To: Richard N. Sifers, Ph.D., Research Director
Moran Foundation Research Awards

From: Dolores López-Terrada, M.D., Ph.D.
Joseph F. Pulliam, M.D.
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Subject: Moran Foundation Research Awards, Progress Report
"Beta-Catenin Mutations in Hepatoblastoma."

Date: July 27, 2005

Dear Dr. Sifers,

We are pleased to report to you our progress in the study of mutations in the *CTNNB1* gene for beta-catenin in the pediatric liver tumor hepatoblastoma as a recipient of the Moran Foundation Research Awards in the 2004-2005 cycle. Our work has been performed under the guidance of Dr. Milton Finegold at Texas Children's Hospital. Other members of our working group include Dr. Adekunle Adesina from the Department of Pathology and Dr. Judy Margolin from the Department of Pediatrics.

A) Progress Report

The long-term goal of our research is the identification of pathogenic factors that determine the prognosis and response to treatment of hepatoblastomas. Hepatoblastomas are the most common malignant liver tumor in early childhood and are characterized by a diversity of epithelial and sometimes mesenchymal tumor cell types. These include pure fetal epithelial tumors, which comprise about 10-15% of hepatoblastomas and in many cases can be treated by surgical resection alone. More aggressive patterns of epithelial morphology include embryonal and small cell hepatoblastoma. These, in particular small cell hepatoblastoma, are associated with a less favorable prognosis. Although the correlation of histology with prognosis is well appreciated, it is unclear how the molecular pathogenesis of these tumors determines both their degree of differentiation and clinical behavior. It is known that activation of the Wnt signaling pathway, in particular through mutations of its central regulator beta-catenin, are frequent in hepatoblastoma. It is also known that the level of activation of Wnt-target gene expression and the *in vitro* malignant phenotype of tumor cells varies with the type of *CTNNB1* mutation. These findings in addition to our preliminary data on gene expression patterns in hepatoblastoma, provided the background for our study of the relationship between beta-catenin mutation, Wnt target gene expression and histological pattern in hepatoblastoma.

The primary questions we asked in this study were:

1) What is the immunohistochemical pattern of beta-catenin expression in hepatoblastoma?
2) What is the corresponding mutational status of beta-catenin in hepatoblastoma?
3) How do these correlate with the WNT signaling gene expression pattern and histology of hepatoblastoma?

SPECIFIC AIMS AND RESULTS FROM THIS STUDY

1) **Beta-catenin protein expression and cellular localization in hepatoblastoma will be determined by immunohistochemistry.** Beta-catenin is normally expressed in a membranous fashion, with cytoplasmic and in particular nuclear translocation indicating constitutive activation of the beta-catenin signaling pathway. Nuclear translocation of beta-catenin can occur as a result of mutations of the beta-catenin gene as well as inactivating mutations of the *APC, Axin* or *GSK-β* tumor suppressor genes.

We successfully developed a technique for beta-catenin immunohistochemistry. We applied this technique to identify frequent nuclear expression of beta-catenin in hepatoblastomas. Nuclear beta-catenin was identified in a subset of pure fetal hepatoblastomas (those with CTNNB1 mutations) and all high-grade embryonal/small-cell hepatoblastomas. As a percentage of cells, the frequency and intensity of nuclear beta-catenin increased with the grade of the tumor in individual cases but was variable from case to case. A pure fetal hepatoblastoma with no identified CTNNB1 mutation showed faint membranous beta-catenin staining accompanied by less than 1% nuclear beta-catenin.

2) **Beta-catenin gene mutations will be determined by RT-PCR and sequencing.** Activating β-catenin mutations including point mutations and deletions for the exon three regulatory region will be determined. The mutational pattern will be correlated with the immunohistochemical profile of the β-catenin determined in specific aim 1.

We successfully developed a technique for RT-PCR and sequencing of CTNNB1 exons three and four. Thus far, we have analyzed thirty-three hepatoblastoma cases and found beta-catenin mutations in twenty-nine (88%). All cases with mutations that were tested by immunohistochemistry showed nuclear accumulation of beta-catenin. Approximately half the cases were obtained from the pediatric section of the Cooperative Human Tissue Network and consist of minute frozen sections of tumor that would be compromised by immunohistochemical staining. We are in the process of evaluating corresponding

paraffin sections for these cases to investigate beta-catenin immunohistochemistry.
CTNNB1 identified mutations included fourteen cases with point mutations in the exon three regulatory domain and fifteen cases with interstitial deletions. Of the deletions, eleven were limited to the coding region of exon three, while three involved the coding regions of both exon three and exon four. A significant pattern emerged in which the large deletions involving exon three and exon four were present only in pure fetal hepatoblastomas (three of six pure fetal hepatoblastomas). By contrast, cases with point mutations or deletions affecting only the coding region of exon three were predominantly high-grade embryonal/small cell hepatoblastomas (twenty-four of twenty-six cases) with only two pure fetal hepatoblastomas in this group. Exon three contains serine and threonine residues that, when phosphorylated by CK1α and GSK3β, lead to ubiquitination and degradation of beta-catenin. Disruption of these residues either by point mutation or deletion leads to cytoplasmic and nuclear accumulation of beta-catenin. By contrast, exon four contains residues that are necessary for the interaction of beta-catenin with BCL9 and BCL9-2, proteins that form a transactivational complex with beta-catenin, Pygopus and the TCF/LEF family of transcription factors leading to the transcription of a number of Wnt target genes. It has been demonstrated that in vitro mutation of certain of these beta-catenin exon four residues will disable the transactivational complex.

3) Beta-catenin immunohistochemistry determined in specific aim 1 and mutational status determined in specific aim 2 will be correlated with the gene expression of the canonical and noncanonical WNT pathway in hepatoblastoma.

We correlated the findings from specific aims 1 and 2 with the ongoing gene expression analysis by quantitative real-time RT-PCR for the expression of genes in the canonical and noncanonical Wnt pathway. Two specific findings have been made. First, tumors with mutations of CTNNB1 show similar levels of nuclear beta-catenin accumulation regardless of the type of mutation (exon three-only versus large deletions involving both exon three and exon four). Second, the tumors with CTNNB1 mutations limited to exon three showed stronger transcription of canonical Wnt target genes such as Axin2 than did tumors with large deletions involving both exon three and exon four. The likely explanation implied in specific aim 2) above is that the deletion of key residues in beta-catenin exon four, which form part of the nuclear transactivation complex for Wnt target gene expression, result in decreased beta-catenin mediated transcription. Thus, tumors with large deletions (exon three and four) of CTNNB1 accumulate nuclear beta-catenin in a similar manner to the tumors with mutations confined to exon three, but do not activate Wnt target gene expression to the same degree because of the deletion of necessary residues that interact with BCL9 and/or BCL9-2. A third finding, which corroborates with recent reports from the literature on liver development, is that CCND1 is not a Wnt target gene in the liver.

B) Publications and presentations related to this project, acknowledging Moran Foundation support.

Articles:

López-Terrada D, Pulliam JF, Adesina A, Margolin JF, Gunaratne PH, Finegold MJ (2005). Evaluation of *CTNNB1* mutations in hepatoblastoma – association of large deletions with better differentiated histologic subtypes. *In preparation for submission to Oncogene.*

Abstracts:

López-Terrada D, Pulliam JF, Adesina A, Margolin JF, Gunaratne PH, Finegold MJ (2004). Studies on Beta-catenin status and Wnt pathway in hepatoblastoma. Baylor College of Medicine Cancer Center Annual Symposium. October 2004.

López-Terrada D, Gunaratne PH, Pulliam JF, Adesina A, Margolin JF, Finegold MJ (2005). Analysis of Beta-catenin status and Wnt pathway in different histologic subtypes of hepatoblastoma. Society for Pediatric Pathology Annual Meeting. San Antonio, Texas, February 2004. *Modern Pathology; 2005 (18):302*

López-Terrada D, Pulliam JF, Gunaratne P, Adesina A, Margolin J, Finegold MJ (2005). Evaluation of *CTNNB1* mutations, beta-catenin (BCAT) immunohistochemistry (IHC) and tissue morphology in hepatoblastoma (HB). Submitted, Association for Molecular Pathology Annual Meeting, Phoenix, Arizona, November 2005.

Presentations:

February 26, 2005 – López-Terrada D, Gunaratne PH, Pulliam JF, Adesina A, Margolin JF, Finegold MJ. Analysis of Beta-catenin status and Wnt pathway in different histologic subtypes of hepatoblastoma. Platform Presentation: Society for Pediatric Pathology Annual Meeting.

C) Status of Project – Active

Our work on hepatoblastoma is ongoing. Currently we are working on the role of additional molecular lesions (mutations or methylation) in other key regulatory genes in the Wnt pathway. We are also investigating the interaction of the Wnt pathway with other developmental pathways in hepatogenesis and oncogenesis.

Society of Pediatric Pathology Annual Meeting, San Antonio, February 26-28, 2004. (accepted for platform presentation)

Analysis of Beta-catenin status and Wnt pathway in different histologic subtypes of Hepatoblastoma. Dolores Lopez-Terrada, Preethi H. Gunaratne, Joseph F. Pulliam, Adekunle Adesina, Judith F. Margolin, Milton J. Finegold. Department of Pathology, Baylor College of Medicine, Department of Pediatric Hematology & Oncology, Texas Children's Hospital, Houston, TX 77030.

Hepatoblastoma represents the most common type of pediatric liver tumor. They are classified into epithelial (56%) or mixed epithelial/mesenchymal (44%), with the epithelial types being further subdivided into fetal, embryonal and small cell subtypes. In completely resected tumors a pure fetal epithelial histology with minimal mitotic activity confers a better prognosis, whereas a small cell undifferentiated histology is associated with a poor prognosis. The molecular pathogenesis of hepatoblastoma is poorly understood. Recent studies have shown activating mutations in the β-catenin pathway in over 90% of hepatoblastomas, leading to nuclear accumulation of β-catenin and transcription of several target genes. Activation of the β-catenin pathway is a central feature of numerous benign and malignant epithelial and mesenchymal neoplasms, and can be associated with good or bad prognosis, in some cases correlating with specific mutations.

In an attempt to explore the role of the Wnt pathway in this disease we analyzed the expression patterns of a number of genes in this pathway, in seven primary hepatoblastomas (three pure fetal type, three mixed epithelial and one epithelial and mesenchymal) using quantitative QRT-PCR. All these cases were sporadic with the exception of one Beckwith-Wiedemann patient. Two of the patients had a history of extreme prematurity. *Dickkopf1* an antagonist of canonical Wnt signaling is significantly overexpressed in two out of three fetal type hepatoblastomas analyzed. *DAAM1* (disheveled associated activator of morphogenesis 1) which is involved in the non-canonical Wnt pathway was over expressed in all three fetal hepatoblastomas.

The pattern of β-catenin protein expression and activating mutations in individual or groups of tumors are crucial to understanding the corresponding differences in their gene expression profiles. In an effort to define the possible role of beta-catenin in the biology of these tumors, we amplified beta catenin transcripts using RT-PCR and carried out sequence analysis of exon 3 of the beta-catenin gene. We also performed immunohistochemical stains for the beta catenin protein. 5 of 7 tumors showed mutations of the beta catenin gene including four cases with deletion of all or part of exon 3 and one case of a recurrent hepatoblastoma with point mutation (G34R) within exon 3. Four (4) tumors with mixed epithelial patterns and one tumor with pure fetal histology showed a gene expression pattern of activated canonical WNT signaling. The other two (2) fetal hepatoblastomas showed wild type β-catenin and no activation of canonical WNT signaling pathway. Our findings are consistent with a relationship between poor histologic phenotype and beta catenin activation in hepatoblastoma and indicate the potential utility of targeted gene expression assays to identify molecular events related to the pathogenesis and prognosis of hepatoblastomas.

Association for Molecular Pathology Annual Meeting. November 11-13, 2005, Phoenix, Arizona

Title: Evaluation of *CTNNB1* mutations, beta-catenin (BCAT) immunohistochemistry (IHC) and tissue morphology in hepatoblastoma (HB).

Authors: Lopez-Terrada D[1], Pulliam JF[1], Gunaratne P[1], Adesina A[1], Margolin J[2], Finegold MJ[1].

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Introduction: HB, the most common pediatric malignant liver tumors, show diverse epithelial/mesenchymal cell types. Pure fetal HB comprise 15% and have better prognoses, while embryonal/small cell HB have worse prognoses. The molecular pathogenesis of HB is poorly understood. HB consistently shows activation of canonical WNT signaling with nuclear BCAT translocation, which may be a poor prognostic marker. We correlated HB canonical WNT signaling with histology, BCAT IHC and *CTNNB1* (gene for BCAT) mutation status. Preliminary results suggested that large *CTNNB1* deletions correlated with low-grade morphology and decreased canonical WNT target gene expression. We therefore compared *CTNNB1* mutations with histology in a larger group of HB.

Methods: Following IRB approval, RNA isolated from the HepG2 cell line and 33 HB was used for RT-PCR with primers F-5'-agcgtggacaatggctactcaa and R-5'-acctggtcctcgtcatttagcagt for a 506 bp amplicon (nucleotides 201-707 of *CTNNB1* ref seq NM_001904, exon-2 to the 3' end of exon-4). RT was performed at 50°C and touchdown PCR with annealing temperatures dropping from 60° to 57°C. Bands extracted from agarose gels were bi-directionally sequenced. IHC with monoclonal anti-BCAT antibody (1:500; Transduction Lab, Lexington, KY) was performed by conventional methods.

Results: *CTNNB1* mutations in embryonal/small cell HB were confined to exon-3. In contrast, *CTNNB1* mutations in 3/6 pure fetal HB and HepG2 showed large deletions including most of exon-3 and exon-4, significantly different by Fisher exact test. BCAT IHC on 7 cases showed < 1% cells with nuclear BCAT in fetal HB areas with no *CTNNB1* mutation, 15% in a fetal case with a large *CTNNB1* deletion, and 18-35% in embryonal/small cell cases with mutations confined to exon 3.

Conclusions: Our results showed different histology but not BCAT IHC pattern in HB by the type of *CTNNB1* mutation. Mutations confined to exon-3 may not affect the exon-4 BCL9-interaction domain essential for WNT target gene transcription that may facilitate the more aggressive phenotype (proliferation, metastasis) seen in embryonal/small cell HB. In contrast, tumors with large deletions extending from exon-3

to part of the BCL9-interaction domain of exon-4 showed low-grade morphology despite comparable nuclear BCAT accumulation. This may be due to decreased expression of canonical WNT target genes in these low-grade tumors compared to more aggressive tumors with *CTNNB1* mutations limited to exon-3.