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The Presence of PIM3 Increases Hepatoblastoma Tumorigenesis and Tumor Initiating Cell Phenotype and is Associated with Decreased Patient Survival

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Abstract

Purpose—Hepatoblastoma is the most common primary liver cancer of childhood and has few prognostic indicators. We have previously shown that Proviral Integration site for Moloney murine leukemia virus (PIM3) kinase decreased hepatoblastoma tumorigenicity. We sought to determine the effect of PIM3 overexpression on hepatoblastoma cells and whether expression of PIM3 correlated with patient/tumor characteristics or survival.

Methods—The hepatoblastoma cell line, HuH6, and patient-derived xenograft, COA67, were utilized. Viability, proliferation, migration, sphere formation, and tumor growth in mice were assessed in PIM3-overexpressing cells. Immunohistochemistry was performed for PIM3 on patient samples. Correlation between stain score and clinical/pathologic characteristics was assessed.

Results—PIM3 overexpression rescued the anti-proliferative effect observed with PIM3 knockdown. Sphere formation was increased in PIM3 overexpressing cells. Cells with PIM3 overexpression yielded larger tumors than those with empty vector. Seventy-four percent of samples expressed PIM3. There was no statistical difference in patient characteristics between subjects with strong versus weak PIM3 staining, but patients with strong PIM3 staining had decreased survival.

Conclusions—PIM3 expression plays a role in hepatoblastoma tumorigenesis. PIM3 was present in the majority of hepatoblastomas and higher PIM3 expression correlated with decreased

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survival. PIM3 warrants investigation as a therapeutic target and prognostic marker for hepatoblastoma.

Keywords

PIM3; Hepatoblastoma; PIM kinases; Cancer stem cells; Survival

Introduction

Hepatoblastoma is the most common primary liver malignancy of childhood and is most common in children younger than 5 years of age [1]. There are few prognostic indicators in hepatoblastoma. Small cell undifferentiated histology portends a poor prognosis whereas pure fetal indicates low-risk disease [2, 3]. Alpha-fetoprotein (AFP) levels correlate with outcomes such that AFP < 100 ng/mL is associated with worse prognosis [4]. Patients older than 5 years of age also have worse outcomes compared to younger patients [5]. However, none of these prognostic indicators are targetable or clinically actionable items.

Proviral Integration site for Moloney murine leukemia virus (PIM) kinases are a family of serine/threonine kinases (PIM1, PIM2, PIM3) that have been implicated in the tumorigenicity, or capacity to form tumors, of multiple cancer types [6–12]. They act through downstream proteins associated with the cell cycle [13–16], migration [10], and apoptosis [17]. Some of the target proteins include the pro-apoptotic protein, BAD [17], and the cell cycle inhibitor, p21 [15], which we have previously shown to play a role in hepatoblastoma. Others have shown that PIM3 plays a role in the development of hepatocellular carcinoma [11, 18], an adult liver cancer.

We have previously demonstrated that PIM3 kinase inhibition decreased tumorigenicity of hepatoblastoma *in vitro* and *in vivo*, indicating a role for PIM3 in maintenance of hepatoblastoma [19]. We sought to determine the effect of PIM3 overexpression on hepatoblastoma cells and evaluate the frequency of PIM3 expression in patient specimens and determine whether expression of PIM3 correlated with patient/tumor characteristics or survival.

Materials and Methods

Cells and cell culture

Cell lines were maintained at 37°C and 5% CO₂. The mixed epithelial human hepatoblastoma cell line, HuH6, was acquired from Thomas Pietschmann (Hannover, Germany) [20] and maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (HyClone, GE Healthcare Life Sciences, Logan, UT), 1 µg/mL penicillin/streptomycin (Gibco, Carlsbad, CA), and 2 mmol/L 1-glutamine (Thermo Fisher Scientific, Waltham, MA). The human embryonal hepatoblastoma patient-derived xenograft (PDX), COA67, was developed as described previously [21]. COA67 cells were maintained in Dulbecco's Modified Eagle's Medium/Ham's F12 supplemented with 2 mmol/L 1-glutamine (Thermo Fisher Scientific), 1 µg/mL penicillin/streptomycin (Gibco), 20 ng/mL epidermal growth factor (EMD Millipore, Billerica, MA), 20 ng/mL beta-fibroblast growth

factor (EMD Millipore), 2% B27 supplement (Gibco), and 2.5 µg/mL amphotericin B (HyClone). Both HuH6 and COA67 cell lines were verified within the last 12 months using short tandem repeat analysis [Heflin Center for Genomic Sciences, University of Alabama, Birmingham (UAB), Birmingham, AL]. Real-time qPCR was performed to assess the percentage of human and murine DNA contained in the COA67 PDX to ensure that the tumor did not harbor murine contamination (TREND RNA/DNA Isolation and TaqMan QPCR/Genotyping Core Facility, UAB, Birmingham, AL).

PIM3 overexpression vector and transfection

The PIM3 overexpression vector, pcDNA3.1/V5-His-*PIM3*, was a generous gift from Dr. Jussi Taipale and was generated by PCR amplification and cloning into the pcDNA3.1/V5-HisC vector [22]. The plasmid was sequenced for verification (Heflin Center for Genomic Sciences, UAB). Empty vector (ev, pcDNA3.1/V5-HisC) was used as a control for comparison. Transfection was carried out using FuGENE® HD Transfection Reagent (Promega, Madison, WI) per the manufacturer's protocol. Briefly, cells were plated on the day prior to transfection. The appropriate plasmid was incubated for 15 minutes at room temperature in OptiMEM™ media (Thermo Fisher Scientific) with FuGENE® HD Transfection Reagent in a 3:2 ratio of transfection reagent to DNA with 7.5 µg DNA per 1×10^6 cells and added to the cells while swirling the flask. Cells were transfected 48-72 hours prior to use in experiments.

PIM3 rescue

HuH6 cells (4×10^5) were transfected with PIM3 or control small interfering RNAs (siRNAs) at 20 nM concentration with Lipofectamine® RNAiMax (Thermo Fisher Scientific). PIM3 siRNA was obtained from Dharmacon (Dharmacon, GE Life Sciences, Lafayette, CO) as an ON-TARGETplus™ SMARTpool and as each of the component siRNAs individually (#8-GGCCGUCGUCGGAUCAGAU, #9-GCAGGACCUCUUCGACUUU, #10-GCGUGCUUCUCUACGAU, and #11-GGACGAAAAUCUGCUUGUG). Control siRNA (siNeg) was obtained from Dharmacon (ON-TARGETplus™ Non-targeting siRNA #1 with the sequence UGGUUUACAUGUCGACUAA). Six days later, cells from each group were transfected with either pcDNA3.1/V5-HisC (ev) or pcDNA3.1/V5-HisC-*PIM3* (PIM3 overexpression vector). After 72 hours, proliferation was assessed as below and lysates were made to perform immunoblotting as below to assess for PIM3 expression.

Immunoblotting

Whole-cell lysates were isolated using radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitors (Sigma Aldrich), phosphatase inhibitors (Sigma Aldrich), and phenylmethanesulfonylfluoride (Sigma Aldrich). Lysates were centrifuged at 14 000 rpm for 1 hour at 4°C. Protein concentrations were determined using Pierce™ BCA Protein Assay reagent (Thermo Fisher Scientific) and separated by electrophoresis on sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels. Antibodies were used according to the manufacturers' recommended conditions. Molecular weight markers (Precision Plus Protein Kaleidoscope, Bio-Rad) were used to confirm the expected size of the proteins of interest. Immunoblots were developed with Luminata Classico or Crescendo Western HRP

Substrate (EMD Millipore). Blots were stripped with stripping solution (Bio-Rad) at 65°C for 20 minutes and then re-probed with selected antibodies. Equal protein loading was confirmed using β -actin. Rabbit monoclonal anti-PIM3 (4165) was from Cell Signaling Technology (Beverly, MA). Mouse monoclonal anti- β -actin (A1978) was from Sigma Aldrich (St. Louis, MO).

Cell viability and proliferation

Cell viability was measured using the alamarBlue® Cell Viability Assay (Thermo Fisher Scientific). Cells (1.5×10^4 per well) were plated in 96-well plates and incubated for 24 hours prior to addition of 10 μ L of alamarBlue® reagent. Absorbance was read at 562 nm (reduced reagent) and 595 nm (oxidized reagent) using a microplate reader (BioTek Gen5, BioTek, Winooski, VT). After subtracting background absorbance of the media alone, reduction of alamarBlue® reagent was calculated according to the manufacturer's protocol. Viability was reported as fold change.

Cell proliferation was measured using the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega). Cells (5×10^3 per well) were plated in 96-well plates and incubated for 24 hours prior to addition of 10 μ L of CellTiter 96® reagent. Absorbance was read at 490 nm using a microplate reader (BioTek Gen5) to detect the formazan product. The background absorbance of the media alone was subtracted and proliferation was reported as fold change.

Cell migration

Cell migration was evaluated utilizing a monolayer wound-healing assay (scratch assay). HuH6 cells were allowed to grow to 80% confluence and a standard scratch was made in the well with a sterile 200 μ L pipette tip. Images of the scratch wound area were obtained at 0, 24, 48, and 72 hours. The area of the wound in pixels was quantified using the ImageJ MRI Wound Healing Tool [http://dev.mri.cnrs.fr/projects/imagej-macros/wiki/Wound_Healing_Tool]. Data were reported as fold change scratch area and compared between groups.

In vivo studies

Six-week old female athymic nude mice (Envigo, Pratville, AL) were maintained in the specific pathogen-free facility with standard 12-hour light/dark cycles and access to chow and water *ad libitum*. Experiments were approved by the Institutional Animal Care and Use Committee (IACUC-09803) and conducted within institutional, national, and NIH guidelines.

HuH6 human hepatoblastoma cells were transfected with ev or PIM3 overexpression vector as above and 2.5×10^6 cells were injected subcutaneously in the flank of six athymic nude mice (ev on left, PIM3 overexpression on right). Tumor volumes were measured twice weekly with calipers and calculated with the standard formula ($\text{width}^2 \times \text{length} / 2$), where the length is the largest measurement. Animals were humanely euthanized with CO₂ and cervical dislocation when IACUC parameters were met.

In vitro limiting dilution sphere assay

Transfected cells (ev or PIM3 overexpression, as above) were plated into 96 well ultra-low attachment plates using serial dilutions with 5000, 1000, 500, 100, 50, 20, or 1 cell per well for HuH6 cells and 50000, 10000, 5000, 1000, 500, 100, 50, or 1 cell per well for COA67 cells with at least 10 replicates per dilution. Cells were plated into Dulbecco's Modified Eagle's Medium/Ham's F12 supplemented with 2 mmol/L 1-glutamine (Thermo Fisher Scientific), 1 µg/mL penicillin/streptomycin (Gibco), 20 ng/mL epidermal growth factor (EMD Millipore), 20 ng/mL beta-fibroblast growth factor (EMD Millipore), 2% B27 supplement (Gibco), and 2.5 µg/mL amphotericin B (HyClone) combined with 50% conditioned medium. Conditioned medium was obtained by removing culture medium in the presence of cells and filtering it to remove cells and debris. Once spheres were present in the most concentrated wells, all wells were assessed for the presence or absence of spheres by a single researcher. Extreme limiting dilution analysis software was utilized to analyze the data (<http://bioinf.wehi.edu.au/software/elda/>).

Patients and tissue samples

Experiments were approved by the University of Alabama, Birmingham, Institutional Review Board (UAB IRB-100930009). Tissues from 23 cases of hepatoblastoma before chemotherapy treatment were obtained from Children's of Alabama between 1977 and 2015. Tissues were fixed in formalin and embedded in paraffin. Of the 23 samples available, 4 were not evaluable by a pathologist and 2 lacked corresponding clinical data, leaving 17 evaluable hepatoblastoma specimens with corresponding patient data (Fig. 1).

Immunohistochemistry

Specimens were sectioned into 6 µm sections and baked at 70°C for one hour on positive slides. Slides were deparaffinized, steamed, quenched with 3% hydrogen peroxide, and blocked with blocking buffer [bovine serum albumin (BSA), powdered milk, Triton X-100, phosphate buffered saline (PBS)] for 30 minutes at 4°C. The primary antibody anti-PIM3 (rabbit polyclonal, 1:300, ab71321, Abcam, Cambridge, UK) was added and incubated overnight in a humidity chamber at 4 °C. After washing with PBS, the secondary antibody for rabbit (R.T.U. biotinylated universal antibody, Vector Laboratories, Burlingame, CA) was added for 30 minutes at 22°C. The staining reaction was developed for 30 minutes at room temperature with VECTASTAIN Elite ABC reagent (PK-7100, Vector Laboratories) and Metal Enhanced DAB Substrate (Thermo Fisher Scientific, Waltham, MA) for 2 minutes. Slides were counterstained with hematoxylin. A negative control (rabbit IgG, 1 µg/mL, EMD Millipore, Burlington, MA) was included with each run. Stained slides were evaluated by a pathologist (E.M.M.) blinded to the patient data. A qualitative stain score of 0, 1+, or 2+ was assigned, with 0 and 1+ considered weak PIM3 staining and 2+ considered strong PIM3 staining.

Statistical analysis

Experiments were repeated at least in triplicate. Variable distributions were presented using standard descriptive statistics. Age at diagnosis was described in months. Children's Oncology Group (COG) was the staging system used to define extent of disease at the time

of initial surgery. COG Stage data were missing for four subjects and AFP levels were missing for five subjects. Data were analyzed using a t-test for numeric data or χ^2 test for categorical data to assess the correlation between PIM3 staining and clinical and pathologic parameters. Survival analysis was completed using Kaplan-Meier estimates and was compared using the log-rank test. A p-value ≤ 0.05 was considered statistically significant.

Results

PIM3 kinase overexpression did not have any significant phenotypic effect on hepatoblastoma cell viability, proliferation, or motility

PIM3 overexpression was achieved using transient transfection of HuH6 and COA67 cells with a pcDNA3.1/V5-HisC-based expression vector and confirmed by immunoblotting (Fig. 2A). There was no significant change in viability or proliferation in the PIM3-overexpressing HuH6 or COA67 cells compared to the empty vector controls (Fig. 2B-E). Additionally, there was no significant change in motility based on scratch assay in PIM3-overexpressing HuH6 cells compared to empty vector control (Fig. 2F). The scratch assay measured the ability of cells to migrate and served as a measure of the cells' invasion of local tissues and the motility necessary for metastasis.

PIM3 overexpression rescued the phenotype observed with PIM3 kinase knockdown in hepatoblastoma

Despite PIM3 overexpression not affecting hepatoblastoma cells *in vitro*, we have previously demonstrated that PIM3 knockdown, knockout, and inhibition decreased hepatoblastoma tumorigenicity [21]. It therefore appears that the presence of PIM3 is sufficient to induce a tumorigenic phenotype. To further demonstrate that PIM3 expression was directly responsible for the tumor phenotype observed, a rescue experiment was performed. HuH6 cells were transfected with siNeg or siPIM3 followed by empty vector or PIM3 overexpression plasmid. Both PIM3 knockdown and overexpression were observed by immunoblotting (Fig. 3A, Supplementary Fig. 1 for decreased exposure). Whereas proliferation was lower in siPIM3 cells than siNeg cells as previously demonstrated [21], PIM3 overexpression in knockdown cells returned the level of proliferation back to the baseline observed in cells transfected with siNeg and empty vector (Fig. 3B).

Sphere formation in PIM3 overexpressing hepatoblastoma cells

Sphere formation is a marker for the presence of stem cell like cancer cells (SCLCCs). HuH6 cells overexpressing PIM3 formed spheres at significantly lower cell number than those with the empty vector control ($p < 0.01$, Fig. 4A), indicating an increase in SCLCCs in PIM3 overexpressing cells. COA67 cells overexpressing PIM3 had a trend toward forming spheres at lower cell numbers than empty vector controls, but this did not reach significance ($p=0.07$, Fig. 4B).

PIM3 kinase overexpression increased *in vivo* tumor growth in a mouse model of hepatoblastoma

With the knowledge that PIM inhibition decreased tumor growth *in vivo* [21], we wished to assess whether PIM3 overexpression had an effect on tumor growth *in vivo*. We injected

HuH6 cells transfected with PIM3 overexpression plasmid or empty vector plasmid and assessed tumor growth over time. Tumors with ectopic PIM3 expression were significantly larger than empty vector control tumors ($p < 0.05$, Fig. 5).

Patient and tumor characteristics

To determine the frequency of PIM3 expression in patient samples and the effect of PIM3 expression on clinical characteristics, we examined tumor samples from a single institution. The clinical features of the subjects included in this study are described in Table 1. There was a 1.4:1 ratio of males to females (10 males and 7 females). The median age at diagnosis was 10 months (range 0.5-63 months). The majority of subjects (53.8%) had Stage I hepatoblastoma followed by 23% with Stage IV, 15% with Stage III, and 8% with Stage II disease.

Expression of PIM3 in patient samples

Fourteen specimens (73.7%) were positive for PIM3 expression. Overall, 82.4% exhibited weak PIM3 staining (stain score 0 or 1+) and 26.3% exhibited strong PIM3 staining (stain score 2+). Example images of specimens with each stain score are shown in Figure 6 along with the number and percent of specimens with each stain score.

Correlation between PIM3 expression and clinical and pathologic features

There was no statistical difference in sex, COG Stage, histologic subtype, AFP, or age between subjects with weak versus strong PIM3 staining (Table 2). When excluding the somewhat equivocal 1+ stain scores, there remained no significant difference between those expressing higher PIM3 expression (2+ stain score) versus those lacking PIM3 expression.

Survival analysis

We compared the strong PIM3 expressing subjects to those with weak PIM3 expression for the survival analysis. Those with strong PIM3 staining had significantly decreased survival compared to those with weak PIM3 staining (Fig. 7, $p = 0.024$). Only one subject with weak PIM3 staining died and this subject had Stage IV disease. There were two other subjects with weak PIM3 staining who had Stage III/IV disease and both were alive at last follow up (161 and 53 months). Two subjects with strong PIM3 staining survived; one had an unknown COG Stage and one had Stage III disease. Of those with strong PIM3 staining, the lowest observed Stage was Stage II and that subject died 82 months after diagnosis.

Discussion

We have previously shown that PIM3 is important for tumor formation in hepatoblastoma by demonstrating that PIM3 knockout, knockdown, and inhibition abrogated hepatoblastoma tumorigenicity [21]. In the present study, we assessed the effect of PIM3 overexpression *in vitro* and in a mouse model of hepatoblastoma and PIM3 expression in patient tumors. *In vitro*, PIM3 overexpression was not found to significantly affect hepatoblastoma viability, proliferation, or migration. We have previously shown that hepatoblastoma cells express PIM3 at baseline whereas normal liver tissue does not [21]. These data support the idea that the presence of PIM3 alone confers a tumorigenic phenotype but that increasing expression

of PIM3 does not significantly alter these characteristics over a relatively short time in a tissue culture environment. However, when PIM3 overexpression HuH6 cells were compared to the control transfected with empty vector *in vivo*, tumors were larger in the mice bearing PIM3 overexpressing cells. The difference between these results indicates that it takes a longer time to observe an increase in tumorigenicity with PIM3 overexpression, so PIM3 must play a role in not only tumor initiation, but in tumor maintenance as well.

A potential mechanism for the increase in tumorigenicity with PIM3 overexpression is that PIM3 may regulate SCLCCs. In this study, we provide evidence for this postulation by showing that PIM3 overexpression yielded sphere formation at lower cell numbers. These findings indicate an increase in SCLCCs with PIM3 overexpression. Others have similarly found that PIM3 regulates stemness in cancer cells in pancreatic cancer. Li *et al.* demonstrated that PIM3 induced stemness via activation of the STAT3 pathway [23]. The STAT3 pathway has not been studied in hepatoblastoma aside from the understanding that STAT3 signaling is present [24, 25]. Additionally, many of the pathways through which we have already demonstrated PIM3 to play a role in hepatoblastoma tumorigenicity are related to characteristics of SCLCCs. We plan to more thoroughly examine the mechanisms by which PIM3 regulates stemness in hepatoblastoma in the future, including the role of the STAT3 pathway.

A limitation of the *in vitro* portion of this study is the use of only one long-term passaged cell line. However, this is unavoidable as there is a dearth of cell lines available to study hepatoblastoma. We were able to include data from a patient-derived xenograft developed at our institution which were consistent with the findings from the long-term passaged cell line, HuH6.

In the present study, we examined tissue from 19 patients with hepatoblastoma, of which 17 had correlated clinical data. Using immunohistochemistry (IHC), we found that PIM3 was expressed in the majority (73.7%) of specimens, but only stained strongly in 26.3%. Others have used a variety of methods and demonstrated that PIM3 is not expressed in normal liver [18, 26]. These findings indicated that PIM3 may be a good target for treatment as it is not expressed in normal tissues but is expressed in a large proportion of hepatoblastoma tissues.

The patient population in this study was similar to that which has been observed on a larger scale using 606 patients in the Surveillance, Epidemiology and End Results (SEER) database [5]. In our patient population, 58.8% of hepatoblastoma patients were male, which is similar to the 59.4% observed using SEER data. Metastatic disease accounted for 26.9% of patients in the SEER data and 23.1% of patients in our cohort. In a sample comprised of 43 patients with hepatoblastoma, similar findings were observed for sex and age [27]. These similarities to previously published descriptive studies suggested that our findings in a small cohort are likely to translate to a larger population.

Many of these aforementioned characteristics have been demonstrated by others to correlate with outcomes in hepatoblastoma. Higher COG Stage is associated with poor prognosis [28]. Small cell undifferentiated histology is a poor prognostic factor whereas pure fetal histology portends a better prognosis [2, 3]. AFP < 100 ng/mL at diagnosis is associated

with extensive disease at diagnosis, chemoresistance, and poor outcome [4]. Patients younger than 5 years have a 5-year survival of 64% compared to only 20% for those 15-19 years old [5]. However, we found no correlation between PIM3 expression and sex, histologic subtype, AFP, or age. The COG Stage neared significance and with a larger sample size a difference may exist such that patients strong PIM3 staining correlates with higher stage disease.

Finally, we examined the relation between PIM3 expression and survival. Patients expressing high levels of PIM3 had worse survival compared to those with low PIM3 expression. These survival differences did not strictly relate to stage of disease. We realize that one limitation to this study is the small number of samples evaluated. Despite spanning 38 years, we only had 19 evaluable samples, which were reduced to 17 due to a lack of clinical data corresponding to some of the older specimens. Unfortunately, it is challenging to gather tissue from large numbers of patients due to the low incidence of hepatoblastoma. Future directions include the expansion of this project to evaluate PIM3 expression in samples from multiple institutions. By increasing the sample size, we may be able to draw further conclusions about patient characteristics that predict PIM3 expression and by extension, response to PIM inhibition therapy.

Conclusions

These findings suggest that PIM3 expression plays a role in hepatoblastoma tumorigenesis, but overexpression beyond physiologic levels only minimally increases tumorigenesis. PIM3 is present in the majority of hepatoblastomas and higher PIM3 expression appears to be correlated with poor outcomes. Therefore, PIM3 warrants further investigation as a therapeutic target and prognostic marker for hepatoblastoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Biographies

DR. MARTIN: In the mouse models, did you look at in the cells that were injected with PIM3 overexpression? After the tumors were injected, did you look back at the histology or the gene expression of the tumors to see if PIM3 expression was changed due to the tumor microenvironment in the experiment?

DR. STAFMAN: Thank you. So we are looking at histology now. It's a little bit challenging. PIM3 is a little hard to look at, but we haven't shown this yet. We're looking at PCR for PIM3 expression in those right now. PIM3 has been shown to affect the tumor

microenvironment, particularly angiogenesis, so we're going to look at CD31 and markers for angiogenesis. The problem with studying the microenvironment in terms of immune cells is that these are athymic nude mice, and they lack T-cells, which makes them not a good model to examine that.

Dr. MICHAEL P. LAQUAGLIA, NEW YORK, NY: This is a very interesting paper. I had two questions: number one, did you have patients or tumor cells that were metastatic versus localized? And is there a difference? And number two, how does it all tie in with the Wnt pathway in hepatoblastoma?

DR. STAFMAN: Great question. For the first question about metastatic cells, we unfortunately only have cells from primary tumors right now. We do have a patient drive xenograph program and are trying to look at metastatic versus primary, but unfortunately we just can't do that right now.

And regarding Wnt, it plays a large role in hepatoblastoma. We have not examined the relationship between PIM3 and Wnt, but that would very interesting to do.

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Figure 1.

Flowchart of samples included in the study. Twenty-three tissue samples from patients with hepatoblastoma were available from 1977 to 2015. Four samples were excluded as they were not evaluable by a pathologist and 2 lacked corresponding clinical data. The final cohort for correlation of PIM3 expression with clinical and pathologic parameters and outcomes totaled 17.

Figure 2.

PIM3 kinase overexpression did not affect hepatoblastoma cell viability, proliferation, or motility. Cells were transfected with pcDNA3.1/V5-HisC-based expression vector containing the *PIM3* gene. (A) PIM3 overexpression in the HuH6 and COA67 cell lines was confirmed by immunoblotting. Cells transfected with the PIM3 vector had higher PIM3 expression than cells treated with FuGene transfection reagent only or empty vector (ev). Viability was assessed with the alamarBlue® Cell Viability Assay. There was no difference in viability between cells transfected with ev or PIM3 vector in (B) HuH6 or (C) COA67 cells. Proliferation was assessed with the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay and no difference was observed between cells transfected with ev or PIM3 vector in (D) HuH6 or (E) COA67 cells. (F) A standard scratch assay was performed to assess motility using HuH6 cells. There was no difference in scratch area at 24, 48, or 72 hours between cells transfected with ev and PIM3 vector.

A

B

Figure 3.

PIM3 overexpression rescued the anti-proliferation phenotype observed with siRNA knockdown. HuH6 cells were transfected with siNeg or siPIM3 followed by PIM3 overexpression vector or empty vector (ev). (A) Immunoblotting confirmed decreased PIM3 expression in siPIM3 cells and increased expression of PIM3 in cells transfected with PIM3 overexpression vector. (B) Cells transfected with siPIM3 followed by ev exhibited decreased proliferation as observed previously, but overexpression of PIM3 in cells with PIM3 knocked down returned the proliferation level to baseline.

Figure 4. Sphere formation in PIM3 overexpressing cells. (A) PIM3 overexpression significantly increased sphere formation in HuH6 cells ($p < 0.01$). (B) There was a trend toward increased sphere formation with PIM3 overexpression in COA67 cells ($p=0.07$).

Figure 5. PIM3 kinase overexpression increased tumor growth in a xenograft model of hepatoblastoma. HuH6 human hepatoblastoma cells were transfected with transfection reagent only (FuGene), empty vector (ev), or PIM3 overexpression vector and 2.5×10^6 cells were injected subcutaneously in the flank of 6 athymic nude mice (ev on left, PIM3 overexpression on right). Tumor growth was followed and tumors were measured twice weekly with calipers to calculate tumor volumes. Cells overexpressing PIM3 formed significantly larger tumors than control cells transfected with empty vector, reported as tumor volume $\pm$ SEM ($p < 0.05$).

Figure 6. Immunohistochemical staining of PIM3 in hepatoblastoma specimens. Example images of negative (0), 1+, and 2+ PIM3 staining. IgG was used as a control. Number and percent of specimens with each stain score are listed above the respective images.

Figure 7. Kaplan-Meier survival curve. The relation between PIM3 expression as assessed by immunohistochemistry and survival in patients with hepatoblastoma. Patients with strong PIM3 expression had significantly worse survival than those with weak PIM3 staining of their tumors ($p=0.024$).

Table 1.

Clinical and pathologic features of hepatoblastoma subjects.

Characteristic	Number of subjects (%)
Sex	
Male	10 (58.8%)
Female	7 (41.2%)
COG Stage	
I	7 (53.8%)
II	1 (7.7%)
III	2 (15.4%)
IV	3 (23.1%)
Histologic subtype	
Embryonal	2 (11.8%)
Fetal	3 (17.6%)
Small cell undifferentiated	1 (5.9%)
Mixed	11 (64.7%)
AFP	
<100	1 (8.3%)
>100	11 (91.7%)
	Median (range)
Age (months)	10 (0.5-63)

Table 2.

Correlation of PIM3 expression with clinical and pathologic characteristics. There was no significant correlation between PIM3 expression and sex, COG Stage, histologic subtype, AFP, or age. Comparisons were made between weak PIM3 staining and strong PIM3 staining.

Characteristic	Stain score		p-value
	Weak	Strong	
Number of subjects (%)			
Sex			0.59
Male	8 (67%)	2 (40%)	
Female	4 (33%)	3 (60%)	
COG Stage			0.09
I	7 (70%)	0 (0%)	
II	0 (0%)	1 (33.3%)	
III	1 (10%)	1 (33.3%)	
IV	2 (20%)	1 (33.3%)	
Histologic subtype			0.83
Embryonal	1 (8.3%)	1 (20%)	
Fetal	2 (16.7%)	1 (20%)	
SCU	1 (8.3%)	0 (0%)	
Mixed	8 (66.7%)	3 (60%)	
AFP			0.55
<100	1 (11.1%)	0 (0%)	
>100	8 (88.9%)	3 (100%)	
3-year OS			0.11
Survival	9 (90.0%)	2 (50.0%)	
No survival	1 (10.0%)	2 (50.0%)	
Age in months [median (range)]	9 (2-42)	15.5 (0.5-63)	0.69