**Cancer Research Foundation Young Investigator Progress Report**

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**Project Title:** Utilizing hypoxia-response models to identify novel neuroblastoma therapy.

**Background:** Outcome for children with high-risk neuroblastoma is poor, with survival rates of less than 50% despite intensive, multi-modality therapy. There is extensive evidence that most solid tumors of adults and children often outgrow their blood supply and subsequently must adapt to oxygen tensions as low as 0.5%. A hypoxia gene signature that is prognostic of outcome in children with neuroblastoma has been identified, suggesting that the transcriptomic changes induced by hypoxia promote clinically aggressive neuroblastoma growth. Hypoxia frequently confers a malignant phenotype by stabilizing hypoxia inducible factor (HIF)-1α and HIF-2α. In many cancers, these transcription factors regulate redundant cellular functions promoting tumor progression. However, unlike most tumors, evidence suggests HIF-1α may decrease neuroblastoma growth while HIF-2α promotes aggressive behavior. Recent studies demonstrate that these proteins also play distinct roles in the regulation of cancer stem cells and tumor phenotype. A deeper understanding of the disparate effects of HIF-1α and HIF-2α on the molecular mechanisms responsible for the growth and progression in hypoxia is needed to determine how drugs that inhibit HIF activity can be used most effectively.

**Hypothesis and aims:** I hypothesize that elucidation of the pathways affected by hypoxia will lead to the discovery of new biomarkers and may ultimately direct us to improved risk stratification strategies and new therapeutic targets.

**Current Status:**

**Neuroblastoma cells exhibit a heterogeneous growth response to hypoxia**

We analyzed transcriptome data from diagnostic neuroblastoma tumors and hypoxic neuroblastoma cell lines to identify genes whose expression levels correlate with poor patient outcome and are involved in the hypoxia response. By integrating a diverse set of transcriptome datasets, including those from neuroblastoma patients and neuroblastoma derived cell lines, we identified nine genes (*SLCO4A1*, *ENO1*, *HK2*, *PGK1*, *MTFP1*, *HILPDA*, *VKORC1*, *TPI1*, and *HIST1H1C*) that are up-regulated in hypoxia and whose expression levels are correlated with poor patient outcome in three independent neuroblastoma cohorts. Analysis of 5-hydroxymethylcytosine and ENCODE data indicate that at least five of these nine genes have an increase in 5-hydroxymethylcytosine and a more open chromatin structure in hypoxia versus normoxia and are putative targets of hypoxia inducible factor (HIF) as they contain HIF binding sites in their regulatory regions. Four of these genes are key components of the glycolytic pathway and another three are directly involved in cellular metabolism. We experimentally validated our computational findings demonstrating that seven of the nine genes are significantly up-regulated in response to hypoxia in the four neuroblastoma cell lines tested. This compact and robustly validated group of genes, is associated with the hypoxia response in aggressive neuroblastoma and may represent a novel target for biomarker and therapeutic development.

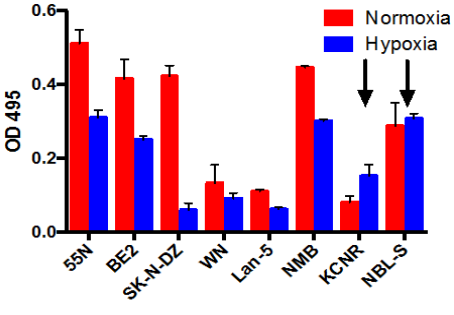


**Figure 1: Assays of proliferation and apoptosis.** Proliferation (panel A) of neuroblastoma cells in hypoxia (red) vs. normoxia (blue). All cell lines show decreased proliferation in hypoxia except for those with arrows.

In addition, I have conducted growth assays which demonstrate that six neuroblastoma cell lines, including SK-N-BE2, exhibited decreased proliferation after 48 hours of exposure to 1% oxygen (**Figure 1A**). In contrast, the proliferation of two neuroblastoma cell lines (NBL-S and KCNR) was not decreased in these conditions. To determine if HIF-1α and/or HIF-2α contribute to the disparity in neuroblastoma cell line growth in hypoxia, I conducted Western blot analyses. As shown in **Figure 2**, the pattern of HIF-1α and HIF-2α expression differed in these cell lines. In cell lines that exhibit inhibited proliferation in hypoxic conditions (SK-N-BE2 and

La1-55n), HIF-1α was up-regulated by hypoxia. Although a modest increase in HIF-2α was also seen in SK-N-BE2 after 6 hours of hypoxia, minimal to no change in the level of HIF-2α is detected in these cells after 48 hours. In contrast, the growth of NBL-S cells is not modified by hypoxia, and these cells express high levels of HIF-2α after 48 hours of culture in hypoxic conditions and much less HIF-1 α activation. SK-N-DZ exhibit high levels of apoptosis after 48 hours of culture in hypoxic conditions. Interestingly, these cells express high levels of HIF-2α after 6 hours of exposure to hypoxia, whereas higher levels of HIF-1α and lower levels of HIF-2α are detected at 48 hours. Thus, an inverse relationship between HIF-1α and HIF-2α levels was detected in these cells after exposure to hypoxia and a correlation between the pattern HIF-1α and HIF-2α protein levels and cellular response to hypoxia was observed. Specifically, inhibited proliferation was observed in neuroblastoma cells cultured in hypoxia that expressed HIF-1α, whereas hypoxia did not impair the growth of neuroblastoma cells with high levels of HIF-2α.

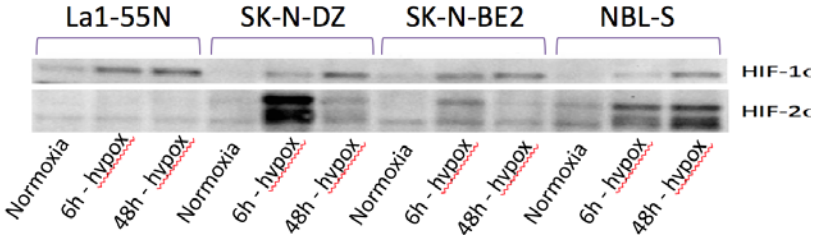


**Figure 2: Western blot showing HIF-1α and HIF-2α**. HIF-1α is predominantly expressed in neuroblastoma cells which grow poorly in 48 hours of hypoxia compared to the NBL-S line which expresses more HIF-2α and grows well in these conditions.

In order to determine what biologic pathways are differentially regulated in cells that grow well in hypoxia compared to those that do not, I have performed RNAseq on the NBL-S and SK-N-BE2 cell lines. RNA was extracted from each cell line grown in 48 hours of normoxia and hypoxia prior be sequenced. 50bp single end reads were aligned Tophat2 v2.0.13 and counts read with HTSeq. Pathway analysis of genes that were uniquely up or down regulated in only one of the two cell lines was then performed. The top overexpressed pathways in the SK-N-BE2 cell line which is HIF-1α driven and grows poorly in hypoxia were those related to metabolism (Cori Cycle, $p = 6.51x10^{-8}$; cholesterol biosynthesis; $p = 1.32x10^{-7}$, and glycolysis; $p = 2.2x10^{-7}$) while those that were down regulated belonged to cell cycle pathways (DNA strand elongation; $p = 7.66x10^{-8}$, S Phase; $p = 2.82x10^{-7}$, DNA Replication; $p = 6.58x10^{-7}$) consistent with growth assays. Conversely, the NBL-S line showed upregulation in pathways related to extracellular matrix organization (ECM-receptor interaction, $p = 8.57x10^{-7}$; ECM proteoglycans; $p = 3.48x10^{-6}$, and ECM organization; $p = 4.02x10^{-6}$) and down regulation of pathways involved in metabolism and signal transduction (cholesterol biosynthesis, $p = 5.29x10^{-11}$; small molecule metabolism; $p = 9.59x10^{-6}$, and signal transduction; $p = 2.13x10^{-5}$) suggesting that these mechanisms may be important for allowing this cell line to thrive in hypoxia and warrant further exploration using patient tumor samples.

In addition to the above efforts to understand the biologic consequences of hypoxia in this model, I have also begun work to evaluate the effects of modulating HIF-1α and HIF-2α expression in both the NBL-S and SK-BE-2 cell lines. I have transduced shRNA against each of these genes individually into each cell line. While I expect to have full confirmation of successful reduction in the expression of each gene in both cell lines. Once these I have validated successful expression knockdown, I will then evaluate the phenotype of these transfected cells with the hypothesis that decreased expression of HIF-1α will lead to increased growth of SK-N-BE2 cells in hypoxia while decreased expression of HIF-2α in NBL-S cells will attenuate hypoxia growth.

With the support of the CRF, I have been able to hire a laboratory technician and the supplies necessary to conduct the research described. This has led to several manuscripts, talks at national, and international meetings, and two additional grants. I plan to incorporate this data in an application for an NIH K award in the coming year. The specifics of these accomplishments are described below.

***Publications:***

1. **Applebaum MA**, Jha AR, Kao C, Hernandez K, DeWane G, Salwen HR, Chlenski A, Dobratic M, Mariani CJ, Godley LA, Prabhakar N, White K, Stranger BE, Cohn SL. Integrative genomics

reveals hypoxia inducible genes that are associated with a poor prognosis in neuroblastoma patients. In revision in Oncotarget, October 2016.

2. **Applebaum MA**, Vaksman Z, Lee SM, Hungate EA, Henderson TO, London WB, Pinto N, Volchenboum SL, Park JR, Naranjo A, Pearson AD, Hero B, Stranger BE, Cohn SL, Diskin SJ. Second Malignancies in Patients with Neuroblastoma: A Report from the International Neuroblastoma Risk Group Project. In revision in Eur. J. Cancer. October 2016.

3. Chlenski A, Dobratic M, Salwen HR, **Applebaum MA**, Guerrero LJ, Miller R, DeWane G, Solomaha E, Marks JD, Cohn SL. Secreted Protein Acidic and Rich in Cysteine (SPARC) induces lipotoxicity in neuroblastoma by regulating transport of albumin complexed with fatty acids. In revision in Oncotarget. September 2016.

4. **Applebaum MA**, Hungate EA, Skol AD, Vaksman Z, Diamond M, McDaniel L, Volchenboum SL, Stranger BE, Diskin SJ, Maris JM, Onel K, Cohn SL. Genetic variants are associated with MYCN-amplification in neuroblastoma. Manuscript in preparation.

***Presentations:***

Oral Abstract. “Genetic Variants in *BARD1* and *KIF15* are Associated with *MYCN*-Amplification in Neuroblastoma.” Advances in Neuroblastoma Research Congress. Cairns, Australia.

Plenary session. “Second Malignancies in Patients with Neuroblastoma: A Report from the International Neuroblastoma Risk Group Project.” Advances in Neuroblastoma Research Congress. Cairns, Australia.

Oral Abstract. “Genetic Variants in *KIF15* are Associated with *MYCN*-Amplification in Neuroblastoma.” Children’s Oncology Group Annual Meeting. Atlanta, GA. Selected as one of top young investigator abstracts.

***Grants and Awards:***

NIH K12-CA139160, Paul Calabresi Career Development in Clinical Oncology Award. Total direct cost: $130,000/yr. Annual salary recovery or effort: 80%. Project period: 3/1/16-2/28/18.

University of Chicago Cancer Center Auxiliary Board Physician Partnership. Total direct cost: $50,000/year. Project period: 5/1/16-4/30/19.

Conquer Cancer Foundation of ASCO Young Investigator Award. “Defining the genetic mechanisms of the hypoxia-response in aggressive, therapy resistant neuroblastoma.” Total direct cost: $50,000/year. Project period: 11/1/16-10/31/17.

Lerner Family Foundation Early Career Travel Award, Advances in Neuroblastoma Research Congress.

Merit Award for top junior faculty abstract - University of Chicago Department of Pediatrics Research Symposium.

Merit Award for top clinical oral abstract by a young investigator – Advances in Neuroblastoma Research Congress, Cairns, Australia.