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Long-term exposure to sorafenib of liver cancer cells induces resistance with epithelial-to-mesenchymal transition, increased invasion and risk of rebound growth ☆

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ABSTRACT

Sorafenib leads to a survival benefit in patients with advanced hepatocellular carcinoma but its use is hampered by the occurrence of drug resistance. To investigate the molecular mechanisms involved we developed five resistant human liver cell lines in which we studied morphology, gene expression and invasive potential. The cells changed their appearance, lost E-cadherin and KRT19 and showed high expression of vimentin, indicating epithelial-to-mesenchymal transition. Resistant cells showed reduced adherent growth, became more invasive and lost liver-specific gene expression. Furthermore, following withdrawal of sorafenib, the resistant cells showed rebound growth, a phenomenon also found in patients. This cell model was further used to investigate strategies for restoration of sensitivity to sorafenib.

1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most common malignancy worldwide [1]. When HCC is diagnosed at an early stage, patients are eligible for curative treatment and the five-year survival rates may reach 70%. However, the majority of HCC patients (60–70%) are diagnosed in a more advanced stage and face a grim prognosis [2]. Sorafenib represents the current standard of care for patients with vascular invasion or extra hepatic spread (Barcelona Clinic Liver Cancer (BCLC) stage C) [3]. Sorafenib is a multikinase inhibitor and targets both angiogenesis (the serine-threonine kinases Raf-1 and B-Raf) and proliferation (the vascular endothelial growth factor (VEGF) receptors and the platelet-derived growth factor beta (PDGFB) receptor) on tumor cells, endothelial cells and pericytes [4]. *In vitro* sorafenib has an antiproliferative effect on liver cancer cell lines and in xenograft models it was shown that sorafenib inhibits tumor growth rather than inducing tumor shrinkage [4,5]. In two recent phase III trials in patients with advanced HCC an overall survival benefit of three months compared to placebo was demonstrated [6,7]. Although these results are encouraging, the use of sorafenib is hampered by two phenomena. First of all, up to 80% of patients treated with sorafenib suffer from side effects. The most important grade 3 adverse events include hand-foot syndrome, diarrhea, hypertension and fatigue [8,9]. In the SHARP trial 26% in the sorafenib arm needed dose reductions, 44% had short-term "drug holidays" and 11% had to permanently discontinue the treatment due to drug-related adverse events [6]. The second phenomenon is that patients who initially respond to therapy eventually will show progression. The time to radiologic progression is delayed by sorafenib, only a small percentage of patients show a partial response, but complete responses are rare. Therapy is most often

Abbreviations: Δlog, (^{2}log fold change); ACTB, β-actin; ATCC, American type culture collection; BCLC, Barcelona Clinic Liver Cancer; BrdU, bromodeoxyuridine; CDH1, E-cadherin; DAVID, database for Annotation, Visualization and Integrated Discovery; EMT, epithelial-to-mesenchymal transition; ERK, extracellular-signal-regulated kinase; FCS, fetal calf serum; HCC, hepatocellular carcinoma; IC_{50}, 50% inhibitory concentration; KRT19, keratin 19; MAPK, mitogen-activated protein kinase; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PDGF, platelet-derived growth factor; PDGFB, platelet-derived growth factor beta; PI3K, phosphatidylinositol 3-kinase; RAF, proto-oncogene serine/threonine-protein kinase; VEGF, vascular endothelial growth factor; VIM, vimentin; WEM, williams medium E; XTT, (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide).

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stopped at progression, although it is known from the literature that tumor growth is even more rapid after abrogation of anti-angiogenic therapy [10,11].

The effects observed in patients treated with sorafenib suggest the development of resistance [12]. The molecular basis for this resistance is only partly elucidated. To be able to study this acquired resistance we developed several HCC cell lines resistant to sorafenib following continuous exposure. Furthermore we investigated gene and protein expression, functional behavior and *in vitro* invasion in this model. We used these cells to test strategies to overcome this resistance. Recently, Chen et al. [13] reported the development of sorafenib resistance in Huh7 cells and investigated PI3K/Akt signaling. The PI3K/Akt signaling pathway is working parallel to the sorafenib-targeted Raf/Ras/MAPK signaling pathway [14] which makes it an attractive target. We investigated reversal of sorafenib resistance *in vitro* by combination treatment of sorafenib with Akt pathway inhibitor LY294002. We also investigated the possible involvement of drug transporters. Finally, we studied the effect of sorafenib withdrawal (seen clinically: dose reduction, drug holidays or discontinuation of therapy) on growth and gene expression.

2. Materials and methods

2.1. Cell culture and compound

HepG2 human hepatoblastoma cells (HB-8065) and WRL-68 human embryonic liver cells (CL-48) were obtained from ATCC (Rockville, MD, USA). The human hepatocellular carcinoma cell line Huh-7 was obtained from Health Science Research Resources Bank (Sennan-shi, Osaka 590-0535, Japan). HepG2 cells were grown in Williams Medium E (WEM, Invitrogen) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 20 mU/mL insulin, 50 nM dexamethasone, 100 U/mL penicillin, 100 μg/mL streptomycin, 2.5 μg fungizone, 50 μg/mL gentamycin and 100 μg/mL vancomycin (=WEM-C). WRL-68 cells were grown in Eagle's Minimum Essential Medium (MEM, Invitrogen) supplemented with 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. Huh-7 cells were grown in Dulbecco's Modified Eagles Medium (DMEM, Invitrogen) supplemented with 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin.

Sorafenib (Bayer HealthCare, Leverkusen, Germany) was dissolved in 100% dimethylsulfoxid (DMSO) (Acros Organics, New Jersey, USA). LY294002-hydrochloride was from Sigma–Aldrich (St Louis, USA) and Vismodegib (GDC-0449) was obtained from (Selleck Chemicals LLC, Houston Tx, USA).

2.2. Development of sorafenib resistance

First, we determined the IC_{50} of HepG2, Huh-7 and WRL-68 cells for to sorafenib. Cells were seeded in a 96-well plate and treated with increasing doses of sorafenib. After three days cells were incubated with XTT-assay and cell viability was determined (described below). Next, we cultured HepG2, Huh-7 and WRL-68 cells in 6-well plates at 75×10^3 cells per well and incubated the cells with sorafenib concentrations just below their IC_{50}. During the following weeks, we slowly increased the sorafenib dose with 0.25 μM per time. Over several months we developed for HepG2 two cell lines resistant to sorafenib (HepG2S1 and HepG2S2). This was repeated starting from the parental HepG2 cells and a third resistant line was isolated (HepG2S3). For both WRL-68 and Huh7 a single sorafenib-resistant line was developed; WRLS1 and Huh7S1 respectively. After establishment, these resistant cell lines were continuously cultured in the presence of sorafenib.

2.3. XTT-assay

Cell viability and proliferation was measured with the "Cell Proliferation Kit II (XTT)" (Roche Applied Science, Penzberg, Germany). All experiments were performed at least three times with every condition in triplicate. XTT solution was added to the wells at a concentration of 0.3 mg/mL and after 4 h of incubation with XTT assay, the metabolic activity as an indirect measure for cell number, was quantified spectrophotometrically at dual wavelength (490–655 nm). Results were expressed relative to control conditions.

2.4. BrdU-assay

For proliferation measurements, cells were cultured with bromodeoxyuridine (BrdU). After incubation, the wells were treated with Anti-BrdU antibody during 1 h and with the secondary horse-radish peroxidase conjugate. Immunodetection was performed, after tetra-methylbenzidine solution treatment, with a spectophotometric plate reader at dual wavelength (450–540 nm) according the manufactures instructions (Calbiochem, La Jolla, USA).

2.5. Western blot

Cells were washed with phosphate buffered saline (PBS) and lysed in buffer (50 mMol/L Tris pH 8.0, 150 mMol/L NaCl, 0.01% SDS, 1% NP40, 0.5% Na-desoxycholaat, 1 mMol/L PMSF, 1 mMol/L NaF, 1 mMol/L Na_3VO_4) containing protease inhibitors (Complete Protease Inhibitor Cocktail, Roche). Fifty micrograms of protein per condition was loaded on 4–10% gels. Samples were separated and transferred onto a nitrocellulose membrane. Membranes were incubated with 5% non-fat milk powder in PBS to avoid non-specific binding. Blots were subsequently incubated overnight with primary antibodies (Supplemental Table 1) followed by the corresponding horseradish peroxidase-conjugated secondary antibody. Blots were developed with enhanced chemiluminescence reagent (ECL, Amersham, Roosendaal, The Netherlands). Afterwards membranes were stripped and incubated with β-actin (1:10.000) to confirm equal protein loading.

2.6. Immunocytochemistry

Cells were grown on thermanox cover slides (Ø 13 mm) and placed in a 24 well plate. After 48 h cells were fixed on the cover slides with acetone and stored at −20 °C. For immunocytochemistry we used the Envision technique of Dako. Cells were incubated for 90 min with the primary antibody E-cadherin (CDH1) (1:50) or vimentin (VIM) (1:50). As secondary antibody Envision monoclonal anti-mouse antibody was used (Dako, Glostrup, Denmark). Finally, the staining was executed with 3-amino-9-ethylcarbazole (AEC) followed by contra-staining with heamatoxylin. To evaluate the staining we used a semi-quantative quickscore, which combines positivity (P) and intensity (I) [15]. Positivity was scored as: 1 = 0–4%, 2 = 5–19%, 3 = 20–39%, 4 = 40–59%, 5 = 60–79% and 6 = 80–100%. Intensity was scored as: 0 = negative, 1 = weak, 2 = intermediate and 3 = strong. The final score was the total of P + I and has a range of 1–9.

2.7. Microarray

HepG2S1 cells were grown at 1×10^6 in 25 cm^2 tissue flasks ($n = 10$). Five flasks were cultured with 6 μM sorafenib and five flasks were withdrawn from sorafenib. In parallel HepG2 cells were grown at $1x10^6$ in 25 cm^2 tissue flasks ($n = 5$), also without sorafenib exposure. After 72 h, cells were harvested with Trizol (Invitrogen, Merelbeke, Belgium) treatment and RNA was isolated with the RNeasy Kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. The RNA quality was assessed with the Agilent 2100 BioAnalyzer (Agilent, Palo Alto, CA). Microarray analysis was performed on three representative samples of all three conditions. The Affymetrix Human Gene 1.0 ST Array (Affymetrix) was used as platform. Microarray data was analyzed with the Limma package from Bioconductor (http://www.bioconductor.org) [16]. Differentially expressed genes were assessed using a moderated *t*-test. The resulting *p*-values were corrected for multiple testing with Benjamini–Hochberg to control false discovery rate [17]. For selecting differentially expressed genes a cut-off of Δlog (^{2}log fold change) >+1 or <−1 and a corrected $p < 0.05$ was applied. Pathway analysis, with gene-annotation enrichment analysis, functional clustering and BioCarta & KEGG pathway mapping, was performed with the bioinformatics tool DAVID (Database for Annotation, Visualization and Intergration Discovery, DAVID bioinformatics Resources version 6.7, http://david.abcc.ncifcrf.gov/) [18].

2.8. Quantitative real-time PCR

All culture conditions were performed in quadruple, after five days of stimulation the cells were collected for RNA isolation. After Trizol (Invitrogen, Merelbeke, Belgium) treatment, RNA was isolated with the RNeasy Kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. One microgram of cellular RNA was reverse transcribed into cDNA using SuperScript II reverse transcriptase and random hexamer primers (Invitrogen Life Technologies, USA). The PCR reaction was carried out in a mixture that contained appropriate sense- and anti-sense primers and a TaqMan MGB probe in TaqMan Universal PCR Master Mixture (Applied Biosystems, Foster City, USA) (Supplementary Table 2). Beta-2-microglobulin was used as housekeeping gene. Real-time PCR amplification and data analysis were performed using the A7500 Fast Real-Time PCR System (Applied Biosystems). Each sample was assayed in duplicate in a MicroAmp optical 96-well plate. The ΔΔCt-method was used to determine relative gene expression levels.

2.9. Invasion assay

Invasion studies were performed using BD BioCoat Matrigel Invasion Chambers (BD Biosciences, Bedford, MA). Cells (7.5×10^4) were added to the top inserts in FCS free WEM-medium. The bottom chambers were filled with medium containing 10% FCS, serving as a chemo attractant. After two days, the non-invaded cells were removed with a cotton swab and the assays were fixed and stained using Diff Quick