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Novel patient-derived xenograft and cell line models for therapeutic testing of pediatric liver cancer

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Authors' contributions

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jhep.2016.04.009>.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Abstract

Background & Aims—Pediatric liver cancer is a rare but serious disease whose incidence is rising, and for which the therapeutic options are limited. Development of more targeted, less toxic therapies is hindered by the lack of an experimental animal model that captures the heterogeneity and metastatic capability of these tumors.

Methods—Here we established an orthotopic engraftment technique to model a series of patient-derived tumor xenograft (PDTX) from pediatric liver cancers of all major histologic subtypes: hepatoblastoma, hepatocellular cancer and hepatocellular malignant neoplasm. We utilized standard (immuno) staining methods for histological characterization, RNA sequencing for gene expression profiling and genome sequencing for identification of druggable targets. We also adapted stem cell culturing techniques to derive two new pediatric cancer cell lines from the xenografted mice.

Results—The patient-derived tumor xenografts recapitulated the histologic, genetic, and biological characteristics—including the metastatic behavior—of the corresponding primary tumors. Furthermore, the gene expression profiles of the two new liver cancer cell lines closely resemble those of the primary tumors. Targeted therapy of PDTX from an aggressive hepatocellular malignant neoplasm with the MEK1 inhibitor trametinib and pan-class I PI3 kinase inhibitor NVP-BKM120 resulted in significant growth inhibition, thus confirming this PDTX model as a valuable tool to study tumor biology and patient-specific therapeutic responses.

Conclusions—The novel metastatic xenograft model and the isogenic xenograft-derived cell lines described in this study provide reliable tools for developing mutation- and patient-specific therapies for pediatric liver cancer.

Lay summary—Pediatric liver cancer is a rare but serious disease and no experimental animal model currently captures the complexity and metastatic capability of these tumors. We have established a novel animal model using human tumor tissue that recapitulates the genetic and biological characteristics of this cancer. We demonstrate that our patient-derived animal model, as well as two new cell lines, are useful tools for experimental therapies.

Keywords

Pediatric liver cancer; Patient-derived xenograft; Therapeutic testing

Introduction

Pediatric liver cancer is a rare (~3 patients per million) but often aggressive disease of very young children. Hepatoblastoma (HB) accounts for more than two-thirds of cases, with most of the remainder being hepatocellular carcinoma (HCC) [1], and the average age at diagnosis is 18 months, with only 5% of new HB cases diagnosed in children who are older than four years of age [2]. Worse, the incidence of HB in the US doubled between 1975 and 1995 [1], which is likely to be due to the improvement in survival of premature infants of very low birth weight; one of the major risk factors for HB [3–7]. Patients with high-risk tumors presenting with multifocal lesions and metastatic disease have an overall survival of only 50–65% [2], and current treatment is a punishing regimen of surgery and chemotherapy (cisplatin, doxorubicin, vincristine, or 5-fluorouracil). It is clearly necessary to develop more targeted, less toxic therapies. To do this, however, we need physiologically relevant models.

Although some groups have reported animal models that develop tumors with the histomorphological characteristics of HB, genetic mouse models of liver cancer are all accompanied by hepatocellular carcinoma (HCC) [8,9]. These liver tumors do not manifest until adulthood even when induced during gestation [8], and the extent to which they reflect pediatric liver tumors is questionable. Thus far, the most promising approach to modeling pediatric liver cancer has been the development of patient-derived xenografts obtained by directly injecting primary tumor cells into the subcutis of nude mice [10–12]. These models represent a certain degree of tumor heterogeneity, but their utility is limited by the fact that the resulting tumors are encapsulated (rather than invasive), and so far subcutaneous HB have not been shown to form metastases. Another type of tumor model, often used for *in vivo* drug studies, involves the injection of human HB cell lines (rather than primary tumor cells) into the subcutaneous tissue or splenic capsule of immune-deficient mice [13]. These models are also limited, however, because unlike the primary tumors, the cell lines are usually monoclonal and have been selected for their ability to grow in tissue culture.

Cell lines are nonetheless preferable to animal models for some applications, including high-throughput screening. Unfortunately, only a few pediatric liver cancer cell lines have been described to date: Huh6 [14], HepG2 [15], HepT1 [16], HepT3 [17], Hep293TT [18], HB1 [19], and HepU1/2 [20]. The vast majority of pediatric liver cancer studies have relied on a single cancer cell line, HepG2, which is insufficient to represent the intertumoral heterogeneity of this disease. The over-reliance on HepG2 underscores the need for more representative pediatric hepatoblastoma cell lines to facilitate both basic and translational research.

Here we have sought to overcome the various limitations of extant models. We have developed the first metastatic patient-derived tumor xenograft (PDTX) models of pediatric liver cancer, derived novel cell lines from the patient tumors, and tested small molecule inhibitors targeted to the molecular profiles of two tumors.

Materials and methods

Generation of PDTX

Freshly procured pediatric liver cancer samples were cut into small tissue blocks (~50 mm³) and kept in tissue culture media on ice until use (<5 h). Human samples were obtained with consent of parents and approval from institutional review board (IRB), which conforms to the ethical guidelines of the 1975 Declaration of Helsinki. Animals received human care and the Institutional Animal Care and Use Committee (IACUC) approved all animal experiments.

2–4 months old NSG or FRG mice were anesthetized (isoflurane/oxygen mixture). Midabdominal incision was performed through the skin and musculature. The left lower liver lobe was exposed and a ~2 mm long incision made in the Glisson's capsule, immediately after which we applied human tumor on the incision to effect hemostasis. Engrafted tissue blocks were carefully sealed onto the murine liver using tissue adhesive (Vetbond). When tissue adhesive dried (4–5 min), the abdominal cavity was closed using reabsorbable sutures for the muscle layer and tissue clamps for the skin layer. In the FRG strain, selection pressure for the human xenograft was applied where indicated as previously described [21].

In vivo studies

PDTXs from patient #1 and patient #2 were treated daily with either trametinib (1 mg/kg) and buparlisib (50 mg/kg) (N = 8) or vehicle (solvent without drug) (N = 8). Trametinib was administered (0.2 ml/20 g body weight) by gavage dissolved in 0.5% hydroxypropylmethylcellulose and 0.2% Tween-80 in bidistilled water (pH 8.0). Final dose of trametinib: 1 mg/kg. Buparlisib was dissolved in 10% N-Methyl-2-pyrrolidone (NMP) and 90% PEG300, freshly formulated and administered by gavage (0.1 ml/20 mg body weight) within 1 h. Final dose of buparlisib: 50 mg/kg. Dosing began when tumors reached approximately 0.2 cm³ (range: 0.10–0.37 cm³) drugs and vehicle were administered thereafter every day for two weeks.

Cell lines

HepG2 cell line was purchased from ATCC, Huh6 from Riken and HepT1 was a kind gift from Stefano Cairo. B6 cell lines were generated as follows: harvested tumors from PDTX and patients were minced into small pieces (<1 mm²) with a scalpel, centrifuged at 50 g, resuspended and plated on Matrigel®-coated tissue culture plates. Media was replaced after 24 h and thereafter every second day. Cells were plated and maintained in HCM media (Lonza) supplemented with 3% FBS. B6 cell lines were subcultured (splitting ratio 1:2–4) after 7–10 days when reaching confluency of 80%. Cells were first Incubated for 3–5 min in 2 mg/ml dispase (Invitrogen) at 37 °C until edges of colonies start to detach from the dish. Then dispase was carefully removed, followed by 3 washing steps with DMEM/F12 to remove remaining dispase. Cell clusters were disintegrated manually with a serological pipette sliding over colonies and extruding culture media. Floating colonies were transferred onto freshly coated Matrigel tissue culture dishes. Some B6 cell lines (10⁶ cells) were also used for direct intrahepatic injections instead.

Cell viability by MTT assay

Cells were seeded in 96-well plate and incubated with trametinib (0.001 nM–100 nM), and buparlisib (5 nM–50 μ M) for 72 h. The number of viable cells was determined according to the manufacturer's protocol using an MTT assay (Sigma, cat # M6494) and a plate reader. Additional methods may be found in the Supplementary material.

Results

Establishing an orthotopic patient-derived tumor xenograft for hepatoblastoma

We cut a freshly procured primary tumor specimen from a hepatoblastoma patient into small tissue blocks (~50 mm³) and engrafted them onto the murine livers as shown in Fig. 1A. In brief, we cut through the Glisson's capsule of the murine liver and immediately engrafted the human tumor tissue on top of the incision, attaching the edges of the human tissue onto the mouse liver with surgical tissue adhesive. This technique addresses two needs at once: it immediately stanches the copious bleeding from the mouse liver, and it establishes a prompt blood supply for rapidly proliferating tumor cells within the tissue block (see Materials and methods). Two immunodeficient mouse strains were used as hosts for PDTX; the NSG (NOD.SCID, *Il2rg*^{-/-}) [22–24] and the FRG (*fah*^{-/-}, *rag2*^{-/-}, *Il2rg*^{-/-}) [21,25] strains. After transplantation, we measured human alpha fetoprotein (AFP), a tumor marker of HB and HCC, and human albumin (ALB), which is produced by tumor and normal liver cells. Both AFP and ALB were detectable in murine blood one week after transplantation, with concentrations progressively increasing over the following weeks (Fig. 1B). Levels of AFP were comparable to those detected in human cancer patients, reaching up to 10⁶ IU/ml. In contrast, ALB levels did not approach human values, since this protein is predominantly produced by normal hepatocytes rather than tumor cells.

Animals were sacrificed 4–12 weeks after transplantation. We observed heterogeneous, exophytically growing tumors on the engrafted murine liver lobe (Fig. 1A). Histological evaluation of the xenografts revealed tumor tissue with similar morphological characteristics as the original primary human tumor: mixed epithelial HB with fetal (70%), embryonal (30%) and mesenchymal (~1%) components (Fig. 1C). Immunological markers of pediatric liver tumors such as glypican 3 (GPC3) and nuclear β -catenin (CTNNB1) were detected in both xenograft and primary patient tumors (Fig. 1C). Furthermore, the formation of osteoid in the xenografts (Fig. 1D) demonstrated preservation of the rare mesenchymal components of this HB. Co-staining for von Willebrand factor (vWF) and human nuclei indicated that the tumor vessels were not of human origin (Fig. 1E). Immunostaining for MECA32, a panendothelial mouse-specific antibody (Fig. 1E), confirmed the murine origin of the PDTX vasculature.

The catabolic enzyme fumarylacetoacetate hydrolase (FAH) was expressed in human hepatoblastoma samples (Supplementary Fig. 1). In analogy to previous data with xenotransplanted human hepatocytes [21,25], we hypothesized that the take rate of the human xenograft might be increased in the *fah*^{-/-} FRG strain, since the transplanted cells undergo selection pressure. We found, however, that both acceptor strains had similar take rates: seven out of eleven for the NSG and five out of nine for the FRG strain.

We next explored serial transplantation of the PDX in NSG mice. As for the primary human tumors, we cut small tissue blocks from the xenograft immediately after sacrificing the mouse, 8–12 weeks after tumor implantation. Since the cancer tissue grows exophytically from the murine liver, we could macroscopically distinguish between xenograft tumor and normal mouse liver tissue (Fig. 1A). Serial transplantation of xenografts had a higher take rate (80%) than engraftment of primary patient samples (60%). We serially transplanted the PDX seven times. Histological analysis revealed preservation of both the embryonal and fetal HB tumor components (Supplementary Fig. 2). We could also detect rare mesenchymal components of this tumor in several serially transplanted PDX.

Orthotopic PDX models for common histological subtypes of pediatric liver cancer

Having developed a successful engraftment technique for hepatoblastoma, we next aimed to generate more PDXs from other pediatric cancer patients. Table 1 summarizes clinical patient data and established PDX models. In addition to the PDX described above (Patient #1), we were able to generate five further PDX models (Patients #1, #2, #3, #7, #8 and #12) out of fifteen patient samples. These PDX models represent the most common histological subtypes of pediatric liver cancer: HB (Patients #3, #7 and #8), HCC (patient #12), and hepatocellular malignant neoplasm (Patient #2). This last subtype is a difficult to classify but should be included in the provisional “hepatocellular malignant neoplasm, NOS” group, according to the latest international consensus classification [26]. All children with liver cancer were treated with chemotherapy except the patient with HCC (patient #12). Tumor chemosensitivity, resulting in significant necrosis and generally associated with reduction of AFP, would likely hinder establishment of PDX. Indeed, five out of eight tumor samples from patients with AFP >2000 IU/ml at the time of tissue harvest led to successful PDX, as compared to one out of seven patient samples with AFP <2000 IU/ml (positive predictive value 0.71). Nevertheless, our inability to engraft two diagnostic biopsies with high AFP levels (patient #10 and #15) suggests that there are additional factors influencing the success of engraftment.

As for the first PDX model (patient #1), we analyzed all consecutive PDX for human ALB and AFP (Table 1). Both proteins could be measured in all successful PDX, while neither protein could be detected for tumors that did not take. Again we compared PDX to their primary tumors and confirmed similar characteristics for all different histological subtypes of pediatric liver tumors (Supplementary Fig. 3).

PDX models can recapitulate metastatic disease

Three out of fifteen pediatric cancer patients developed metastatic disease over time (Table 1), but only one out of the three patient samples led to a PDX model (patient #2). In order to determine if PDX models would give rise to metastases, we analyzed multiple organs (liver, lungs, kidney, heart, spleen) grossly and microscopically by H&E staining. We detected intrahepatic metastases in three out of six mice with PDXs (patient #1, #2, #7) (Fig. 2A). In contrast to the human liver, the murine liver lobes do not form one continuous organ: the seven lobes are largely independent, with the left and right upper lobes that are separated by the gallbladder forming a parenchymal connection. Since we engrafted the

PDTX onto the lower left lobes, human tumors found in other lobes can be considered metastatic lesions rather than locoregional expansion of the xenograft.

The same three PDTXs also generated metastases in the lungs (Fig. 2B). Metastasis was observed as early as nine weeks and as late as eleven months after primary engraftment of patient tissue. Interestingly, we observed that some metastases retained the histologic heterogeneity of primary tumor, displaying components of fetal and embryonal HB within the same metastatic lesion (Fig. 2C). This finding has never been reported for clinical samples, but we could confirm data from our xenograft models also in metastatic patient samples (Fig. 2C).

Derivation of new cancer cell lines

Primary tumor patient samples were used not only to establish xenografts but also to derive cancer cell lines. Tumors were minced into small pieces (1 mm) then plated on Matrigel-coated plates (see Materials and methods). Most tumor samples showed heterogeneous outgrowth of epithelial cancer cells and fibroblasts within 1–2 days, but we were not able to derive epithelial cell lines from any of the fifteen pediatric cancer specimens using conventional tissue culture techniques. Instead, the cell lines produced mesenchymal, fibroblast-like cells after subculturing for several passages (Supplementary Fig. 4). In some primary cultures, we observed very dense epithelial colonies resembling embryonic stem cells. Since HB is an embryonal neoplasm that often presents as a congenital tumor and microscopically resembles developmental stages of the liver, we explored whether we could establish tumor lines using techniques designed to culture stem cells. We were able to derive two new cancer cell lines by subculturing in cell clusters that retain cell-to-cell contact (Fig. 3A). Line B6-1 is derived from a hepatoblastoma patient (patient #1), while B6-2 is derived from a patient with hepatocellular malignant neoplasm (patient #2). Cytogenetic analysis of both cell lines revealed karyotypic abnormalities typically seen in hepatic tumors [26] (Supplementary Figs. 5 and 6). Intriguingly, we were able to derive these cell lines only from xenograft samples, not directly from either primary tumor specimen.

We derived several clones of B6-1 and B6-2 cell lines from different PDTX and investigated whether these cell lines were identical to each other and to what extent they represented the corresponding primary tumor. To this end, we injected the different cell line clones into the livers of NSG mice. After ten to sixteen weeks, we sacrificed the animals and analyzed the livers microscopically using H&E staining. Each cell line formed multiple homogenous tumors compatible with clonal selection in tissue culture and/or clonal expansion *in vivo*. Interestingly, each clone formed a morphologically distinct pattern. For instance, a B6-1A clone resembled the embryonal part of the primary tumor, displaying a trabecular and pseudoglandular morphology with angulated, hyperchromatic cells with a high nucleocytoplasmic ratio (Fig. 3B). A B6-1B clone presented with a non-specific cancer morphology with atypia and nuclear pleomorphism with prominent nucleoli (Fig. 3B). For further analysis, we decided to use the cell line clones (B6-1A and B6-2B) that most closely resembled typical pediatric liver tumor morphologies after injection into the murine liver. We subsequently attempted to adapt the two established cell lines to standard tissue culture conditions, e.g., subculturing as single cell suspension and culturing on non-coated tissue

culture dishes, for the sake of convenience and reducing cost. We were able to adapt B6-2 to grow without Matrigel and be subcultured in a single cell suspension (Supplementary Fig. 7) while the B6-1 cell line could not be adapted to subculturing in single cell suspension.

Gene expression profiling of PDTX and hepatic cancer cell lines

We performed RNA sequencing of the xenografts (first passage) and isogenic cell lines derived from patients #1 and #2, pre- and post-treatment primary tumor samples from patient #2, six normal human liver samples, eight additional untreated primary pediatric liver tumors, and three frequently-studied pediatric liver cancer cell lines (HepG2 [15], Huh6 [14] and HepT1 [16]). In addition we did gene expression profiling for five passages (p1, p2, p3, p13 and p20) of the PDTX from #1. The normal liver and untreated tumor samples were obtained from children enrolled on IRB-approved protocols for clinical exome sequencing study (Patient #2) or genomic characterization of pediatric liver cancers (remaining patients) [27].

We first assessed the expression of a set of known liver tumor markers (AFP, DLK1, DKK1 and SALL4) in these samples (Fig. 3C). These genes were invariably much more highly expressed in the primary tumors, cell lines and PDTXs than in normal liver. Then we performed principal component analysis (PCA) for 2438 genes that were expressed and variable – with mean expression greater than 25 fragments per kilobase of transcript per million (FPKM) mapped reads and coefficient of variation greater than 0.3 – across all 27 samples (Supplementary Table 1). PCA revealed three clusters: a normal liver cluster, a primary tumor cluster, and a published-cell-line cluster (Fig. 4). Strikingly, both new cell lines (B6-1 and B6-2) clustered together with primary tumor samples, and not, as might have been expected, with the published cell lines (HepG2, Huh6 and HepT1). The coefficient of variation, across the 20 passages of PDTX#2, was lower than 0.3 for over 90% of HGNC protein coding genes that had average expression greater than 25 FPKM over the 5 passage samples, suggesting that the gene expression program was mostly unchanged across passages (see Supplementary Table 1 for normalized expression values).

Patient-specific treatments inhibit tumor cell growth in cell culture

Clinical whole-exome sequencing of pre-treatment tumor and blood samples from patient #2 had revealed an activating somatic Q61K mutation in the neuroblastoma ras viral oncogene homolog gene (*NRAS*) (Supplementary Table 2). Currently, there is no effective small molecule inhibitor targeting *NRAS*, but it has been proposed that inhibition of the two primary downstream effector pathways, MAPK/ERK and PI3K/AKT/mTOR, could provide an alternative approach to cancers with *NRAS* mutations [28,29]. We therefore evaluated the ability of a pan-PI3K inhibitor, buparlisib (NVP-BKM120), and a MEK-1/2 inhibitor, trametinib (GSK1120212), to inhibit signal transduction in B6-2 cells, which retained the Q61K *NRAS* mutation observed in the isogenic primary tumor (Supplementary Fig. 8).

Buparlisib inhibited AKT phosphorylation and trametinib inhibited ERK phosphorylation in a dose-dependent manner (Fig. 5A). The B6-2 cell line was more sensitive to trametinib (IC₅₀: 0.95 nM) than buparlisib (IC₅₀: 0.50 μM). Because inhibition of the MAPK/ERK pathway in *NRAS*-mutated melanoma [28] and rhabdomyosarcoma [29] can lead to

compensatory PI3K/AKT/mTOR signaling, we evaluated the efficacy of the two drugs in combination in blocking B6-2 cell growth (Fig. 5B). The addition of 50 nM buparlisib added only marginally to the activity of trametinib. Nevertheless, we maintained the combination therapy for subsequent *in vivo* experiments, since signaling and cross-talk between these two pathways might differ between *in vitro* and *in vivo* growth.

Small molecules inhibit tumors with NRAS mutation in mice

In order to determine the best therapeutic endpoint for small molecule inhibitors, we explored magnetic resonance (MRI) and fluorothymidine PET (FLT-PET) imaging of the PDX models. Although both methods were able to detect and monitor a PDX (Fig. 5C), MRI-based volumetry was more reproducible, likely because it is affected by fewer variables (proper injection, vascularization, stability of thymidine analogue, etc.). We then treated the PDX from patient #1 (wild-type *NRAS*) and the PDX from patient #2 (*NRAS* Q61K) with trametinib (1 mg/kg) and buparlisib (50 mg/kg) when tumors reached ~0.2 cm³ (6–8 weeks after engraftment).

The drug cocktail significantly inhibited the growth of tumors with the *NRAS* mutation ($p < 0.01$), while this treatment did not alter the growth ($p > 0.1$) of tumors lacking the *NRAS* mutation compared to the non-treated isogenic control tumors (Fig. 5 and Supplementary Fig. 9). We confirmed reduced proliferation of treated *NRAS* Q61K tumors by pulse chase experiment with EdU, a thymidine analogue (Fig. 5E). Although this aggressive tumor could not be completely blocked *in vivo*, its growth was specifically and significantly inhibited by trametinib and buparlisib. It is unlikely that this therapeutic effect was due to drug toxicity since the weights of the mice did not significantly change in any of the groups throughout this experiment (Supplementary Fig. 10).

This provides proof of principle that orthotopic PDX models provide a physiologically relevant, useful experimental model for assaying therapeutic sensitivity.

Discussion

The presence of metastasis is the most important prognostic factor for pediatric liver cancer patients, yet until now there has been no report of a metastatic PDX model for pediatric liver cancer. Here we have established a series of orthotopic PDX models for the most common types of pediatric liver cancer (HB and HCC) that replicate the molecular profiles and metastatic behavior of the primary tumors.

Orthotopic models have proven more informative than ectopically grown models in other organ systems [30], not only for microenvironment driven formation of metastases [31], but also for the evaluation of drug efficacy [32]. The goal of the present study, however, was not to directly compare ectopic vs. orthotopic PDX, but rather to establish conditions that would most faithfully recapitulate the human disease, and in particular, the formation of metastases. We observed metastases in both the liver and the lung in 50% (three out of six) of the successfully established PDXs. The metastatic disease we observed is similar to human clinical data, where 46% HB and 44% HCC patients develop metastatic disease, primarily in the lung [33,34]. We also demonstrated that the orthotopic PDXs preserve

tumor heterogeneity not only within the primary xenograft but, intriguingly, also within specific metastases. This observation has not been previously reported for pediatric liver cancer patients, but we confirmed this heterogeneity in a metastatic specimen from an HB patient.

Subcutaneously implanted PDTXs seem to preserve some degree of tumor heterogeneity [11,12], although one study reported that after four serial passages in nude mice, tumors underwent a shift from a purely epithelial to a predominately mesenchymal (osteoid) HB phenotype [11]. In our study, we had a similar primary tumor (patient #1), but we could not detect a shift towards a mesenchymal phenotype, even after seven passages. It is conceivable that passaging will eventually lead to reduction of original tumor heterogeneity and clonal composition, biased by faster growing, better adapted cells (clonal evolution) or simply by a biased selection of the tumor piece to passage further. Nevertheless, our study shows that our PDTX models can faithfully recapitulate the human disease, and thus provide a much-needed physiologically relevant tool for exploring the molecular basis of pediatric liver cancers. Most importantly, the mutation- and patient-specific responsiveness to the small molecule inhibitors we tested, demonstrates the usefulness of these PDTX models in a preclinical setting, where they could provide a valuable resource for the clinical management of children with liver cancer and perhaps mitigate the debilitating toxicity (e.g., oto- and nephrotoxicity) of current chemotherapy regimens.

The present study further reveals how pediatric liver cancer cell lines can be derived and maintained from PDTX models. Importantly, the cell lines were contingent on initial establishment of the PDTX, since we were not able to derive cell lines directly from the primary tumor samples. A similar phenomenon has been described previously in colon cancer, where primary tumor samples were used to derive cell lines directly or indirectly by first generating a xenograft and then establishing cell lines from the PDTX [35]. Our study supports the strategy of establishing a PDTX first to facilitate cell line derivation and raises the question of whether this phenomenon is generally applicable for other cancers.

Another crucial component for the successful derivation of pediatric liver cancer cell lines was the use of stem cell culture techniques. Like human pluripotent stem cells, the B6-1 and B6-2 cell lines grow in flat colonies and require cell-to-cell contact. Both pluripotent stem and pediatric liver cancer cell lines are epithelial, known to form tightly packed sheets with abundant cell junctions. When applying stem cell splitting (subculturing) techniques, the majority of these junctions are maintained and, as demonstrated here, allowed successful propagation of hepatic cell lines. Upon injection into the murine liver, our isogenic cell line clones did not always form lesions that resembled the primary tumor; it is thus important to select tissue-culture-adapted clonal cell lines. The cell line clones we selected (B6-1A and B6-2B) not only recapitulate the histological characteristics but also demonstrate a gene expression profile that clusters with the primary human tumor samples, further demonstrating the usefulness of these new cell lines.

In summary, we have devised a method to develop orthotopic PDTXs of pediatric liver cancer that recapitulate the human disease, including metastasis, and provide a resource from which to derive pediatric liver cancer cell lines. Our analyses demonstrate that these

new tools are useful models for pediatric liver cancer, providing a significant step towards more faithful disease modeling both *in vitro* and *in vivo*. These models (and methods) shed light on the biological and clinical complexity of pediatric liver cancers and, most importantly, constitute an excellent platform to test patient-specific therapies, which should allow more rapid translation into the clinic.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PDTX	patient-derived tumor xenograft
FRG	<i>fah</i> ^{-/-} <i>rag2</i> ^{-/-} <i>Il2rg</i> ^{-/-} mouse strain
NSG	NOD.SCID <i>Il2rg</i> ^{-/-} mouse strain
HB	hepatoblastoma
HCC	hepatocellular cancer
NOS	hepatocellular malignant neoplasm
FAH	fumarylacetoacetate hydrolase
ALB	albumin
AFP	alpha fetoprotein
MRI	magnet resonance imaging
FLT-PET	[18F] fluorothymidine positron emission tomography
OLT	orthotopic liver transplantation
NMP	N-Methyl-2-pyrrolidone
FPKM	fragments per kilobase of transcript per million mapped reads
IRB	institutional review board
IACUC	Institutional Animal Care and Use Committee

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Fig. 1. Characterization of a patient-derived tumor xenograft (PDX) model for pediatric liver cancer

(A) Illustration of the transplantation technique for orthotopic PDX. Tumor tissue blocks from patients were engrafted onto incised Glisson's capsule of the murine liver. (B) Human proteins in the murine blood; AFP, a hepatic tumor marker, and albumin, a liver specific marker, expressed from hepatic tumor cells. Quantitative ELISA with human specific antibodies ($n = 3$). (C) Comparison of primary tumor tissue and PDX. Xenograft demonstrates embryonal (arrowhead) and fetal (arrow) tumor components, patchy nuclear β -catenin and glypican 3 (GPC3) staining consistent with the original human tumor. (D) Osteoid within PDX. (E) Immunostaining for human nuclei (red), von Willebrand factor (vWF, in green) and MECA32 (green) a panendothelial mouse-specific antibody demonstrating murine vasculature in the human PDX. (This figure appears in colour on the web.)

Fig. 2. Formation of metastases in PDX models

(A). Multiple intrahepatic metastases (circles and box) from the PDX, depicted PDX from patient #2. (B) Intrapulmonary metastasis from multiple PDX of varying size and different patients. (C) Lung metastases of PDX and patient display heterogeneity with embryonic (arrowhead) and fetal (arrow) hepatoblastoma in the same metastatic lesion. Boxed pictures are higher magnification of the boxed insets in adjacent pictures. Scale bar is 50 μ m. (This figure appears in colour on the web.)

Fig. 3. Characterization of isogenic cell lines

(A) Morphological appearance of B6-1 and B6-2 cell lines in comparison to human iPS cells. (B) B6-1 clones (depict B6-1A and B6-1B) show different morphologies upon injection into the murine liver. Scale bar is 50 μ m. (C) Expression of selected liver cancer markers in normal human liver, pediatric liver cancer, PDTX and B6-1 and B6-2 cell lines. Error bars represent mean $\pm$ SEM. (This figure appears in colour on the web.)

Fig. 4. Principal component analysis (PCA) of PDX, cell line models to clinical samples
 PCA based on the expression profiles of 2438 expressed genes shows formation of three clusters: normal human liver (NL), primary tumor (T), and published cell lines (Huh6, HepT1, HepG2) cluster. PDX and cell line from patient #1 are in red and from patient #2 in blue. For patient #1 we analyzed serial passages of the xenograft model (p2, p3, p13 and p20) and for patient #2 we analyzed the primary tumor before (T#2) and after (T#2p) chemotherapy. (This figure appears in colour on the web.)

Fig. 5. Validation of patient-specific therapy using the B6-2 cells line and isogenic PDTX model
 The tumor from patient #2 contained an activating *NRAS* Q61K mutation, which was detected in cell lines and in the isogenic PDTX. (A) The small molecule inhibitors, buparlisib and trametinib blocked MAPK and PI3K/AKT/mTOR signaling, respectively. (B) Cell viability accessed by the MTT assay upon treatment with trametinib alone or in combination with 50 nM buparlisib. (C) MRI of donating patient #2 and PDTX, showing heterogenic liver tumor. Right panel shows FLT-PET of a mouse bearing PDTX. Incorporation of the PET positive thymidine analogue is indicated in color. (D) MRI-based volumetry of *NRAS* Q61K and *NRAS* wild-type (w.t.) tumors before and two weeks after treatment with buparlisib and trametinib or vehicle only. N = 3 for each of the four groups. (E) Pulse chase experiment with EdU overnight. Non-treated *NRAS* Q61K tumor is displayed in the image with incorporated EdU in pink. DAPI stained nuclei are in blue. Graph represents a quantification (mean $\pm$ SEM) of incorporated EdU in 10 representative sections per group. (This figure appears in colour on the web.)

Table 1

Patient and PDTX characteristics.

Number	Age	Sex	Pathology	Clinical patient description			PDTX		
				Sampling/AFP [UI/ml]	Metastasis	Metastasis	PDTX Y/N (Take rate)	Human AFP/ALB	
#1	15M	male	HB: mixed epithelial, 70% fetal and 30% embryonal and 1% mesenchymal (osteoid), venous invasion	Hemi-hepatectomy/46,761	N	Y	Y (60%)	Y/Y	
#2	7Yrs	male	Hepatocellular malignant neoplasm (NOS) or transitional liver cell tumor	Hemi-hepatectomy/67,580	Y	Y	Y (63%)	Y/Y	
#3	25M	female	HB: mixed epithelial, 70% fetal and 30% embryonal, invasion in portal veins and bile ducts	OLT/471,970	N	N	Y (8%)	Y/Y	
#4	17M	male	HB: epithelial, 50% fetal and 50% embryonal	OLT/1,024	N	n.a.	N	N/N	
#5	16M	female	HB: mixed epithelial, 90% fetal and 5% embryonal and 5% mesenchymal (osteoid formation)	Hemi-hepatectomy/513	N	n.a.	N	N/N	
#6	12M	male	HB: mixed epithelial, 80% fetal and 20 % embryonal, 1% small cell undifferentiated	OLT/424	N	n.a.	N	N/N	
#7	18M	male	HB: mixed epithelial, 65% fetal and 5% embryonal and 30% mesenchymal (teratoid)	OLT/16,895	N	Y	Y (33%)	Y/Y	
#8	2Yrs	female	HB: epithelial, 95% fetal and 5% embryonal, venous invasion	OLT/762	N	N	Y (40%)	Y/Y	
#9	3Yrs	male	HB: mixed epithelial (predominantly fetal) and mesenchymal (osteoid and extramedullary hematopoiesis) extensive portal vein invasion	OLT/1,090	N	n.a.	N	N/N	
#10	2Yrs	male	HB: mixed fetal (90%), focal macrotubercular (10%) and cholangioblastic (rare) patterns	Biopsy/46,434	Y	n.a.	N	N/N	
#11	15M	male	HB: mixed epithelial, 19% fetal and 1% embryonal and 80% mesenchymal, extensive portal venous invasion	OLT/1,581	Y	n.a.	N	N/N	
#12	7Yrs	female	HCC with venous invasion	OLT/5,603	N	N	Y (17%)	Y/Y	
#13	15M	female	HB: well differentiated fetal predominance (100%) with small foci of pleomorphic fetal	Segmentectomy/43	N	n.a.	N	N/N	
#14	1Yrs	male	HB: epithelial, 70% fetal and 30% embryonal	Segmentectomy/201	N	n.a.	N	N/N	

Clinical patient description							
Number	Age	Sex	Pathology	Sampling/AFP [U/ml]	Metastasis	Metastasis	PDX Y/N (Take rate)
#15	2Yrs	female	HB: epithelial, 60% fetal and 40% embryonal	Biopsy and hemi-hepatectomy/ >225,848 and 53,628	N	n.a.	N
							N/N

OLT, orthotopic liver transplantation; AFP, alpha fetoprotein; ALB, albumin; M, months; Yrs, years.