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

From: Dolores López-Terrada, M.D., Ph.D.
Joseph Pulliam, MD

Subject: Moran Foundation Research Awards, Progress Report
**"Beta-Catenin Mutations in Hepatoblastoma."**

Date: August, 2006

Dear Dr. Sifers,

As recipients of the Moran Foundation 2004-2005 Research Award, we are pleased to report our progress in the study of " Mutations in the *CTNNB1* gene for beta-catenin in the pediatric liver tumor hepatoblastoma". Our work has been performed under the guidance of Dr. Milton Finegold at Texas Children's Hospital. Other members of our working group include Preethi Gunaratne, Ph.D. and Adekunle Adesina from the Department of Pathology.

The long-term goal of our research is to study the biology of hepatoblastoma (HB) and the pathogenic factors that may determine prognosis and response to therapy in these tumors. Hepatoblastoma is the most common malignant liver tumor in early childhood and is 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 histological types include embryonal and small cell hepatoblastoma. The presence of a significant small cell component has been specifically associated with a less favorable prognosis. Although the correlation of histology with prognosis is well established, 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 cell lines 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 *CTNNB1* mutation status, Wnt target gene expression pattern and histological type in hepatoblastoma. Our main hypothesis is that a comprehensive analysis of the Wnt signaling pathway correlated with clinical and histologic classification of these tumors will enhance our understanding of hepatoblastoma pathogenesis, which may be relevant for its classification and patient stratification.

In a previous phase of the study, we successfully developed a technique for beta-catenin immunohistochemical detection and identified frequent nuclear expression of beta-catenin in hepatoblastomas. Nuclear beta-catenin was demonstrated in a subset of pure

fetal hepatoblastomas (those with *CTNNB1* gene mutations) and all with embryonal/small-cell components. As a percentage of cells, significant differences where identified in the frequency and intensity of nuclear beta-catenin when fetal component was compared with the areas of embryonal and small cell components. A pure fetal hepatoblastoma with no identified CTNNB1 mutation showed faint membranous beta-catenin staining accompanied by less than 1% nuclear beta-catenin. We also successfully developed a technique for RT-PCR and sequencing of CTNNB1 exons three and four.

During the current period our aims included:

**Aim 1:** To analyze the expression patterns of a set of canonical Wnt pathway genes (including ligands, receptors, co-receptors agonists and antagonists) and to identify a meningful panel useful to evaluate Wnt pathway activation status in hepatoblastoma.

**Aim 2:** To determine the correlation between *CTNNB1* gene status and Wnt target gene expression profiles in different clinical and histologic hepatoblastoma subtypes.

**Aim 3:** To explore the possible contribution of additional canonical Wnt pathway molecules to the malignant phenotype and biological behavior of hepatoblastoma, and in particular the methylation status of *SFRP* genes in different hepatoblastoma subtypes.

## A) Progress Report

**Aim 1: To analyze the expression patterns of a set of canonical Wnt pathway genes (including ligands, receptors, co-receptors agonists and antagonists) and to identify a meningful panel useful to evaluate Wnt pathway activation status in hepatoblastoma and**
**Aim 2: To determine the correlation between CTNNB1 gene status and Wnt target gene expression profiles in different clinical and histologic hepatoblastoma subtypes.**

33 primary hepatoblastomas, 4 hepatic adenomas and 1 cell line (HepG2) were selected, including 27 epithelial hepatoblastomas (6 pure fetal, 21 fetal/embryonal), 6 mixed and three with small cell component. Histological review of the tissue submitted for RNA extraction was performed prior to total RNA isolation. RNA concentration and integrity was determined using a Nanodrop instrument and Agilent 2100 Bioanalyzer. Pooled fetal and normal liver total RNA were used as controls. *CTNNB1* one-step RT-PCR was performed using primers BCAT-F (5'-agcgtggacaatggctactcaa) and BCAT-R-long (5'-acctggtcctcgtcatttagcagt), amplifying from exon-2 to the 3' end of exon-4. Beta-catenin immunohistochemistry was performed using a monoclonal antibody (17C2, Novocastra, 1:200) and expression quantitated in 1000 tumor cells.
From an initial set of 28 canonical Wnt pathway genes (including ligands, receptors, co-receptors agonists and antagonists) 12 Wnt target genes were selected: *Beta-TRCP, BMP-4, CMYC, CYCLIN-D1, EGFR, SOX9, MMP7, MET, NKD, NLK, ITF2-TCF4 UPAR* and *AXIN2*. Expression of these target genes was analyzed by Real time QRT-PCR using SYBR Green. The fold change was normalized to an endogenous control

(18S) and relative to the normal adult liver calibrator by the formula: Fold change = 2-$\Delta\Delta$Ct, where $\Delta\Delta$Ct = (CtTarget - Ct18S) – (Ctcalibrator –Ct18S).
*CTNNB1* mutations were identified in all HB subtypes. Mutations altering or deleting the ubiquitination domain (exon 3) were present in 30 of 33 HB. 21 embryonal/small-cell cases had mutations limited to exon 3. Large deletions (exon 3 to 4), including a portion of the *BCL9*-interaction domain, were seen only in pure fetal cases. The difference in *CTNNB1* mutation type versus morphology was statistically significant (p=0.003, Fisher Exact Test). 1 fetal HB and 4 adenomas showed no *CTNNB1* mutations and a β-catenin membranous pattern. Exon 3 mutations resulted in predominantly nuclear accumulation (Figure1). Two fetal HB cases showed abundant nuclear localization of beta-catenin. Both cases contained large deletions, which may compromise β-catenin function despite increased stability and nuclear localization. This is noteworthy, since nuclear beta-catenin has been proposed to be a poor prognostic indicator in HB.

Quantitative RT-PCR analysis of canonical Wnt target genes expression (Figure 2) demonstrated *CMYC* and *AXIN2* expression variably increased in all cases except HB7 (pure fetal, wild-type *CTNNB*) other wild-type cases (HB8 and HB18) and rare cases with small cell component (HB5). *CMYC* overexpression was only modestly increased in pure fetal cases. *NDK* expression was mildly increased (less than 5 fold) in a subset of cases (HB 2, 3, 19, 26) and significantly only in a small number of cases (HB10, 20, 21, 22, 24, 27) all with fetal and embryonal components, and 4 of them with a significant mesenchymal component (3 with osteoid formation). Expression levels of *CYCLIND1* were elevated in pure fetals (HB6, HB7, HB13) and in some cases with prominent fetal component (HB19, 21, 23-27). *EGFR* expression was generally low, except for a subset of cases (HB 20, 25, 26) showing fetal-embryonal histology.Increased expression fold change was also found in some cases for *MET* oncogene (18 of 22 tumors), *BMP4* (18), *MMP7* (12), *SOX9* (9) and *UPAR* (6). However, differences in the expression of these genes appeared variable regardless of morphology.
Fetal hepatoblastoma cases showed low *EGFR* expression and somehow elevated *UPAR* and *NLK* expression, while hepatoblastomas with significant small cell component (HB3, 5, 12 18, 25) showed lower levels *CYCLIN D1* and *BetaTRCP*, *MET* and *NLK* expression than other histologic subtypes. No significant correlation between *MET* expression levels and pre and post-chemotherapy status was found by QRT-PCR in the post-chemotherapy hepatoblastoma cases (HB20, 22 and 24).

In summary:

- We have identified frequent *CTNNB1* mutations (30/33) in all hepatoblastoma histologic subtypes
- Large deletions of *CTNNB1* involving exon 3 and exon 4 were preferentially found in hepatoblastomas with pure fetal histology. Fetal HBs with wild type (2) and one with a *CTNNB1* point mutations (1) were also identified.
- Hepatoblastomas with large *CTNNB1* gene deletions demonstrated an immunohistochemical beta-catenin and canonical Wnt target gene expression pattern intermediate between that of wild-type *CTNNB1* cases and those with mutations confined to exon 3 of the *CTNNB1* gene

- Nuclear beta-catenin by immunohistochemistry was identified in all mutated cases and mostly those showing embryonal and small-cell components, whoever it was also identified in rare cases with fetal histology
- Expression of canonical Wnt target genes demonstrated that Wnt activation is a common event found in all histologic HB subtypes, however, significant differences in target gene expression patterns were found in HBs with pure fetal histology and those with a small cell component.

Our results are consistent with a differential role of *CTNNB1* mutations confined to exon-3 versus large deletions (from exon-3 to 4). Mutations confined to exon-3 maintain the BCL9-interaction domain, essential for transcription of WNT target genes and may facilitate more aggressive phenotypes. Large deletions including the BCL9-interacting domain, while still resulting in decreased proteosomal degradation of β-catenin, appear not be as capable of facilitating canonical WNT target gene expression. Large deletions are found in pure fetal hepatoblastoma and are associated with β-catenin and Wnt target gene expression pattern considerably different from hepatoblastomas with *CTNNB1* mutations confined to exon-3.

**Aim number 3: Evaluation of the SFRP genes methylation status in different hepatoblastoma subtypes.**

Activation of the canonical Wnt signaling pathway, either normally through binding of Wnt ligand to the Frizzled/LRP co-receptor or pathologically by *CTNNB1* mutation, results in the expression of numerous Wnt target genes, including those that provide feedback inhibition of the Wnt pathway. Among these are the *SFRP* genes, which encode a family of secreted Frizzled-related proteins that compete with Frizzled for binding of Wnt in the extracellular space, thereby blocking Wnt signaling. Decreased expression of *SFRP* genes due to methylation of *SFRP* promoters was recently identified in colorectal cancer. Restoration of SFRP expression in colorectal cancer cell lines reduced Wnt signaling by 60% even in the presence of activating mutations in *CTNNB1* or homozygous deletion of *APC*, an intracellular Wnt inhibitor.
We have analyzed the expression of canonical Wnt pathway genes in hepatoblastoma by quantitative real-time RT-PCR. Although in general we found evidence of activation of Wnt target genes, we were surprised to find markedly decreased expression of *SFRP2* (a known Wnt target gene), as well as decreased expression of *SFRP1, SFRP4* and *SFRP5*. We therefore examined the methylation status of the *SFRP* genes in hepatoblastoma. Methylation of *SFRP* genes was found only for *SFRP2* and only in one case (HB16), indicating that methylation may be a rare cause of decreased *SFRP* gene expression in hepatoblastoma and that other causes for decreased *SFRP* gene expression are present (Figure 3). It is interesting that HB16 also demonstrated abnormal loss of *MEG3* imprinting due to methylation, indicating that while methylation may be rare in hepatoblastoma, it is present in multiple pathways and may therefore contribute to disease pathogenesis in select cases. Alternatively, the CpG islands for *SFRP* promoter methylation important in colorectal cancer may not be identical to those that regulate *SFRP* gene expression in the liver. A broader screening technique that includes a greater

range of CpG islands, such as bisulfite-sequencing or bisulfite-pyrosequencing, may detect alternate methylation sites leading to decreased *SFRP* gene expression in hepatoblastoms.

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

Manuscripts:

López-Terrada D, Pulliam JF, Adesina A, Margolin JF, Gunaratne PH, Finegold MJ (2005). Correlation of *CTNNB1* mutation status, canonical Wnt pathway activation and tissue morphology in hepatoblastoma (HB). *In preparation for submission to Hepatology.*

Adesina A, Nguyen Y, Guanaratne G, Pulliam P, Lopez-Terrada D, Margolin J, Finegold MJ. FoxG1 is overexpressed in Hepatoblastoma. *submitted to Human Pathology*

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:

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. February 26, 2005.

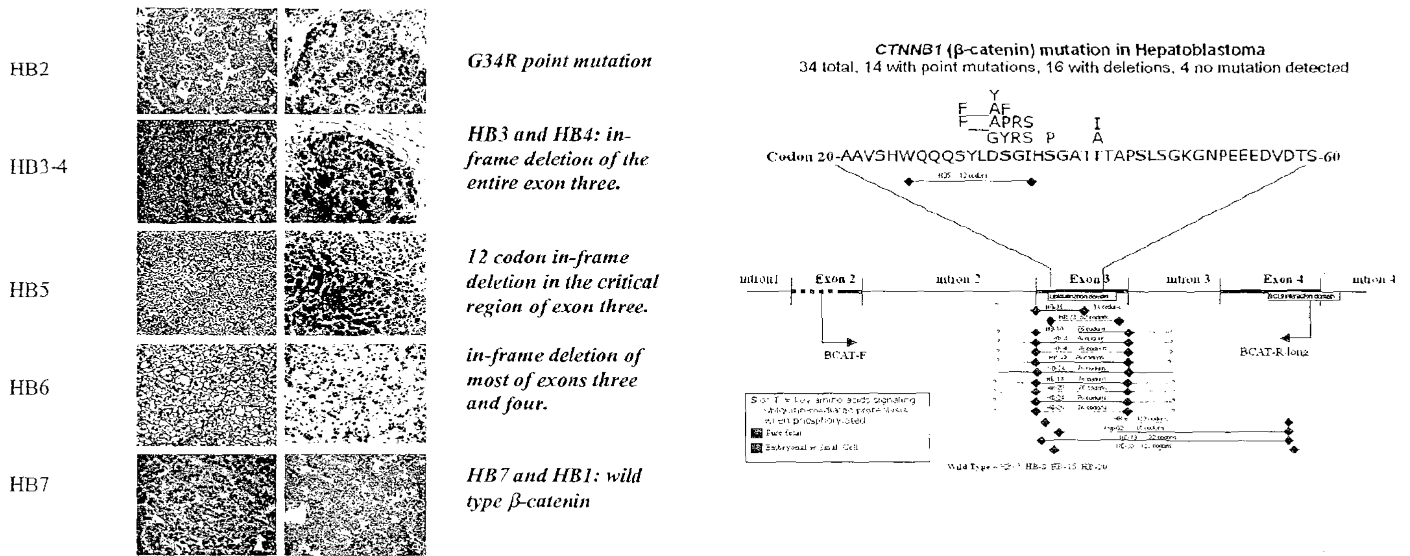
López-Terrada, D.; Gunaratne, P. H.; Pulliam, J. F.; Adesina, A.; Judith F. Margolin J. F.; Finegold, M. "Evaluation of CTNNB1 gene and canonical WNT pathway activation in hepatoblastoma: correlation with histologic subtypes"

FASEB meeting on Liver Biology, Development and Disease, Snowmass, Colorado July 22-27, 2006.

## C) Status of Project – Active

Our work on hepatoblastoma is ongoing. We are currently working on evaluating the expression of newly described, liver-specific Wnt target genes in our tumors, including Gluthamine synthetase (GS) and Gpr49 (G-protein coupled receptor 49.
We are also investigating the role of additional molecular lesions (mutations or methylation) in other key regulatory genes in the Wnt pathway and other pathways that have been demonstrated to interact with the Wnt pathway during hepatogenesis and liver regeneration, in particular the Notch signalling pathway.

## Figure 1. Beta-catenin immunohistochemistry and mutation analysis




## Figure 2. Expression of canonical Wnt target genes by QRT-PCR

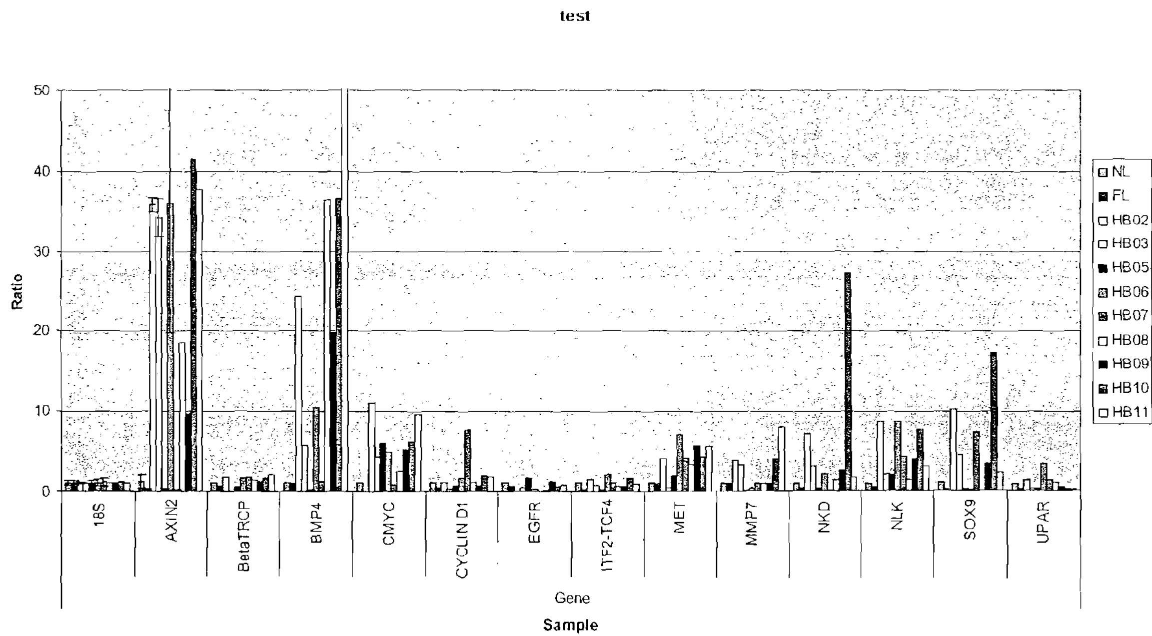


**Figure 3. Quantitative RT-PCR for Wnt Pathway Inhibitors in Hepatoblastoma Normalized to 18S rRNA and Normal Liver (ΔΔCt method)**

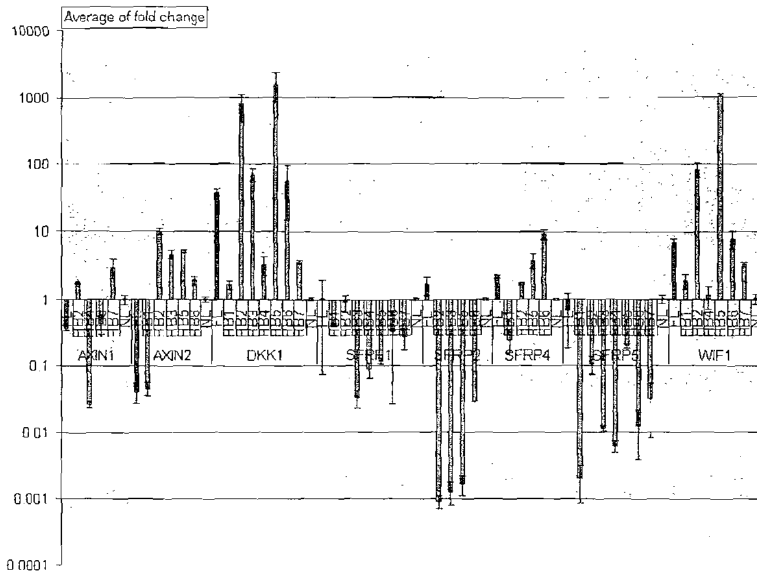



**Table 1 Results of Methylation Specific PCR for *SFRP* Genes**

| HB # | histology subtype | SFRP1 Methylation Specific PCR | SFRP2 Methylation Specific PCR | SFRP4 Methylation Specific PCR | SFRP5 Methylation Specific PCR | MEG3 Methylation Specific PCR |
|---|---|---|---|---|---|---|
| HB1 - fetal area | mixed epithelial - fetal area only | U | U | U | U | nl = U/M |
| HB2 | fetal/embryonal | U | U | U | U | nl = U/M |
| HB4 | fetal/embryonal/small cell | U | U | U | U | nl = U/M |
| HB5 | fetal/embryonal/small cell | U | U | U | U | nl = U/M |
| HB6 | pure fetal | U | U | U | U | nl = U/M |
| HB7 | pure fetal | U | U | U | U | nl = U/M |
| HB8 | fetal/embryonal | U | U | U | U | nl = U/M |
| HB9 | fetal/embryonal | U | U | U | U | nl = U/M |
| HB10 | fetal/embryonal/osteoid | ND | U | U | U | nl = U/M |
| HB11 | fetal/embryonal/osteoid | U | U | U | U | nl = U/M |
| HB12 | fetal/embryonal/small cell/osteoid | U | U | U | U | nl = U/M |
| HB13 | pure fetal | U | U | U | U | nl = U/M |
| HB14 | fetal/embryonal | U | U | U | U | nl = U/M |
| HB15 | fetal/embryonal | U | U | U | U | nl = U/M |
| HB16 | not favorable histology (CHTN) | U | M | U | U | abnl = M only |

U=Unmethylated M=Methylated nl=normal ND=not determined