIHL is driven in part by irreversible damage to mechanosensory hair cells in the cochlea. Cisplatin exposure promotes DNA damage, apoptosis, reactive oxygen species (ROS) accumulation, lipid peroxidation, and inflammation, making antioxidant-based strategies a promising approach for inner-ear protection. Superoxide dismutases (SODs) are a family of endogenous antioxidant enzymes that neutralize superoxide radicals and protect cells from oxidative injury. Importantly, cisplatin reduces SOD enzymatic activity, and this loss of antioxidant defense has been mechanistically implicated in cochlear oxidative damage and hearing loss. To address this vulnerability, we developed a targeted AAV-based gene therapy platform to overexpress compartment-specific SOD isoforms in cochlear hair cells. Using intra-cisterna magna delivery in neonatal gerbil pups, we achieved transgene expression of SOD1, SOD2, or SOD3 in the developing cochlea. Three weeks after injection, periweanling animals underwent a clinically relevant cisplatin regimen consisting of 3 mg/kg cisplatin administered for 4 consecutive days, followed by 10 days off, repeated for 3 cycles. Auditory function was then assessed to determine whether SOD upregulation could mitigate CIHL. We found that all three SOD isoforms significantly reduced cisplatin-induced hearing loss, with the mitochondrial isoform SOD2 providing particularly robust otoprotection. These findings support oxidative stress as a key therapeutic target in CIHL and identify AAV-mediated SOD upregulation as a promising long-term otoprotective strategy. Ultimately, this approach could provide a durable co-treatment for pediatric cancer patients, preserving cochlear function and improving quality of life for survivors.

Development of Sensitive Blood-Based Biomarker Panels for Early Detection and Monitoring of Pediatric Diffuse Intrinsic Pontine Glioma / High-Grade Glioma

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Pediatric diffuse intrinsic pontine glioma (DIPG) and high-grade glioma (HGG) are among the most aggressive childhood brain tumors, with limited treatment options and a critical need for minimally invasive biomarkers to enable real-time disease monitoring. This project focuses on the development and validation of highly sensitive, multiplex PCR-based assays for the detection of circulating tumor DNA (ctDNA) in plasma and cerebrospinal fluid (CSF) from pediatric patients. Leveraging droplet digital PCR (ddPCR) and advanced mutation-selective chemistries, including SuperSelective primer technology, we have established a liquid biopsy platform capable of detecting rare tumor-associated mutations in the presence of high background wild-type DNA. Initial monoplex assays demonstrated highly specific detection of key driver mutations, including *H3F3A* K27M and *HIST1H3*B K27M, commonly identified in pediatric CNS tumors. Building on this foundation, ongoing work is focused on the development and optimization of multiplex ddPCR assays to simultaneously detect multiple clinically relevant alterations, including mutations in *ACVR1* and *TP53*, as well as copy number changes and structural variants such as BCAS1–BRAF fusions. To date, plasma and limited CSF samples have been successfully collected from multiple enrolled patients under an IRB-approved protocol, supporting the feasibility of biospecimen acquisition and processing in this population. Preliminary analysis of patient plasma cfDNA demonstrates detectable tumor-associated genomic alterations, including findings consistent with PTEN loss, highlighting both the promise and current sensitivity limitations of plasma-based ctDNA detection in pediatric CNS tumors. Collectively, this work explores the platform for a clinically actionable liquid biopsy in pediatric patients with brain tumors. This approach has the potential to complement imaging, enable longitudinal molecular monitoring, and improve clinical decision-making for children with CNS malignancies.

Chronic Histone Deacetylase Inhibitor Treatment of Ewing Sarcoma Cells Identifies Potential Novel Therapeutic Pathways

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Aims: Histone deacetylase inhibitors (HDACi's) have multiple effects on cancer cells, including inhibition of growth in vitro, and in tumor xenografts, and modest therapeutic efficacy in some malignancies. As Ewing Sarcoma (ES) cells classically contain the EWS-FLI1 translocation fusion gene, which dramatically alters epigenetic regulation, we hypothesized that chronic treatment of ES cell lines with HDACi's will result in changes in the ES cell gene expression program that may alter their malignant phenotype.

Methods: The ES cell line A673 was chronically treated with increasing amounts of Entinostat (class I HDACi), up to 1.5μM and then studied for basic growth properties and gene expression, and by ATAC-seq.

Results: We observed reductions in cell growth rates, colony formation, and anchorage-independent growth associated with altered expression of hundreds of genes including genes associated with neural, mesenchymal and neural crest differentiation. Immunofluorescence studies suggest that marker genes from these lineages are broadly expressed across both untreated and treated. We observed changes in lineage-determining transcription factors, including a marked decrease in RNA and protein expression of Sox1, an inhibitor of neural differentiation. Expression of the tyrosine kinase, RET, was reduced at both RNA and protein levels. We hypothesized that Ret may play a role in the malignant phenotype of ES and that HDACi-induced reduction in Ret expression might sensitize A673 cells to Ret inhibitors. Co-treatment of A673 cells with Entinostat resulted in over two-fold increased to Cabozantinib, a partially selective Ret tyrosine kinase inhibitor.

Next Steps: We are studying additional biological effects of chronic HDACI treatment, associated with metastasis and changes in vivo growth. We are currently testing the requirement for Sox1 for ES cell growth, survival and differentiation and continuing to study Ret as a potential therapeutic target, We are extending these studies to additional ES cell lines (SK-ES) and additional HDACIs (Panibinostat).

PTEN-loss Confers Dependence on the Guanylate Synthesis Enzyme IMPDH in T-Cell Acute Lymphoblastic Leukemia

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Loss of the tumor suppressor PTEN is common in T-cell acute lymphoblastic leukemia (T-ALL), and is associated with poor prognosis. PTEN-loss drives robust activation of AKT/mTORC1 signaling to promote leukemic cell growth. We find that PTEN-loss in T-ALL confers dependence on the guanylate nucleotide synthesis enzyme inosine 5'-monophosphate dehydrogenase (IMPDH) for cell growth and viability. This metabolic vulnerability is dependent on sustained mTORC1 signaling and can be exploited using clinically approved IMPDH inhibitors to selectively kill PTEN-deficient T-ALL cells, and extend survival in genetic and xenograft T-ALL models in mice. Mechanistically, IMPDH inhibitors cause early DNA replication stress, followed by DNA damage. In contrast to treatment with mTORC1 inhibitors, these events culminate in robust and selective cell death in PTEN-deficient T-ALL cells. These findings reveal a targetable metabolic vulnerability in T-ALL, which could provide rationale for repurposing clinically approved IMPDH inhibitors.

Project Update: AYA Cancer Survivor and Parent Communication and Transition Readiness

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Phase 1: Completed cognitive interviews with 5 adolescent and young adult cancer survivors and 5 parents. Cognitive interviews reviewed the survey instrument, generating feedback for changing items, replacing/adding scales. Cognitive interviews occurred from November 2023 until March 2024.

Phase 2: Collected survey data from 54 survivors, 43 parents, resulting in 38 complete dyads. Participants were recruited from the Rutgers Cancer Institute of New Jersey LITE Clinic May 2024 until March 2025.

Current status: Spring 2026, prepared data for dyadic analysis; working with Rutgers University Biostatistics and Epidemiology Services (RUBIES) for sophisticated statistical analyses.

Methotrexate Induces Alterations in One-Carbon Metabolism and in Plasmalogen and Phosphatidylcholine Levels in CSF Of Juvenile Rats

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Background: Although typically curative, treatment for pediatric acute lymphoblastic leukemia (ALL) is associated with neurotoxicity and leads to chemotherapy-related cognitive impairment (CRCI) in 40–70% of survivors, significantly impacting their quality of life. Methotrexate (MTX), a key component of ALL chemotherapy regimens, is a major contributor to CRCI. Using cerebrospinal fluid (CSF) metabolomics, our previous work showed that pediatric patients undergoing chemotherapy exhibited alterations in lipid metabolism, particularly phosphatidylcholines. However, because ALL chemotherapy involves multiple agents, the independent contribution of MTX to these metabolic alterations remains unclear.

Experimental procedures: Using a juvenile rat model designed to isolate MTX-specific effects within a pediatric-relevant context, we administered six intraperitoneal (0.5 mg/kg per dose) and four intrathecal (1 mg/kg per dose) MTX injections between three and eight weeks of age. To characterize MTX-induced metabolic changes, we performed CSF metabolomics at the time of the first intrathecal injection and at the fourth. Five weeks after the last intrathecal injection, we assessed spatial and visual memory using object placement (OP) and novel object recognition (OR) behavioral tests, respectively.

Results: MTX-treated rats exhibited spatial and visual memory impairments compared with controls. In accordance with our previous results and MTX's mechanism of action, determinants of one-carbon metabolism were downregulated in the CSF of MTX-treated animals between the timepoints tested. This includes S-adenosylmethionine (SAM, FC = 0.36, p-adj = 0.02) and methionine (FC = 3.24E-06, p-adj = 6.41E-06). In contrast, transsulfuration pathway metabolites such as cystathionine (FC = 3.98, p-adj = 0.001) and cysteine (FC = 2.81, p-adj = 0.02) were upregulated. Last, MTX treatment also induced alterations in lipid metabolism compared to controls, with a significant over-representation of 8 plasmalogens and 9 phosphatidylcholines, consistent with our findings in the CSF of pediatric ALL patients undergoing chemotherapy.

Conclusions: Despite more than eight decades of clinical use, important aspects of MTX-induced neurotoxicity remain poorly understood. Preclinical models therefore remain instrumental for defining MTX's contribution to chemotherapy-related neurotoxicity. This work, together with our recent findings in humans, underscores the utility of CSF metabolomics for investigating MTX-associated neurotoxicity and provides insight into metabolic pathways, potentially contributing to CRCI.

Inhibition of JNK Preserves the Ovarian Reserve, Fertility, and Hormones During DNA-Damaging Chemotherapy

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Primary ovarian insufficiency (POI), infertility, early menopause, and endocrine disorders due to hormone deficiency are major long-term side effects for pediatric, adolescent, and young adult female cancer patients receiving gonadotoxic chemotherapy therapies. Current strategies to preserve fertility and ovarian endocrine functions remain limited due to concerns of feasibility, efficacy, and safety. Here, we identify c-Jun N-terminal kinase (JNK) as a previously unrecognized upstream regulator of DNA damage response (DDR) in oocytes of primordial follicles in the ovary following DNA-damaging anti-cancer therapy. Both pharmacological inhibition and oocyte-specific deletion of JNK prevented chemotherapy-induced oocyte apoptosis, primordial follicle loss, and POI. Long-term studies revealed that JNK inhibition preserved the ovarian reserve, estrous cyclicity, and fertility after DNA-damaging chemotherapy. Mechanistically, chemotherapy-induced DNA damage selectively activated JNK in oocytes of primordial follicles, promoting the activation of oocyte transcription factor TAp63α and triggering oocyte apoptosis, leading to POI. In a clinically relevant breast cancer mouse model, JNK inhibition protected ovarian reserve without compromising the anti-tumor efficacy of chemotherapy. Taken together, these findings establish JNK inhibition as a promising strategy for developing ovarian protectants to preserve fertility and ovarian endocrine functions in young female cancer survivors.

Expression of Novel Immune Checkpoints HHLA2 and B7x on Circulating Tumor Cells (CTCs) of Pediatric, Adolescent and Young Adult Solid Tumors

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The poor outcomes observed in high-risk pediatric solid tumors often stem from the burden of metastatic disease. It is well recognized that tumor cells originating from the primary site can infiltrate surrounding tissues, enter the bloodstream, and colonize distant organs, thereby initiating metastasis. The utility of liquid biopsies from peripheral blood has emerged as a promising avenue of comprehending the intricate process of metastasis and an area of active investigation. In addition, novel immunotherapies are under development for cancer treatment, particularly for pediatric high-risk and metastatic cases. HHLA2 and B7x are the newest members of the B7/CD28 family immune checkpoint regulators, which typically facilitate tumor immune escape by suppressing the host immune system's ability to recognize and eliminate tumors. These checkpoints are widely expressed across various adult cancers. HHLA2 has been shown to be overexpressed in the tumor microenvironment of patients in a number of adult cancers. Within pediatrics it has been investigated in osteosarcoma, with associated inferior outcome, as well as pediatric Hodgkin Lymphoma. Clinical trials in adult cancer patients have shown efficacy with antibodies to B7/CD28 family member immune checkpoints. However, the expression of HHLA2 and B7x on CTCs of pediatric solid tumors remains poorly understood. Here we describe the initial investigations of HHLA2 and B7x expression on circulating tumor cells of pediatric solid tumors, in effort to begin to understand their role in the metastatic cascade. We employed state-of-the-art techniques to assess immune checkpoints HHLA2 and B7x on Circulating Tumor Cells (CTCs) using the CellSieve size-based microfilitration and our developed HHLA2/B7x multiple cocktailed assay kits. The high porosity of CellSieve precision microfilter allows rapid and efficient capture of CTCs from blood with low leukocyte contamination. The blood specimens were pre-filtered through CellSieve microfilters. Captured cells were further analyzed by immunofluorescence staining with the fluorescently labeled antibody cocktail containing anti-Vimentin antibody (a biomarker for CTC), anti-CD45 antibody (a biomarker for lymphocyte), and DAPI, plus our checkpoint antibodies (HHLA2 and B7x). In addition, to validate the HHLA2/B7x multiple cocktailed assay kits, we used known cultured cell lines expressing these checkpoints as positive controls: A204 and AsPC1 for HHLA2, SK-BR-3 and MD-MB-468 for B7x. H460 served as a negative control for HHLA2, and MD-MB-231 as a negative control for B7x. Blood specimens were collected from ten patients with pediatric cancers, including osteosarcoma, Ewing sarcoma, chordoma, rhabdomyosarcoma, lung carcinoma, hepatocellular carcinoma, neuroblastoma, and ovarian germ cell tumor. The CTCs were identified in all ten patients with pediatric cancers. Furthermore, we evaluated HHLA2 and B7x expression on CTCs in all ten patients with pediatric cancers. Our findings revealed HHLA2 expression on CTCs in 40% of patients (4/10) and B7x expression in 33.3% of patients (1/3). Normal control blood samples were also tested to confirm that the positive signaling was not due to background noise.