Research Article

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

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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.
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Abbreviations: PDTX, patient-derived tumor xenograft; FRG, *fah*$^{-/-}$ *rag2*$^{-/-}$ *Il2rg*$^{-/-}$ mouse strain; NSG, NOD.SCID *Il2rg*$^{-/-}$ 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.

Introduction

Pediatric liver cancer is a rare (~3 patients per million) but often aggressive disease of very young children. Hepatoblastoma (HB)

Cancer

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 mm^3) 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 cm^3 (range: 0.10–0.37 cm^3) 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 mm^2) 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 (10^6 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 μM) 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 mm^3) 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$^{-/-}$)