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.

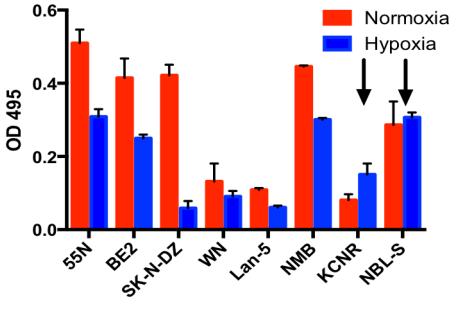
Hypothesis and aims: I hypothesized that elucidation of the pathways affected by hypoxia will lead to the discovery of new biomarkers and may ultimately direct us to improved risk stratification strategies and new therapeutic targets.

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. The Cohn and Godley laboratories have jointly shown global increases in 5-hydroxymethylcytosine, an epigenetic marker of active gene transcription, in neuroblastoma cells cultured in hypoxic conditions (Mariani *et al*. Cell Rep. 2014). Analysis of 5-hydroxymethylcytosine and ENCODE data indicate that at least five of these nine hypoxia induced genes have increased 5-hydroxymethylcytosine deposition and a more open chromatin structure in hypoxia versus normoxia. Further, these genes 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. These results were accepted for publication prior to the start of my Cancer Research Foundation funding period (Applebaum *et al*. Oncotarget. 2016).

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



Recently, 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-1α 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. Interestingly, in a publically available cohort of 709 patients with neuroblastoma tumor expression of HIF-1α was correlated with poor outcomes while expression of HIF-2α was higher in lower-risk tumors with better outcomes (**Figure 3**). These unexpected data suggest an alternate interpretation of the HIF patterns seen in cell lines, namely that HIF-1 is activating pathways that accelerate growth in a pure hypoxic environement leading to eventual metabolic exaustion and death, while HIF-2 actually slows down cellular division and prevents cell death.

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.

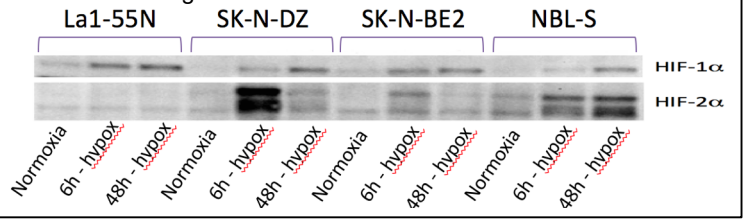
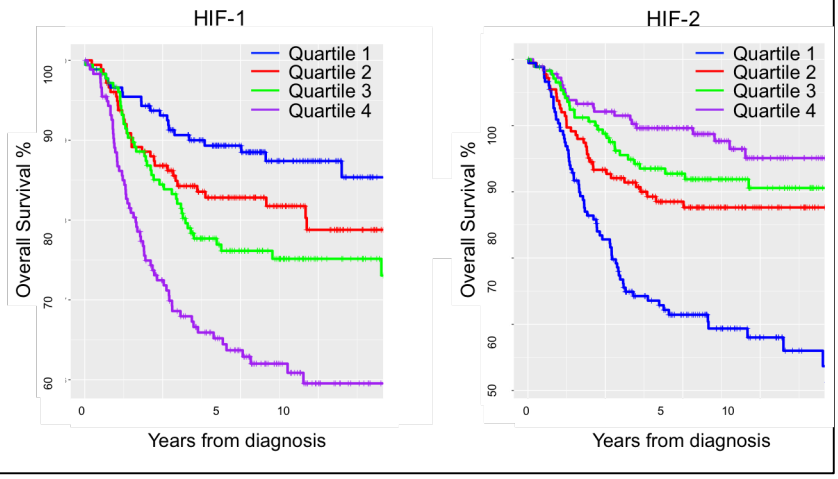


Figure 3: Western blot showing HIF-1α and HIF-2α. High expression of *HIF-1α* in patient tumors corresponded to advanced stage disease and worse outcomes whereas higher *HIF-2α* was seen in low stage tumors and improved survival.

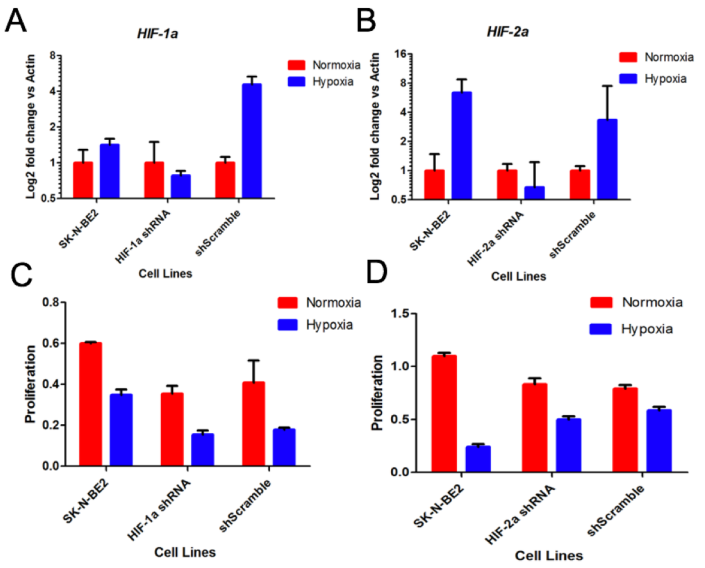


In order to determine what biologic pathways are differentially regulated in cells that grow well in hypoxia compared to those that do not, I have performed RNA-seq on the NBL-S and SK-N-BE2 cell lines. RNA was extracted from each cell line grown in 48 hours of normoxia and hypoxia prior be sequenced. 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, $p = 6.51 \times 10^{-8}$; cholesterol biosynthesis; $p = 1.32 \times 10^{-7}$, and glycolysis; $p = 2.2 \times 10^{-7}$) while those that were down regulated belonged to cell cycle pathways (DNA strand elongation; $p = 7.66 \times 10^{-8}$, S Phase; $p = 2.82 \times 10^{-7}$, DNA Replication; $p = 6.58 \times 10^{-7}$) consistent with growth assays. Conversely, the NBL-S line showed upregulation in pathways related to extracellular matrix organization (ECM-receptor interaction, $p = 8.57 \times 10^{-7}$; ECM proteoglycans; $p = 3.48 \times 10^{-6}$, and ECM organization; $p = 4.02 \times 10^{-6}$) and down regulation of pathways involved in metabolism and signal transduction (cholesterol biosynthesis, $p = 5.29 \times 10^{-11}$; small molecule metabolism; $p = 9.59 \times 10^{-6}$, 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-N-BE2 cell lines. I have successfully reduced the expression at the mRNA and protein levels of both of these genes in SK-N-BE2 cells (**Figure 3A and 3B**). Interestingly, consistent with the expression data from patient tumors, knockdown of HIF-1α led to less proliferation while cells with decreased HIF-2α had greater proliferation in hypoxia compared to parental lines (**Figure 3C and 3D**). One significant challenge in these experiments has been consistency of results and I have spent the past several months generating single clone knockdowns in multiple cell lines and have begun to utilize CRISPR against these HIF targets. Once these I have validated successful expression knockdown, I will then evaluate the phenotype of these cells to determine if my initial hypothesis was correct or if HIF-1α actually promotes aggressive tumor growth as is seen in other tumor types.

Figure 4: shRNA knockdown of A) HIF-1α and B) HIF-2α in the SK-N-BE2 cell lines. C) Inhibition of HIF-1a appears to inhibit the growth of neuroblastoma cells. D) Inhibition of HIF-2a appears to promote the growth of neuroblastoma cells in hypoxia



With the support of the Cancer Research Foundation, 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. Importantly, this project has led me to explore epigenetic mechanisms affected by the HIF response. Preliminary data from this project and additional experiments in epigenetics were incorporated into a submission for a K08 to the NCI. This submission was positively reviewed, receiving an impact score of 24 and is likely to be funded. Thus, this grant has been instrumental to the further advancement of my career and progression towards becoming an independent investigator.

Key Findings:

- Neuroblastoma cell lines have heterogeneous patterns of HIF-1α and HIF-2α expression in response to hypoxic conditions.
- Increased levels of hypoxia induced HIF-2α induction is associated with increased rates of proliferation whereas decreased proliferation is observed in neuroblastoma cell lines with hypoxia induced HIF-1α expression.
- Increased cellular metabolism induced by HIF-1a expression may lead to cell death in extreme hypoxic conditions

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 confer a poor prognosis in neuroblastoma patients. Oncotarget. 2016 Nov 22;7(47): 76816-26. PMID: 27765905. PMCID: PMC5340231. *Denotes co-first authors.

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. Neuroblastoma survivors are at increased risk for second malignancies: A report from the International Neuroblastoma Risk Group Project. Eur J Cancer. 2017 Feb 72:177-185. PMID: 28033528. PMCID: PMC5258837.

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. Oncotarget. 2016 Nov 22;7(47): 77696-706. PMID: 27776337. PMCID: PMC5363614.

4. Hungate EA*, **Applebaum MA***, Skol AD, Vaksman Z, Diamond M, McDaniel L, Volchenboum SL, Stranger BE, Diskin SJ, Maris JM, Onel K, Cohn SL. Evaluation of genetic predisposition for MYCN-amplified neuroblastoma. J Natl Cancer Inst. 2017 Oct 1;109(10). PMID: 29117357. PMCID: pending. *Denotes co-first authors.

5. **Applebaum MA**, Desai A, Glade-Bender J, Cohn SL. Emerging and investigational therapies for neuroblastoma. Expert Opin Orphan Drugs, 201 Apr;5:355-368. PMID: 29062613. PMCID: PMC5649635.

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.

Invited seminar. “Second Malignancies in Patients with Neuroblastoma.” International Neuroblastoma Conference. Houston, TX.

Plenary session. “Whole Genome Analysis of the Epigenetic Mark 5-Hydroxymethylcysteine Reveals Differential Profiles in Low-, Intermediate-, and High-Risk Neuroblastoma.” AACR Conference on Pediatric Cancer Research. Atlanta, GA.

Grants and Awards:

NIH K08-CA226237-01. “Elucidating transcription regulation by epigenetics in neuroblastoma.” Total direct cost: $190,000/year. Project period: 4/1/18-3/31/22. Role: PI.

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: 3/1/16-3/31/18.

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

Cancer Research Foundation Young Investigator Award. Total direct cost: $35,000/year.
Utilizing Hypoxia-Response Models to Identify Novel Neuroblastoma Therapy. 12/01/15-11/30/17.

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. Project period: 11/1/16-10/31/17. Role: PI

And have an additional grant pending:
1. Pablove Foundation. “Epigenetic biomarkers of aggressive neuroblastoma.” Total direct cost: $50,000/year. Proposed project period: 8/1/18 – 7/31/19. Role: PI

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.