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Research Article

Anti-tumor activity of sorafenib in a model of a pediatric hepatocellular carcinoma

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ABSTRACT

Background: Treatment outcome of children with pediatric hepatocellular carcinoma (pHCC) is poor. Therefore, we evaluated the tyrosine kinase inhibitor sorafenib in a model of pHCC.
Methods: Cell viability after treatment with sorafenib was evaluated in HC-AFW1 cells (pHCC) using MTT assay and compared to an adult HCC (aHCC) and two hepatoblastoma (HB) cell lines. ERK, pERK, E-cadherin, and vimentin expression were investigated using Western Blot. Sorafenib (60 mg/kg) was administered orally to NOD.Cg-Prkdcscid-IL2rgtmWjl/Sz mice bearing subcutaneous HC-AFW1-derived tumors. Tumor progression, viability, and vascularization were monitored by tumor volume, AFP levels, and CD31 immunostaining, respectively. Sensitization to sorafenib was evaluated using the β-catenin inhibitor ICG001.
Results: Sorafenib reduced cell viability in HC-AFW1 (IC_{50}: 8 μM), comparable to HB cells, however less pronounced in aHCC cells (IC_{50}: 23 μM). Sorafenib inhibited ERK signaling in both, HC-AFW1 cells and -xenografts. In vivo, sorafenib treatment only led to a moderate tumor growth inhibition, although significant reduction of vascularization and tumor growth kinetics was observed. Long-term treatment with sorafenib decreased E-cadherin, but showed no induction of vimentin expression. Combining sorafenib with a β-catenin inhibitor led to an additional reduction of cell viability.
Conclusion: Sorafenib together with inhibitors of the β-catenin pathway might be an effective tool in the treatment of pediatric HCC.

Introduction

Pediatric hepatocellular carcinoma (pHCC) is a rare epithelial liver tumor with a pathophysiology sparsely understood. In the western hemisphere, it mostly arises in livers without abnormalities, although hepatitis, cholestasis, biliary atresia, glycogen-storage diseases, and low birth weight are discussed to be risk factors for the development of pHCC [1,2].

Abbreviations: pHCC, pediatric hepatocellular carcinoma; aHCC, adult hepatocellular carcinoma; ERK, extracellular signal-regulated protein kinase; HB, hepatoblastoma

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In contrast to the typical histology of adult HCC (aHCC), a distinct variant of fibrolamellar HCC and a transitional tumor combining features of hepatoblastoma (HB) and HCC have been described in children. However, so far histology does not seem to have an impact on outcome of this disease [3].

Unlike in aHCC, a certain response to chemotherapy is regularly observed in pHCC [4,5]. Chemotherapy, radical surgery and in some cases liver transplantation are the corner stones of pHCC treatment in the international multicenter protocols for pHCC. However, treatment results of children with HCC are still poor, despite a general increase of survival rates in most solid tumors in this age group [2]. Less than 20% of patients with advanced HCC exhibit a sustained survival. Therefore, alternative treatment strategies such as transarterial chemoembolization (TACE) or the use of kinase inhibitors were considered in pilot studies [6,7]. Other emerging molecular treatment approaches such as apoptosis sensitizers, therapeutic antibodies, and epigenetic agents may also possibly improve the outcome as observed for related tumors [8–10].

Systematic clinical studies are limited due to the low tumor incidence. Thus a preclinical evaluation may enable a rational selection of new therapeutic concepts. Recently, we described the successful establishment of the continuous epithelial cell line HC-AFW1 derived from a pHCC without viral background [11]. This unique model for pHCC renders a systematic analysis of different treatment strategies in this tumor entity possible. Sorafenib is an inhibitor of multiple receptor thyrosine kinases (VEGFR, PDGFRß, Flt-3 and Kit) and Raf kinases (Raf-1, B-Raf) [12]. Inhibiting angiogenesis and signal transduction through the Raf/Mek/ERK pathway, sorafenib was approved for treatment of liver and renal carcinoma in adults [13,14]. Moreover, sorafenib also showed a high anti-tumoral activity in pediatric liver tumors like Hepatoblastoma in preclinical studies [15,16]. A pilot study considered sorafenib in combination with cisplatin and doxorubicin in pHCC [7]. The heterogenic response in this study with few cases demands a better understanding of the biological response of pHCC to sorafenib treatment. The present study was conducted to evaluate the therapeutic potential of sorafenib and resistance development in a preclinical model for pediatric HCC.

Methods

Cell lines and culture conditions

HC-AFW1 was derived from a 5-year old boy suffering from HCC [11]. Huh7, a cell line derived from a differentiated aHCC, was obtained from the Japanese Collection of Research Bioresources (JCRB0403). The HUH6 cell line originated from a mixed HB and was first described by Doi [17]. The cell line HepT1 was derived from a multifocal embryonic HB first established by Pietsch et al. [18]. All cells were grown in Dulbecco's modified Eagle's medium (DMEM; Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum and 1% L-glutamine on plastic culture dishes (Greiner, Essen, Germany). Cells were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C and were proved to be Mycoplasma negative.

Preparation of sorafenib

Sorafenib tosylate salt (Selleck Chemicals, Houston, TX) was used for all experiments. For in vitro studies sorafenib was dissolved in 100% DMSO and diluted in DMEM to the required concentration with a final DMSO concentration of less than 0.1%. For animal studies sorafenib was dissolved in a Cremophor EL/Ethanol (50:50, Cremophor EL; Sigma, Germany) solution at 100 mg/ml and stored at 4 °C. This stock solution was renewed every 3 d. Through dilution in 50% glucose, solutions with the final doses were prepared immediately before administration.

Viability assay

Tumor cells (10,000 cells/100 µl) were cultured in 96-well plates. After 48 h, sorafenib (Selleck Chemicals, Munich, Germany) was added to the cells at different concentrations of up to 30 µM. Incubation lasted for 72 h. Cell viability was assessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide]-assay. All assays were performed 3 times in quadruplicates. Percentages of viability were calculated through normalization between background of cultures without cells and untreated cultures as control experiments. Dose dependent viability curves were computed by sigmoidal curves with variable slope to determine IC_{50}.

Western blot

Cells were cultured at a density of 50,000 cells/cm². Sorafenib was added for 30 min, following a 24 h culture without FCS. Cells were stimulated with 10% FCS for 10 min before lysis or were left untreated. ERK and phosphorylated ERK (pERK) were assessed using electrophoresis and western blot as previously described [15,16].

Resistance to sorafenib has been reported to be associated with epithelial–mesenchymal transition (EMT) in HCC [19,20]. Therefore expression of E-cadherin (1:10, clone 67A4, Bühring, University of Tuebingen, Germany) and vimentin (1:200, DAKO, Hamburg, Germany) was detected in HC-AFW1 cells treated with 5 µM sorafenib over 20 d. β-catenin expression was detected using a polyclonal antibody (1:2000, Zytomed Systems, Berlin, Germany). GAPDH served as control for protein load (1:200, Santa Cruz, Heidelberg, Germany).

Animal experiments

All animal studies were conducted according to the criteria outlined in the "Legislation for the protection of animals used for scientific purposes" (European Commission, directive 2010/63/EU revising directive 86/609/EEC), and were approved by the local Government's ethical authority for animal experiments (Regierungspraesidium Tuebingen, No.K2/11). NOD.Cg-Prkdcscid IL2rgtmWjl/Sz (NSG) mice were purchased from Charles River (Sulzfeld, Germany) and bred in our facility. 10^6 HC-AFW1 cells (in 200 µl PBS) were injected into the flank of 4–6 weeks old mice (24–30 g), kept in filter-top cages at 22 °C and 60% humidity. Sterilized food and water were accessible ad libidum. Tumor volumes ($V=4/3\pi \times l/2 \times w/2 \times h/2$) were measured every 2 d. Treatment was initiated when the tumors reached a volume of 300 mm^3. Sorafenib tosylate was administered orally once every day with a dosage of 60 mg/kg bodyweight ($n=6$). Animals ($n=6$) were sacrificed at d25 or when tumor volume exceeded 2 cm^3. Sigmoidal curves with variable slopes of the tumor volumes were used to describe each tumor growth over 25 d. Blood samples were taken from the retrobulbar plexus on d0 and after sacrificing. Levels of AFP were determined by ELISA (DRG Instruments GmbH, Marburg/Lahn, Germany), which was carried out according to the manufacturer's protocol. Tumors were explanted and prepared for further analyses.