

Mechanisms of Sorafenib Resistance in HCC Culture Relate to the Impaired Membrane Expression of Organic Cation Transporter 1 (OCT1)

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Introduction: Sorafenib, an FDA-approved drug for advanced hepatocellular carcinoma (HCC) treatment, encounters resistance in many patients. Deciphering the mechanisms underlying sorafenib resistance is crucial for devising alternative strategies to overcome it.

Aim: This study aimed to investigate sorafenib resistance mechanisms using a diverse panel of HCC cell lines.

Methods: HCC cell lines were subjected to continuous sorafenib treatment, and stable cell lines (Huh 7.5 and Huh 7PX) exhibiting sustained growth in its presence were isolated. The investigation of drug resistance mechanisms involved a comparative analysis of drug-targeted signal transduction pathways (EGFR/RAF/MEK/ERK/Cyclin D), sorafenib uptake, and membrane expression of the drug uptake transporter.

Results: HCC cell lines (Huh 7.5 and Huh 7PX) with a higher IC₅₀ (10 μM) displayed a more frequent development of sorafenib resistance compared to those with a lower IC₅₀ (2–4.8 μM), indicating a potential impact of IC₅₀ variation on initial treatment response. Our findings reveal that activated overexpression of Raf1 kinases and impaired sorafenib uptake, mediated by reduced membrane expression of organic cation transporter-1 (OCT1), contribute to sorafenib resistance in HCC cultures. Stable expression of the drug transporter OCT1 through cDNA transfection or adenoviral delivery of OCT1 mRNA increased sorafenib uptake and successfully overcame sorafenib resistance. Additionally, consistent with sorafenib resistance in HCC cultures, cirrhotic liver-associated human HCC tumors often exhibited impaired membrane expression of OCT1 and OCT3.

Conclusion: Intrinsic differences among HCC cell clones, affecting sorafenib sensitivity at the expression level of Raf kinases, drug uptake, and OCT1 transporters, were identified. This study underscores the potential of HCC tumor targeted OCT1 expression to enhance sorafenib treatment response.

Keywords: hepatocellular carcinoma, HCC, cholangiocarcinoma, CCA, tyrosine kinase inhibitors, TKI, organic cation transporter-1, OCT1, organic cation transporter-3, OCT3, sorafenib resistance cell lines, SR huh 7

Introduction

Hepatocellular carcinoma (HCC) stands as the sixth most prevalent cancer globally and the fourth leading cause of cancer-related mortality.¹ Predominantly arising from chronic liver conditions, including cirrhosis induced by viral and non-viral factors such as alcohol and non-alcoholic fatty liver disease, HCC exhibits a rising incidence in the United States, propelled by the obesity epidemic and metabolic syndromes.² While early-stage HCC is amenable to curative interventions like surgical resection or liver transplantation, advanced cases present a clinical challenge.

Sorafenib, an oral multi-kinase inhibitor, has gained FDA approval for treating advanced HCC, targeting RAF kinase signaling pathways pivotal in tumor progression and angiogenesis.³ Despite its efficacy, the survival benefits for sorafenib-treated HCC patients remain modest due to prevalent resistance mechanisms.^{4,5} The reasons underlying HCC resistance to sorafenib are not fully understood.

Two primary resistance mechanisms—preexisting and acquired—contribute to sorafenib treatment failure.⁶ Preexisting resistance manifests when tumors exhibit no response to chemotherapy from the onset, potentially involving activation of various signaling pathways such as EGFR, c-MET, IGFR, Wnt/B-catenin, or PI3K/Akt/mTOR.^{7–12} Conversely, adaptive or acquired resistance occurs when tumors initially respond but later develop resistance, with mechanisms including cancer stem cells, epithelial-mesenchymal transition (EMT), platelet-tumor microenvironment interactions, hypoxia, inflammation, cytokine expression, and cellular autophagy.^{13–19}

Since the clinical trials using MAPK inhibitors are not providing significant benefits to HCC patients, a more thorough understanding of the tumor resistance mechanisms is needed to identify the biological means of resistance to improve sorafenib's therapeutic effects in curing liver cancer.²⁰

Success in anticancer drug therapies hinges on the membrane expression of drug transporters facilitating drug uptake. Sorafenib, like many anticancer drugs, relies on specific transporters for cellular entry. Notably, the organic cation transporter (OCT1 and OCT3) and efflux transporters (MDR1/P-glycoprotein, MRP2, BCRP) play crucial roles in sorafenib response in HCC. Alterations in the expression of these transporters impact drug accumulation and, consequently, chemotherapy response. Among these transporters, organic cation transporter-1 (OCT1) emerges as a key player, strongly expressed in the sinusoidal membrane of the human liver.²¹ Increased expression of uptake transporters and decreased expression of efflux transporters favor the accumulation of the drug and better chemotherapy response. In humans, 395 membrane-soluble carrier transporters are organized into 52 families.²² Soluble carrier family 22 member-1 (SLC22A1), known as OCT1, is strongly expressed in the sinusoidal membrane of the human liver and is responsible for hepatic uptake of small, hydrophilic, positively charged organic molecules.²³ The expression of SLC22A1 variants in HCC has been claimed to affect sorafenib sensitivity, but the mechanisms are unknown.²⁴

In this study, we found that there are intrinsic differences in responses to sorafenib treatment among human hepatoma cell lines. A stable, resistant HCC cell culture was developed via long-term treatment with sorafenib. We found that decreased sorafenib uptake due to the impaired OCT1 membrane expression is one of the mechanisms of resistance. Moreover, the research demonstrates that restoring OCT1 expression in HCC culture via adenoviral gene delivery enhances sorafenib uptake, offering a promising avenue to overcome drug resistance.

Materials and Methods

Cell Culture, Chemotherapy Drugs, Antibodies, Plasmids

The human hepatoma cell lines (Huh 7, Huh 7.5.1, Huh 7.5, Huh 7 PX, SK Hep1, Huh 7AF, HLE, HLF, HepG2) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), non-essential amino acids, sodium pyruvate and 1% penicillin plus streptomycin has been described previously.²⁵ Cells were maintained at 37°C incubator with 5% CO₂. Huh 7AF, Huh 7 PX, Huh 7.5.1 cells were obtained from Jean-Michel Pawlotsky Laboratory, France. These two cell lines have been sequenced for rs12979860 IL28B genotype. Huh 7.5 was obtained from Charlie Rice Laboratory, Rockefeller University. This cell line supports high-level HCV replication. HLF and HLE were obtained from Ludwig Wilkens lab, Institute of Pathology, University of Bern, Bern, Switzerland. These cell lines have been authenticated by Wilken lab.²⁶ These cell lines were obtained through a Material Transfer Agreement (MTA) signed by the technology transfer office at Tulane University Health Science Center, New Orleans. Tulane Office of Technology Transfer Office has given ethical approval to use these cell lines for research purposes only. SK-Hep1, HepG2 cells, and Huh 7 cells were purchased from ATCC.

Drugs

We purchased sorafenib (catalog number 10,009,644) and metformin (catalog number 13,118) from Cayman Chemicals, quinine hydrochloride (catalog number Q1125), hygromycin (catalog number H3274) from Sigma, [³H] labeled cytidine

(catalog number MT-615) and [^3H] labeled sorafenib catalog number MT-1907 from Moravek Biomedicals, Brea, CA. Antibodies to human OCT1 (Anti-SLC22A1, 2C5, catalog number Ab107246) were purchased from Abcam. Antibodies to CNT1 (catalog number SC4860), ENT1 (catalog number. SC-377283, F-12) and ENT2 (catalog number SC373872, A-8), ERK1 (catalog number SC-93), pERK (catalog number SC-7383) were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. RAF family antibody sampler kit (catalog number 2330), EGFR (Cat No.4267S, pEGFR (catalog number 2234S), and AMPK and ACC antibody sampler kit (catalog number 9957s) were from Cell signaling. Antibodies to MEK (catalog number Ma5-15,162) and pMEK (catalog number MA5-15,118) were from Thermo Scientific. Recombinant human epidermal growth factor (EGF) ligand (catalog number 236-EG) was purchased from RD Systems, Minneapolis, MN.

Drug Treatment and MTT Assay

A stock solution of sorafenib (30mM) was prepared by dissolving a 10 mg vial in 500 microliters of 100% dimethyl sulfoxide (DMSO). A 100 μM sorafenib working solution was prepared in phosphate-buffered saline (PBS) (pH 7.2) for in vitro studies. The final concentration of DMSO in the working stock solution was less than 0.001% (v/v). The working solution was sterilized with a 0.2 μm syringe filter, aliquoted, and stored at -20°C until use. A stock solution of metformin was prepared by dissolving 50 mg in 100 mL of PBS (the stock concentration is 500 $\mu\text{g/mL}$). The stock solution was sterilized with a 0.2 μm syringe filter, aliquoted, and stored at -20°C until use. The effect of sorafenib treatment on HCC cell proliferation was measured by MTT assay as described previously.²⁷ The percentage of cell viability was determined by comparison with untreated controls. The concentration of each chemotherapy drug that showed a 50% reduction in cell viability (called the IC_{50}) was determined.

Isolation of Sorafenib-Resistant HCC Cell Line

HCC cell lines were seeded in a 100-mm plate at a density of 2×10^4 cells and allowed to attach by overnight incubation. The next day, cells were replaced with growth medium supplemented with 5 $\mu\text{g/mL}$ of sorafenib. Treated cells were split at a ratio of 1:2 once per week. During the cell split, one dish was stained with Giemsa dye, and the other dish was cultured in a growth medium supplemented with sorafenib. The culture medium was changed at 3-day intervals, and treatment was continued identically for five weeks. Cells were stained with Giemsa dye when they became confluent, using the manufacturer's protocol. Briefly, the cell culture media was removed, and cells were washed with 5 mL PBS. Cells were fixed using 5 mL of methanol for 5 minutes. After that, methanol was aspirated and incubated with 5 mL of Giemsa dye diluted in deionized water for 24 hours. The next day, plates were repeatedly washed with deionized water to remove the background staining and air-dried. Culture treated with solvent was used as a control. Cell colonies that survived sorafenib treatment were isolated, and stable sorafenib-resistant cell lines (SR Huh 7.5 and SR Huh 7 PX) were developed.

Sorafenib Uptake Assay

Sorafenib uptake of HCC culture was measured using a previously described protocol with minor modifications.²⁸ HCC cell lines were harvested after trypsin-EDTA treatment and washed twice with phosphate-buffered saline, pH 7.2. Briefly, 1×10^6 cells were suspended in 1mL of growth medium in sterile, round-bottom polystyrene tubes with caps. Sorafenib uptake assays were initiated by the addition of 100 μL of cell culture medium supplemented with radio-labelled [^3H] sorafenib or [^3H] cytidine (as a control) at 37°C and 4°C . After 5 minutes of incubation, cells were washed with 1 mL of ice-cold PBS to stop the reaction. After this step, cells were washed twice with 1 mL of ice-cold PBS. Cell pellets were treated with 100 μL of lysis buffer (10 mmol/L Tris HCL (pH 8.0), 10 mmol/L NaCL, 1.5 mmol/L MgCl_2 , and 0.1% NP-40 (v/v)). A 40- μL aliquot of soluble protein lysate was mixed with 1mL of scintillation fluid, and radioactivity was measured using a liquid scintillation analyzer (PerkinElmer, Walton, MA). Uptake values were expressed as counts per minute per cell. Radioactivity associated with tubes incubated at 4°C reading was considered background nonspecific reading. The specific uptake was determined by subtracting the nonspecific reading from the total reading at 37°C .

Metformin Uptake

Metformin uptake assay was performed using a standard protocol described earlier.²⁹ Briefly, SR Huh 7.5 and SS Huh 7.5.1 cells (2 X10⁴ cells) were plated into six-well tissue culture plates, supplemented with DMEM with 5% FBS and allowed to adhere by incubation at 37°C overnight, and then treated with 250µM metformin. After one hour, cells were harvested, washed twice with PBS, and cell lysates were prepared for Western blot analysis using AMPK sampler kit.

Stable Expression of OCT1

SR Huh 7.5 cell cultures in 100mm plates at a 50–60% confluence was transfected with OCT1 expression plasmid (pExp-OCT1) and control pcDNA3.1 plasmid using TurboFect (Thermo Scientific). After 72 hours, cells were cultured in the medium containing hygromycin (500µg/mL) for selection of stable OCT1 expression. G-418 (500µg/mL) was used for selection of control vector expression. After several weeks of selection, drug-resistant cell colonies were isolated, and stable cell lines were prepared.

Adenoviral Expression of OCT1

Recombinant adenovirus with cytomegalovirus (CMV) promoter-driven expression of OCT1 and LacZ was obtained from Henriette E. Meyer Zu Schwabe Dissen, Basel, Switzerland.³⁰ The recombinant adenovirus was propagated in HEK293A cells and the virus titer was determined using a standard protocol.

Western Blot Analysis

Cells were serum starved for 24 hours and then treated the next day with increasing concentrations of sorafenib for 2 hours. After this step, cells were washed in PBS three times and lysed in ice-cold RIPA lysis buffer. The total protein content in the lysate was quantified using a protein assay kit (Bio-Rad Laboratories). Twenty micrograms of protein from each sample were used for Western blot analysis using a standard protocol in the laboratory.

Immunohistochemical Stains for OCT1 and OCT3 in HCC Tissues

The expression of OCT1 and OCT3 was performed using paraffin fixed tissue section of HCC cases available from a previous study in the Department of Pathology.³⁰ This does not involve any patient contact therefore ethical clearance is not required. Five-micron tissue sections were prepared from paraffin-embedded HCC tissues. Immunostaining of the paraffin-embedded tissue sections of HCC was carried out using a standard method established in the laboratory. Hematoxylin and eosin (H&E)-stained sections of all specimens including cancer and non-cancer areas of the liver tissue were examined by three pathologists (SNT, TW, and KM). Antigen retrieval and immunostaining were performed using a commercially available kit (BioCare Medical). Tissue sections were deparaffinized for 15 min at 50–60 °C then incubated in xylene twice for 5 min. The tissue sections were rehydrated by sequential treatment with 100%, 95%, and 80% alcohol. Endogenous peroxidase quenching was carried out by incubation with 3% hydrogen peroxide and 100% methanol for 5 min. The slides were placed in a plastic Coplin Jar with Reveal Decloaker RTU (BioCare Medical) for 25 min at 95°C in a steamer for heated antigen retrieval. Following this step, the slides cooled down at room temperature for 20 min. The tissue sections were rinsed in deionized distilled water and marked with a PAP pen. The slides were incubated with a blocking sniper (BioCare Medical) for 10 min and incubated with a primary antibody (OCT1 or OCT3) (1:1000 dilution) overnight. After the primary antibody incubation, slides were washed 3 times in Tris-buffered saline (TBS) (pH 8.0), and incubated with a MACH 4 mouse probe (BioCare Medical, UP534) for 20 min and MACH 4 HRP Polymer (BioCare Medical, MRH534) for 30 min each, then washed 3 times using TBS. Finally, tissue sections were treated with diaminobenzidine (DAB) chromogen (Dako Cytomation, Carpinteria, CA) for 1–5 min. The slides were then counterstained with hematoxylin for 30s and Tacha's Bluing Solution (BioCare Medical, HTBLU) for 30s, dehydrated with 95% and 100% alcohol, mounted, and observed by light microscopy. Immunohistochemical staining of HCC tissue sections was examined by two pathologists (TW and KM). Scores were assigned to the intensity and percentage of positive staining of all the slides used in this study. Score 0 means negative staining, score (+) when 1–10% of cells were positive, score (++) when 10–50% of cells were positive, and score (+++) when 50–100% of cells were positive. Discrepancies were resolved by a consensus between the two pathologists using a multiheaded microscope in the

Pathology Department, Tulane University Health Sciences Center. H&E-stained sections of all specimens including cancer and non-cancer cases were examined by the same pathologists following the immunohistochemical evaluation.

Statistical Analysis

All experiments were done at least 3 times in triplicate. All results were expressed as mean $\pm$ SE (standard error) and $n=3$. A comparison between two groups was performed with a Student's *t*-test. We assume that all measurements have normal probability distributions, which is expected for these types of data. The P-value for the Student's *t*-test was significant when $p<0.05$.

Results

Development of sorafenib-resistant HCC cell lines

To study the intrinsic differences in cellular cytotoxicity, a panel of HCC cell lines with different genetic backgrounds was treated with an increasing concentration of sorafenib (0 to 20 μ M) or with solvent alone. MTT assay determined the proliferation of HCC cell lines in the presence of sorafenib. The IC₅₀ of different HCC lines were compared (Figure 1A). The concentrations of sorafenib used in our in vitro analysis are biologically relevant ranges because the plasma concentration of patients who receive 400 mg of sorafenib twice a day was reported to be 5 μ M.^{31,32} MTT assay results show that sorafenib treatment caused a concentration-dependent decrease in growth of all HCC cell lines (Huh 7, Huh 7.5, Huh 7 PX, Hep-1, Huh 7AF, HLE, HLF, HepG2 and Huh 7.5.1) with a very broad IC₅₀ that ranged from 2 to 10 μ M (Figure 1B). We found that HepG2 and HLF, HLE cell lines are the most sensitive to sorafenib with a low IC₅₀ of 2 μ M and Huh 7 PX, Huh 7.5, Hep-1 cell lines are more resistant to sorafenib with a higher IC₅₀ of 10 μ M, demonstrating the drug sensitivity variation among HCC cell lines. These results indicate HCC cell lines' heterogeneity in their initial response to sorafenib treatment and that variation range of the amount of sorafenib could affect the initial treatment response. Based on this MTT assay data, a long-term sorafenib treatment of HCC culture was performed to verify whether the development of drug resistance could be related to the intrinsic differences in the initial response of HCC cells to sorafenib. For this purpose, HCC cells were cultured in a growth medium supplemented with clinical concentrations of sorafenib (5 μ M) with a regular media change at 3-day intervals. Also, sorafenib-treated cells were split when they became confluent. The growth and proliferation of HCC cell lines in the presence and absence of sorafenib were measured over five weeks. We observed that most of the HCC cell lines died during the course of treatment, except two HCC cell lines that continued to increase in the presence of sorafenib and formed viable cell colonies (Figure 1C). Individual resistant cell clones from these HCC cell lines were isolated and stable sorafenib-resistant lines (SR Huh 7.5, SR Huh 7 PX) were prepared. MTT assay confirmed the sorafenib sensitivity of resistant (SR Huh 7.5) and sensitive cell line (SS Huh 7.5.1) (Figure 1D). Resistant cell line (SR Huh 7.5) did not show any evidence of cellular cytotoxicity when treated with increasing concentrations of sorafenib, as compared to sensitive cells. The drug resistance mechanism is specific to sorafenib since another chemotherapy drug doxorubicin was able to induce cellular cytotoxicity in both the susceptible and resistant cell line (Supplemental Figure 1). To identify whether there was a defect in the expression of oncogenic kinases (BRAF or CRAF) that could explain the mechanisms of resistance, we treated sorafenib sensitive and sorafenib resistant cell lines with increasing concentrations of sorafenib (0–20 μ M) for 2 hours. Cell lysates were used to compare the expression of phosphorylated and unphosphorylated forms of all molecules involved in the MAPK kinases (EGFR/RAF/MEK/ERK/CYCLIN-D) determined by Western blot analysis. Sorafenib treatment showed a concentration-dependent inhibition of BRAF, CRAF, and downstream MEK/ERK pathways in the sensitive HCC cell line (SS Huh 7.5.1). In contrast, the resistant cell line (SR Huh 7.5) treated with sorafenib did not show inhibition of BRAF or CRAF (Figure 2). Interestingly, we found that higher-level expression of EGFR, and down-stream tyrosine kinase expression (BRAF, CRAF MEK, ERK and Cyclin D) in the resistant cell line (SR Huh 7.5) as compared to the sensitive cell line (SS Huh 7.5.1).

Sorafenib Resistance of HCC is Related to Impaired Uptake

The success of anticancer drug treatment is dependent upon intracellular drug accumulation and uptake, which in turn is directly related to the membrane expression of the drug transporters.²³ Increased expression of uptake transporters favors

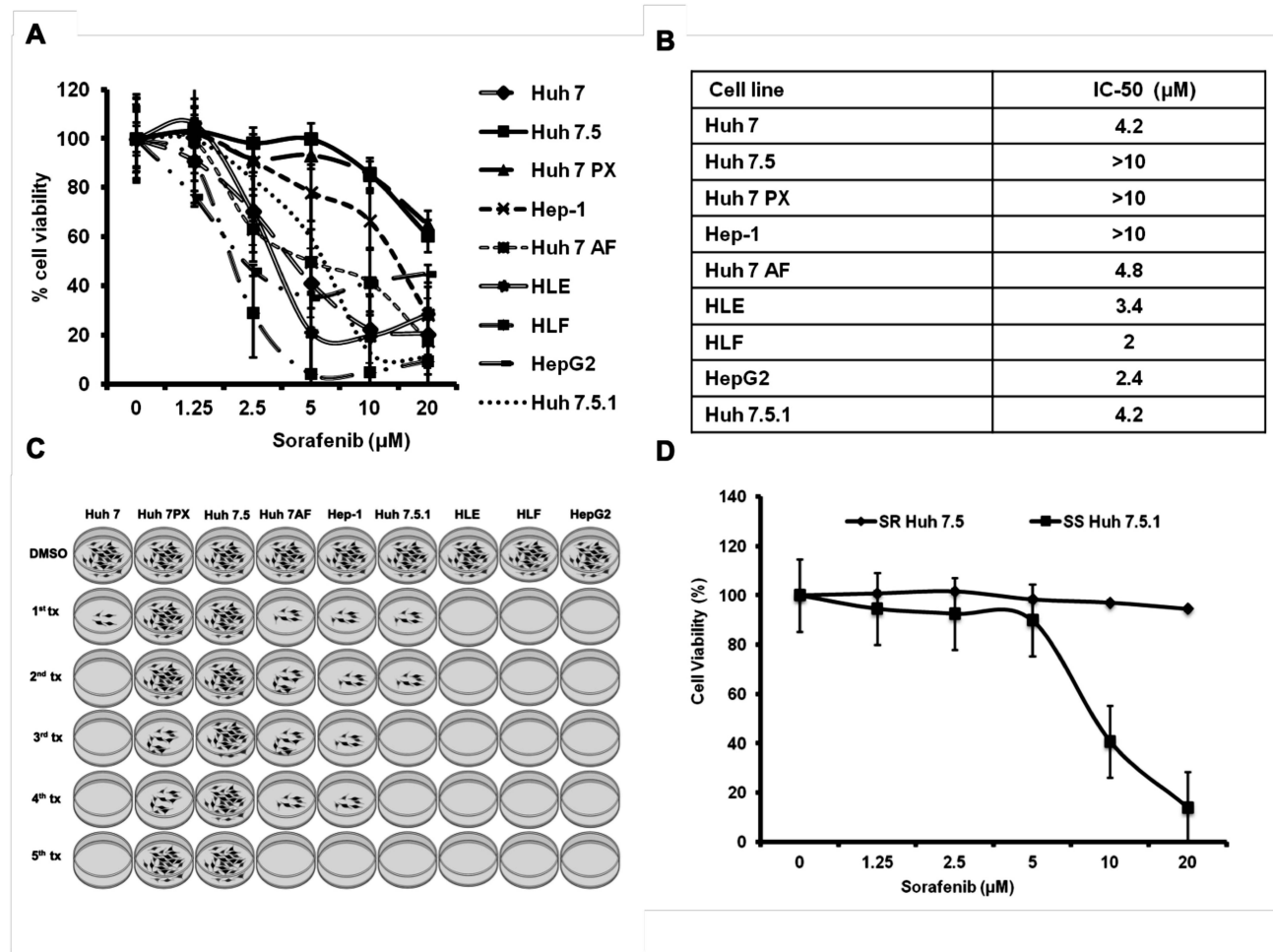


Figure 1 Variation in the sorafenib-induced cellular cytotoxicity among hepatoma cell lines. HCC cell lines were seeded on 24 well plates (2×10^4 cells/well) in triplicate. After 24 hours, cells were treated with five different concentrations of Sorafenib. After 72 hours of incubation, cell proliferation was determined by MTT assay. **(A)** Represents the percentage of cell viability of nine different HCC cell lines after sorafenib treatment is shown. **(B)**. Comparison of IC50 values among nine different HCC cell lines tested. **(C)**. Diagram illustrating generation of sorafenib-resistant HCC cell line in culture. Nine different HCC cell lines were cultured in growth medium supplemented with sorafenib for five weeks. HCC cells that survived sorafenib treatment over five weeks were selected as resistant lines. **(D)**. MTT assay shows a dose-dependent effect of sorafenib on cell viability of sensitive cell line (SS Huh 7.5.1) and resistant (SR Huh 7.5) HCC cell line. Sorafenib-resistant and sensitive HCC cells were seeded in 24 well plates and treated the next day with increasing concentrations of sorafenib. After 72 hours, cell proliferation was measured by MTT assay.

the accumulation of the drug in cancer cells, resulting in better chemotherapy response. The OCT1 located at the basolateral membrane of hepatocytes is one of the transporters involved in sorafenib uptake.²³ We wondered whether sorafenib response to HCC tumor cells depends on the membrane expression of OCT1. To address this question, we examined the uptake among all HCC cell lines using ³H-labeled sorafenib and correlated these results with the MTT assay results. The uptake assay results of all HCC cell lines are shown in Figure 3A. We observed a marked variation in sorafenib uptake (mediated by OCT1) among all ten HCC cell lines compared to cytidine uptake mediated by ENT1 transporter. Among all the cell lines, Huh 7.5 cell sorafenib uptake was significantly less as compared to the remaining susceptible lines. There was no difference between the sensitive and resistant cells in the uptake of ³H-cytidine mediated by the ENT1 transporter.³³ Flow analysis experiments showed that doxorubicin uptake mediated by another transporter called SLC22A16 is not significantly different among all HCC cell lines³³ (Supplemental Figure 2). The uptake assay results were verified using another drug whose uptake is mediated by OCT1. A search for an alternative verification method revealed that OCT1 mediates metformin uptake. The resistant cell line and sensitive cell lines were exposed to metformin for one hour, and the OCT1-dependent uptake of metformin between HCC and primary human hepatocytes was examined by measuring the phosphorylation of AMPK and acetyl-CoA carboxylate (ACC) by Western blot analysis. The resistant cell line (SR Huh 7.5) showed significantly less AMPK and ACC phosphorylation compared to the

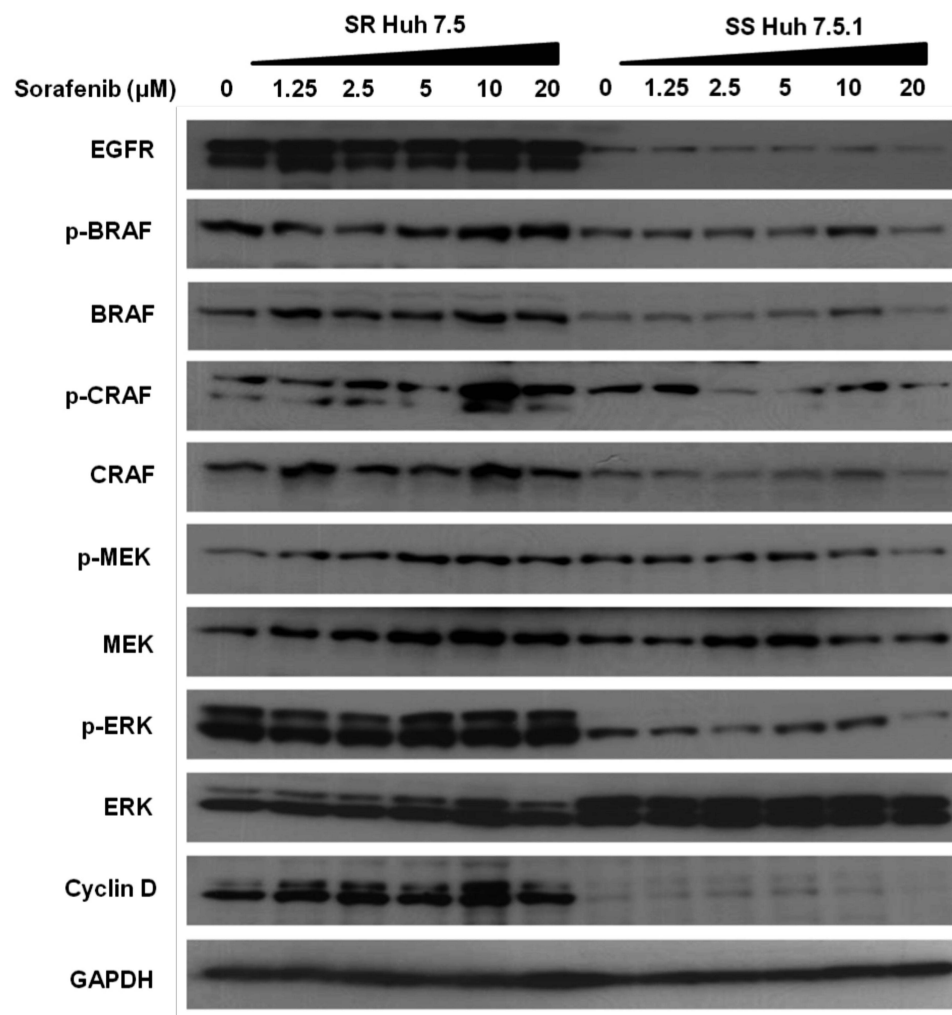


Figure 2 Western blot analysis showing the expression of EGFR and RAS/RAF/MEK/ERK pathway between sorafenib sensitive and resistant HCC cell lines after sorafenib treatment. Cell lines were serum starved overnight and then treated with increasing concentrations of sorafenib for an additional 2 hours. Constitutive basal expression and phosphorylation status of BRAF, CRAF, MEK, and ERK proteins were examined by Western blot analysis. GAPDH level was measured as a loading control. Twenty micrograms of proteins were separated on SDS-PAGE and Western blotting was performed using antibodies to EGFR, BRAF, CRAF, MEK, ERK, and Cyclin D.

sensitive cell line (SS Huh 7.5.1). The results of sorafenib uptake and metformin uptake were consistent among all HCC cell lines ([Supplemental Figure 3](#)). Western blot analysis was performed to determine expression levels of OCT1 in all HCC cell lines. We found that OCT1 expression is absent in the resistant cell line (SR Huh 7.5) and HepG2 cells. OCT1 expression was very weak in HEP-1, Huh 7AF, HLE, and HLF. A strong OCT1 expression was seen in Huh 7, Huh 7PX, and Huh 7.5.1 as shown in [Figure 3B](#). We found that two bands for OCT in Hep-1 cells, one mature and truncated 50kD. To verify whether the lack of membrane expression of OCT1 in HCC cell lines could be due to improper glycosylation, total cell lysates prepared from all HCC cell lines and primary human hepatocytes were examined for the expression of OCT1 by Western blot analysis. We found that the glycosylated mature form of OCT1 has a molecular weight of 70kD and is detectable in primary human hepatocytes, whereas most of the HCC cell lines show 50kD OCT1 protein, indicating improper glycosylation or the presence of splice variants as described earlier.²⁴ OCT3 expression strong in Huh 7.5, Huh 7PX, and Hep-1. Other cell lines show negative OCT3. The expression of other transporters (CNT1, ENT1, and ENT2) did not show any change except HepG2 cell line showed negative for CNT1 and ENT1. The original Western blot for [Figure 3B](#) is presented in [Supplemental Figure 4](#). An earlier study demonstrated that there is a strong relationship between transporter activity, sorafenib uptake, sensitivity, and subcellular localization among OCT1 splice variants.²⁴ We examined the membrane expression of OCT1 by immunostaining using all HCC cell lines. Primary human hepatocytes

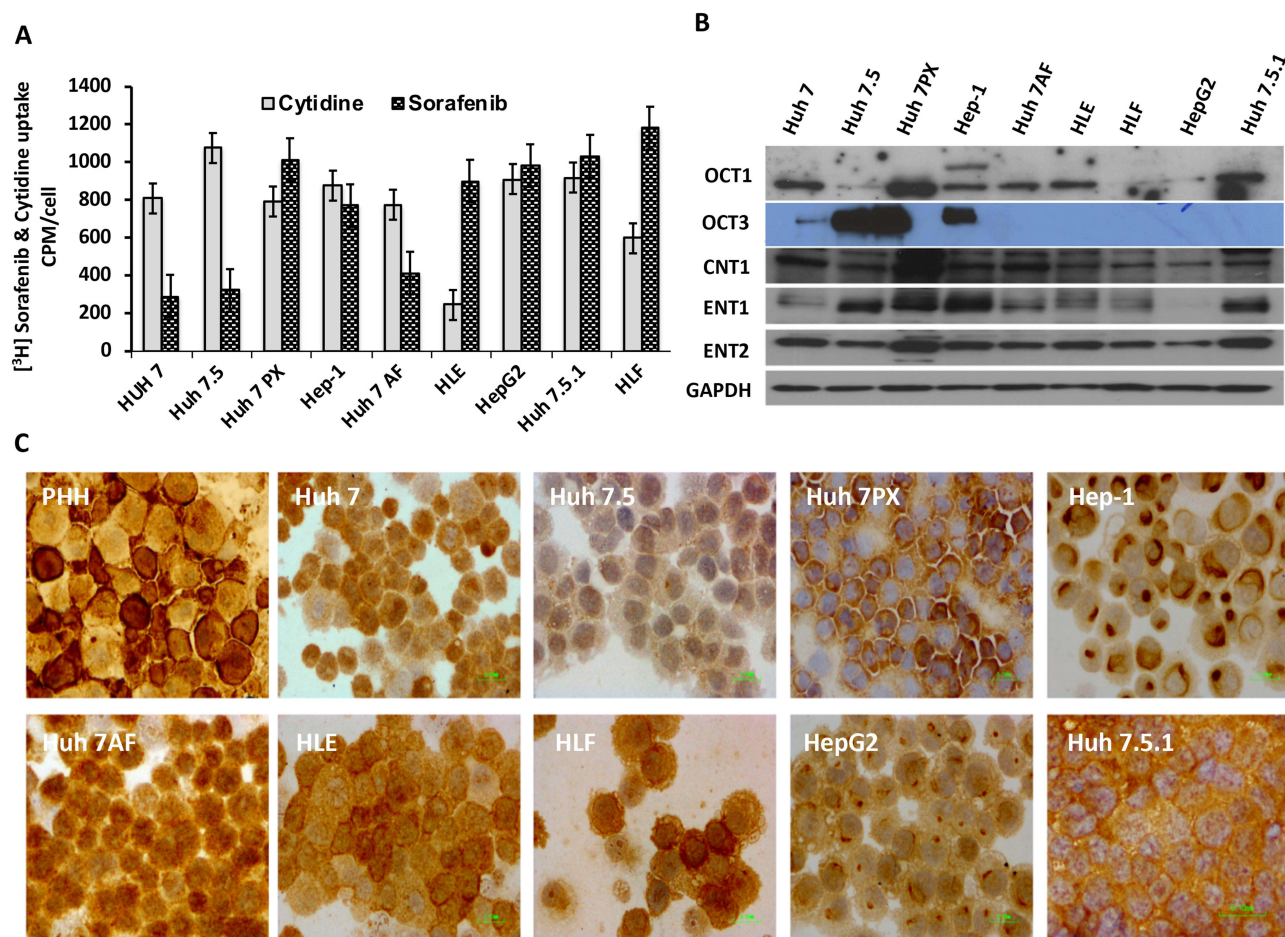


Figure 3 Impaired membrane expression of OCT1 reduced sorafenib uptake in resistant HCC cell line (SR Huh 7.5). Sorafenib uptake between resistant and sensitive cell lines were initiated by the addition of 100 μ L of cell culture medium supplemented with radiolabeled sorafenib [3 H] (specific activity of this is 2.7Ci/mmol) or [3 H] cytidine (as a control) at 37°C and 4°C. After 5 minutes of incubation, cells were washed twice with 1 mL of ice-cold PBS. Cell pellets were treated with 100 μ L of lysis buffer. 40- μ L aliquot of soluble protein lysate was mixed with 1 mL of scintillation fluid, and radioactivity was measured using a liquid scintillation analyzer. Uptake values were expressed as counts per minute per cell. Specific uptake was determined by subtracting the non-specific reading at 4°C from the total reading at 37°C. (A) Show the sorafenib uptake values between sensitive and resistant cell lines. (B) Western blot shows the expression of drug transporter OCT1, OCT3, CNT1, ENT1, ENT2, and GAPDH expression between all sensitive and resistant cell lines. (C) Immunostaining shows the differences in the membrane expression of OCT1 among primary human hepatocytes (PHHs), and sorafenib resistant (SR Huh 7.5) and all HCC cell lines used in this investigation.

showed a strong membranous expression of OCT1 (Figure 3C). However, most of the HCC cell lines except Huh 7.5 (sorafenib resistant) showed positive OCT1 staining in the cytoplasm or in the endoplasmic reticulum (ER), but the membrane expression of OCT1 was reduced in most of the HCC cell lines. Only Huh 7.5 showed negative for OCT in Western blot as well as immunostaining. We selected Huh 7.5 cells for the next set of analysis since it lacks the OCT1 membrane expression completely (Figure 3C). These results indicate that the lack of membrane expression of OCT1 could be the reason for the loss of OCT1 activity in HCC. These analyses revealed impaired OCT1 membrane expression in HCC cell lines compared to non-transformed primary human hepatocytes.

Stable Expression of OCT1 Improves Sorafenib Uptake and Overcomes the Resistance

We performed experiments to confirm whether impaired OCT1 expression is the cause of HCC resistance to sorafenib. The resistant line Huh 7.5 (SR Huh 7.5) was transfected with OCT1 or empty expression plasmid. We prepared a sorafenib-resistant cell line with stable OCT1 expression. Immunostaining verified that most of the stably transfected (SR Huh 7.5-OCT1) cells showed excellent membrane expression of OCT1 compared to the sorafenib-resistant cell line (Figure 4A). Stable transfected SR Huh 7.5-OCT1 cells show the expression of mature forms of OCT1 that are detectable

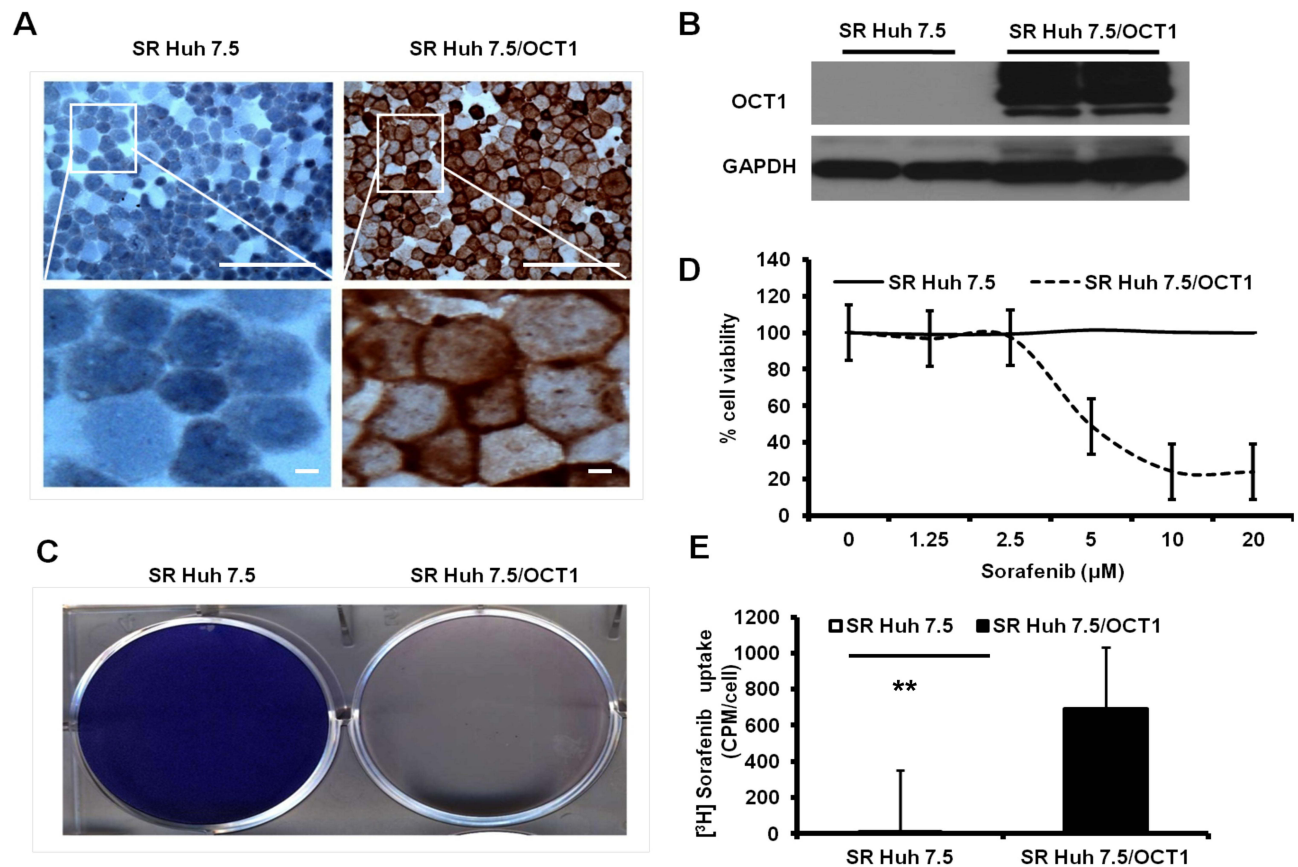


Figure 4 Stable expression of OCT1 in a resistant HCC cell line enhanced sorafenib uptake and induced cytotoxicity. (A). Immunostaining shows a very clear membrane expression of OCT1 in stable transfected cells. No membrane staining was present in the sorafenib-resistant cell line (SR Huh 7.5). (B). Western blot where samples were run in duplicate confirms absence of OCT1 expression in sorafenib-resistant cell line. (C). Giemsa staining of HCC culture treated with sorafenib induced efficient cell killing after one week. (D). MTT assay results of resistant HCC cell line with or without OCT1 expression after sorafenib treatment. (E). Sorafenib uptake between resistance HCC cell line with or without OCT expression (** $p < 0.002$).

by Western blot analysis (Figure 4B). We found multiple bands cross-reacted with OCT1 antibody when it was overexpressed by transfection. This is probably due to various extents of OCT1 glycosylation. An excellent membrane expression of OCT1 in the SR Huh 7.5 cells prompted us to test its sensitivity to sorafenib treatment. For this experiment, sorafenib-resistant (SR Huh 7.5) cells with or without stable OCT1 expression were treated with sorafenib (5μM), and cell survival was compared. Results of this analysis revealed that OCT1 expression induced sorafenib cytotoxicity and killed all HCC cancer cells examined by Giemsa staining (Figure 4C). Cell viability of SR Huh 7.5 cells with or without OCT1 expression after sorafenib treatment was determined after 72 hours by MTT assay (Figure 4D). Sorafenib uptake assay showed that OCT1 expression improved sorafenib uptake significantly (Figure 4E). The role of OCT1 activity in the mechanisms of sorafenib uptake and resistance was verified in the pretreatment of sensitive cells (SS Huh 7.1) with quinine an OCT1 inhibitor. We found that pretreatment of sensitive cells with quinine inhibited sorafenib uptake in the sensitive cell line and induced sorafenib resistance. In contrast, similar treatment did not alter sorafenib uptake in the resistant cell line (Supplemental Figure 5). Taken together, these results support the conclusion that stable OCT1 expression overcomes sorafenib resistance by inducing its uptake and cellular cytotoxicity.

Adenoviral Gene Delivery of OCT1 Overcame Resistance

We performed another confirmatory experiment to verify whether adenoviral-mediated gene transfer of OCT1 could overcome sorafenib resistance in the cell culture model. Recombinant adenovirus carrying OCT1 gene under CMV promoter (Figure 5A) was obtained as a gift from our collaborator.³⁰ The initial experiment was performed using Western blot analysis to verify the expression of OCT1 in SR-Huh-7.5 cells after infection with AdCMV-OCT1. The AdCMV-

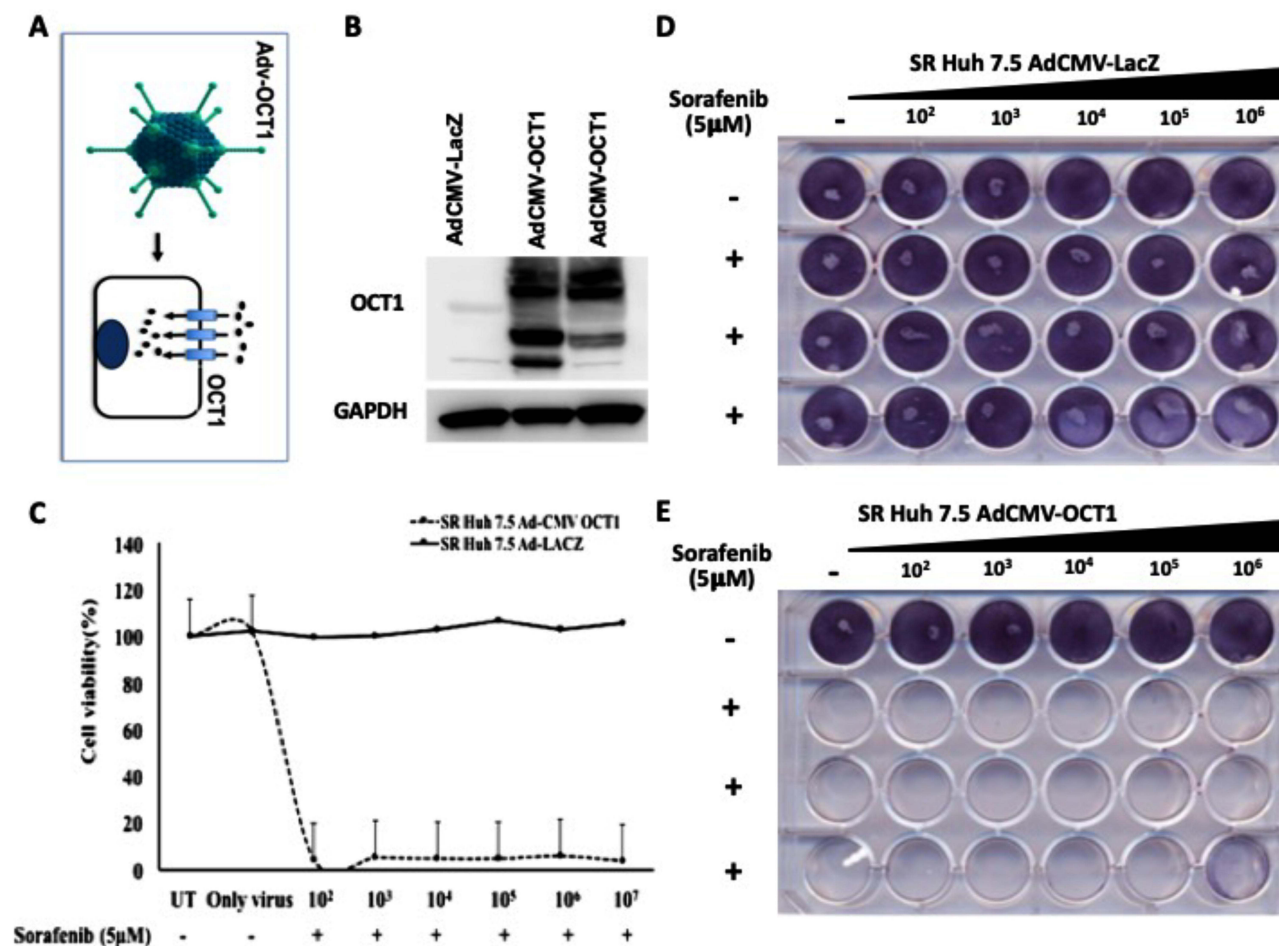


Figure 5 Sorafenib cytotoxicity of SR Huh 7.5 cells after adenoviral gene transfer of organic cation transporter-1 (OCT1). (A). Adv-CMV construct. (B). Western blot showing robust expression of OCT1 in SR Huh 7.5 cells after infection with recombinant AdCMV-OCT1. AdCMV-LacZ infected cells show negative OCT1 expression. Only baseline expression of truncated OCT1 (C). Sorafenib cytotoxicity after OCT1 expression and lacZ gene expression by recombinant adenovirus. In brief, 1×10^5 SR Huh 7.5 cells were seeded in 24-well plate and then infected with AdCMV-OCT1 or AdCMV-LacZ. After 24 hours of infection, cultures were treated with sorafenib for an additional 24 hours. Cytotoxicity was measured by MTT assay and cell colony assay. (D). Cell colony assay showing cell proliferation in the presence of sorafenib after infection with AdCMV-LacZ. (E). Cell colony assay showing SR Huh 7.5 cell proliferation in the presence of sorafenib after infection with AdCMV-OCT1.

OCT1 construct expresses fairly high levels of OCT expression in SR Huh 7.5 cells (Figure 5B). As a control, we used recombinant adenovirus expressing LacZ gene. No OCT1 expression was detected with control adenovirus-infected cells. A faint truncated OCT1 was detected in Ad CMV LacZ-infected cells. An equal number of SR Huh 7.5 cells (1×10^4) were seeded in 24 well plates. One set of sorafenib resistance (SR Huh 7.5) cell line was infected with adenovirus-OCT1 (AdCMV-OCT1) and another set was infected with adenovirus expressing LacZ gene (AdCMV-LacZ) for 24 hours and cell viability was assessed by MTT assay (Figure 5C). The impact of OCT1 expression on the long-term proliferation of sorafenib resistant cell line (SR Huh 7.5) was determined by cell colony assay. Cells were cultured in 24 well plates and then treated with sorafenib (5μM) for an extended time with regular media change after 3 days. Cell colony assay shown in Figure 5D indicates that SR Huh 7.5 cells infected with AdCMV-LacZ showed increased proliferation. In contrast, SR Huh 7.5 cells infected with AdCMV-OCT1 improved sorafenib cytotoxicity and overcame resistance (Figure 5E).

Impaired expression of OCT1 in human hepatocellular carcinoma

Sorafenib, Regorafenib, Lenvatinib, and Cabozantinib are the tyrosine kinase inhibitors (TKIs) approved for HCC treatment. Membrane expression OCT1 and OCT3 expression are critical for the success of TKIs. To verify whether the impaired expression of OCT1 in the sorafenib resistance in HCC culture also occurs in HCC developed in humans.

The expression of OCT1 was examined using leftover paraffin-embedded HCC tissue specimens used in a previous study by immunohistochemistry. Without knowledge of experimental outcomes, two independent pathologists evaluated the membrane OCT1 staining. The intensity was measured using a scale where a score of 0 represented no staining, a score of 1 for having weak staining, a score of 2 for medium staining, and a score of 3 for strong staining. The intensity score was multiplied by the percentage of cells in the tumor and surrounding non-tumorous liver that showed OCT1 positive staining (0–100%), a method described previously.^{34–36} We performed a semi-quantitative assessment of OCT1 expression in 20 human HCC cases and surrounding non-HCC areas was conducted. A summary of the expression pattern of OCT1 is shown in [Supplemental Table 1](#). In most of the HCC cases, the membrane expression of OCT1 was significantly reduced compared to the non-tumorous liver ([Figure 6](#)). Among the 20 HCC samples OCT1 expression was localized to the membrane of hepatocytes in the non-tumorous liver, whereas most of the tumor show negative OCT1 expression. The results of this investigation confirm that membrane expression of OCT1 is impaired in most HCC tumors compared to the non-tumorous liver. This was consistent with all 20 HCCs examined showing most of the tumor areas showed impaired membrane expression. The results of this investigation confirm that membrane expression of OCT1 is impaired in the

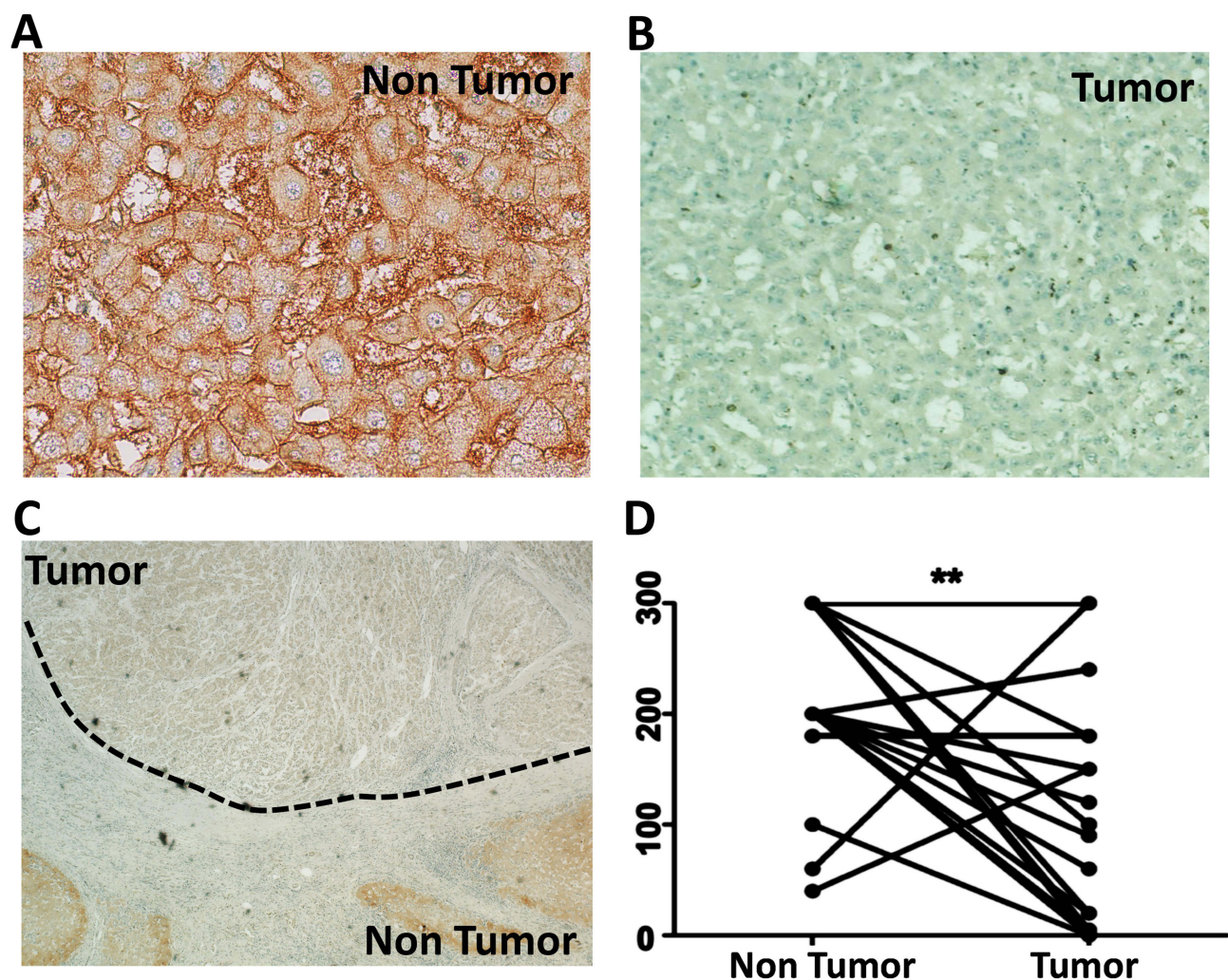


Figure 6 Expression of organic cation transporter-1 (OCT1) in human hepatocellular carcinoma and surrounding non-tumorous hepatocytes. The expression of OCT1 was confirmed using formalin-fixed, paraffin-embedded samples of 20 human HCC samples by immunohistochemistry using a standard protocol. Immunohistochemical staining of OCT1 was evaluated by two independent pathologists (Dr. Moroz and Dr. Wu), who scored staining pattern as 0 having no staining at all, 1 with weak staining, 2 with medium staining, and 3 with strong staining. By multiplying the intensity of score and the proportion of positive cells (0–100%), a semi-quantitative score ranging from 0–300 was calculated. (A). Show OCT1 expression in non-tumorous hepatocytes in the cirrhotic liver (Case number 16, magnification of 40X) (B). Negative OCT1 expression in hepatocellular carcinomas (tumor) (C). Low magnification images (4X, case number 16) showing expression of OCT1 in non-tumorous hepatocytes and HCC tumors show negative OCT1 expression. (D). The staining scores for OCT1 are significantly lower in HCC tissues than the corresponding non-tumorous liver (** $p < 0.0022$).

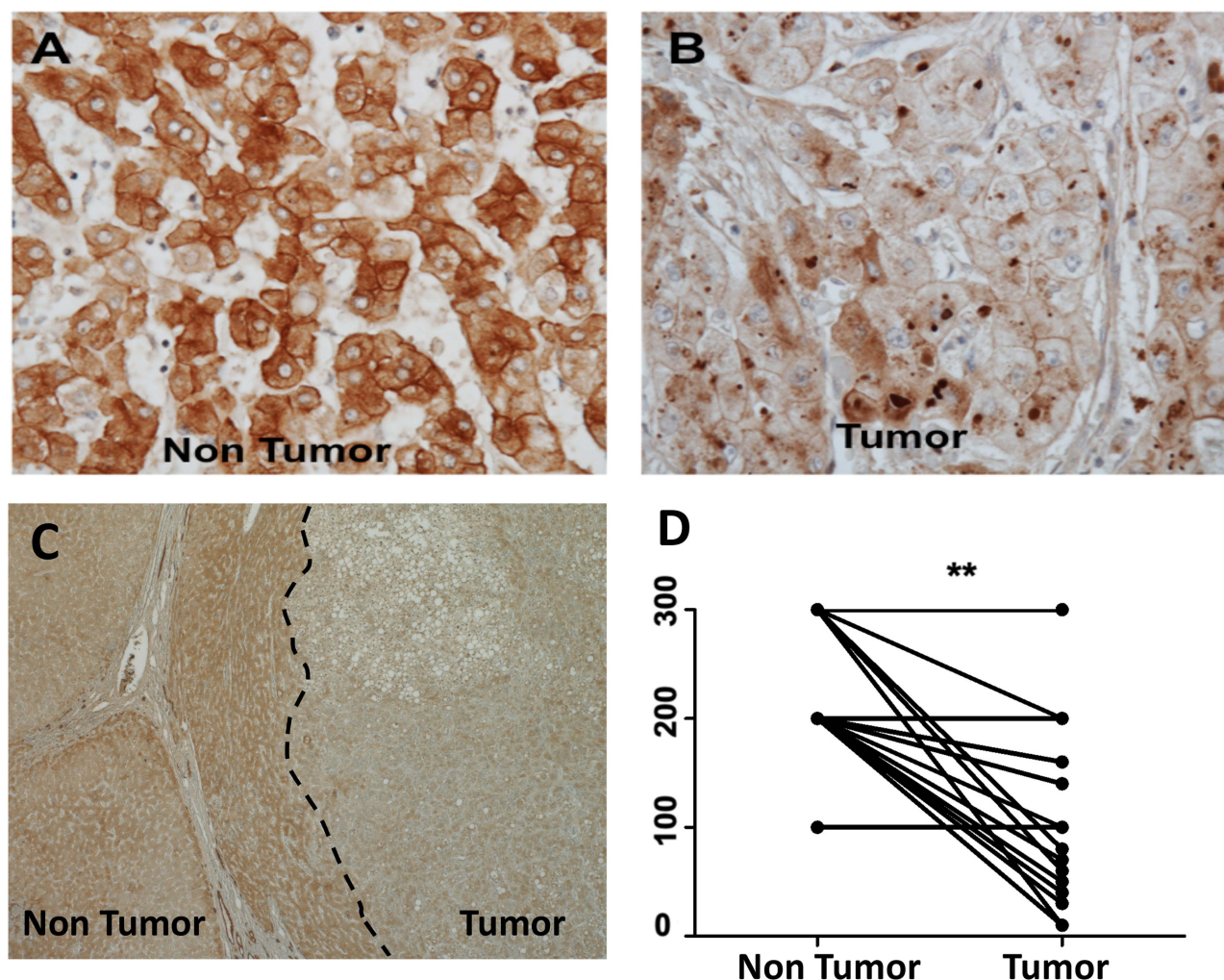


Figure 7 Expression of organic cation transporter-3 (OCT3) in human HCC. Immunostaining of OCT3 in HCC and surrounding non-tumorous area in the 20 human liver specimens. (A) Representative images of OCT3 staining by immunostaining and hematoxylin counterstaining showing a strong membrane expression in non-tumorous cirrhotic liver (Case number 9, magnification of 40X). (B). Representative image showing loss of OCT3 expression in HCC (Case number 9, magnification of 40X) (C). Low magnification images (case number 9, magnification of 4X) show expression of OCT3 in non-tumorous cirrhotic hepatocytes, and most of the HCC tumors show reduced OCT3 expression. (D). The staining intensity scores for OCT3 expression in 20 HCCs and surrounding non-tumorous cirrhotic livers (** $p < 0.001$).

majority of HCC tumors compared to the non-tumorous liver. We provide in vitro and in vivo evidence indicating that impaired expression of OCT1 is one of the intrinsic HCC resistance mechanisms of sorafenib. The expression of OCT3 in HCC is associated with sorafenib treatment response. Immunostaining of OCT3 was also performed in the same set of HCC samples. Staining quantification was performed using the identical protocol described for OCT1. We found OCT3 membrane expression is also decreased in most of the HCC samples. In contrast, the membrane expression of OCT3 is better preserved in the surrounding non-tumor cirrhotic livers (Figure 7). The expression pattern of OCT3 is shown in all 20 HCCs and is summarized in [Supplemental Table 2](#). In conclusion, we found impaired expression of OCT1 and OCT3 in HCC developed in human cirrhotic livers.

Discussion

Sorafenib functions as a multi-targeted tyrosine kinase inhibitor with broad spectrum of actions. Its mechanism of action involves the inhibition of tumor cell proliferation by targeting the RAF kinases (ARAF, BRAF, and CRAF) suppressing the RAS/RAF/MEK/ERK signaling pathway. Furthermore, sorafenib hinders angiogenesis by targeting c-KIT, FLT3, Vascular endothelial growth factor receptors (VEGFRs) and platelet-derived growth factor receptor beta (PDGFR- β).³⁷ Activation of the RAS/RAF/MAPK pathways in HCC provide the rationale for the use of sorafenib for liver cancer

treatment.^{38,39} Sorafenib received FDA approval in 2007 for the treatment of advanced-stage HCC. Sorafenib systemic therapy improves the overall survival of patients with advanced HCC, maintaining its status as the first-line systemic therapy for individuals who are suitable for immunotherapy. Despite the emergence of new TKIs approved for HCC treatment, none of the compounds show higher performance than sorafenib.³⁹ The enduring effectiveness of sorafenib underscores its significance in the therapeutic landscape for advanced-stage HCC. However, developing drug-resistant mechanisms of HCC is one of the limitations of its clinical utility. Understanding the resistance mechanisms of sorafenib and other TKI at the molecular level may help to improve its chemotherapy efficacy and allow biomarker development for the early selection of patients who will benefit from sorafenib chemotherapy and those who will not. The activity of sorafenib relies on the inhibition of activity of tyrosine kinase intracellularly. Multiple cellular mechanisms that inhibit sorafenib activity in HCC include i) imbalances in drug uptake and export; ii) increased drug metabolism; iii) inactivation of drug active target; iv) alteration of cellular DNA repair pathways; v) imbalances in pro-survival and pro-apoptotic factors; vi) tumor microenvironment, and vii) tumor cell plasticity and stemness.^{40–42} Among the cellular targets, drug uptake, and drug efflux transporters are the first line of targets because the expression of these transporters determines the intracellular drug concentrations in the tumor. Tumor cells can resist anticancer drugs if the drug uptake is impaired due to the loss of expression of transporters. The activity of sorafenib depends on the level of drug entry and intracellular access to the transmembrane domain of tyrosine kinases of tumor cells. We selected OCT1 because it is expressed in the liver and implicated in sorafenib uptake. We did not select ABC transporters are responsible for drug efflux since ABC transporter expression is significantly low in HCC developed in cirrhotic liver.²⁴

Our study highlights some key observations that could contribute to understanding the sorafenib treatment of HCC. First, we found that cell lines with IC₅₀ = ten values are more frequently resistant to cell killing after sorafenib treatment. Two HCC cell lines (Huh 7.5 and Huh 7PX) grew permanently in the presence of sorafenib. Our study shows that the mechanisms of sorafenib resistance could be related to differences in the expression of tyrosine kinases. The expression of RAF kinases (EGFR, BRAF, CRAF, MEK, ERK, and CYCLIN-D) is high in sorafenib-resistant cell lines. The heightened baseline activation observed in resistant cell lines may be attributed to the presence of TKI genetic variants or altered gene expression, associated with TKIs. This overactivation of the signaling pathways contributes to increased proliferation and worse outcomes for HCC patients with sorafenib.⁴³ Our study findings align with the hypothesis that the response of HCC to sorafenib is intricately tied to tumor heterogeneity. Tumor heterogeneity is a well-recognized phenomenon, and tumors harbor numerous genetic subclones that differ in their sensitivity to chemotherapy.^{44,45} Sorafenib treatment did not inhibit RAF signaling in resistant cell lines as compared to sensitive cell lines.

Second, we examined sorafenib uptake in correlation with expression of SLC proteins, specially focusing on OCT1 and OCT3. We found that sorafenib uptake and metformin uptake were impaired in resistant HCC cell line as compared to sensitive HCC cell line. Interestingly, doxorubicin induced cytotoxicity in sorafenib resistance cells suggesting that drug uptake mechanism of doxorubicin and sorafenib is different. We found that most HCC cell lines show impaired membrane expression OCT1 and OCT3 compared to primary human hepatocytes. Western blot analysis also revealed that the size of the OCT1 band is smaller in all HCC cell lines compared to primary human hepatocytes. We found two resistant cell lines show abundant expression of OCT3 but most of the sensitive cell lines show negligible expression of OCT3. The other sorafenib resistance cell line (Huh 7PX) and many sensitive HCC cell lines did not show impaired drug uptake suggesting that sorafenib resistance in this cell line occurs OCT1-independent manner. To test the putative role of OCT1 in sorafenib resistance, we performed additional investigation to test whether overexpression of functional OCT1 gene in resistant HCC cell line either by plasmid transfection or by adenoviral-vector mediated gene delivery could make these cell sensitive to sorafenib treatment. As expected, we found stable expression of OCT1 improved sorafenib sensitivity and overcame resistance since cell killing was very efficient after OCT1 membrane expression. We found consistent cellular cytotoxicity induced by sorafenib treatment after OCT1 expression by adenoviral-mediated OCT1 expression.

In the final stage of our investigation, we validated the significance of the OCT1-related intrinsic sorafenib resistance mechanism of HCC cell lines was verified using with HCC developed in human cirrhotic nodules. Immunohistochemical analysis of 20 HCC samples with liver cirrhosis revealed a marked reduction in OCT1 and OCT3 expression in all HCCs

compared to non-tumorous livers. These findings indicate that the diminished expression of OCT1 and OCT3 among HCCs may elucidate one of the intrinsic mechanisms that affect sorafenib treatment response at the level of uptake.

Our findings are consistent with the study of Herraiz et al, who reported that the expression of OCT1 may affect sorafenib treatment.²⁴ Other investigators have also demonstrated the impaired OCT1 expression in HCC.^{46–48} A previous study showed that some OCT1-negative HCC cells still respond to sorafenib in an OCT3-dependent manner, suggesting that OCT1 and OCT3 can compensate each other for sorafenib uptake.⁴⁹ Detecting both OCT1 and OCT3 can be a tremendous prognostic marker for HCC response to sorafenib. The immunostaining data of OCT3 expression is consistent with previous studies indicating that OCT3 expression is lost in HCC compared to non-tumorous hepatocytes.⁴⁹ Due to these reasons, OCT1 is used as a valuable marker for the chemoresistance of esophageal cancer to cisplatin and the treatment of chronic myeloid leukemia using imatinib-based chemotherapy.⁴⁸ The expression of sorafenib efflux transporters ATP-binding cassette (ABC) transporters MDR1/P-glycoprotein, multidrug resistance protein (MRP), and the breast cancer resistance protein (BCRP) decreased in HCC.⁵⁰ Improper function of transporters can lead to chemotherapy resistance.^{51–55} Namisaki et al³⁵ investigated the contribution of drug transporter expression of OCT1, OCT3, and multidrug-resistant 1 (MDR1)/p-glycoprotein, MRP1, MRP2, and BCRP to sorafenib resistance. They found OCT1, OCT3, and MDR1 expression decreased in HCC compared to nontumor tissue of Japanese patients with HCC. Tomonari et al⁵⁶ demonstrated that the drug efflux transporter MRP3 protein plays a role in resistance to sorafenib in HCC cells. Impaired expression of OCT1 in HCC and cholangiocarcinoma (CCA) is associated with sorafenib resistance, as demonstrated earlier by Lozano et al's⁵⁷ study. These authors showed a potential gene therapy strategy of OCT1 using adenovirus vectors using BIRC5 promoter for CCA to overcome sorafenib resistance in a tumor xenograft.

This study has identified several limitations that could serve as avenues for future research. Notably, the investigation did not delve into the significance of OCT3 in sorafenib resistance within HCC culture. While the presence of OCT1 and OCT3 transporters in tumors may predict drug uptake, the study did not explore other potential additive or synergistic mechanisms of chemoresistance. The activities of drug metabolic enzymes or changes associated with the activation of alternative signaling pathways that might impede the RAF/MEK/ERK pathway were not thoroughly examined. Furthermore, the study did not elucidate the mechanisms governing OCT1 expression at transcriptional levels, such as regulation by HNF4 alpha, promoter methylation, and translational levels involving N-linked glycosylation in the extracellular loop. Other investigators reported that epigenetic events due to promoter methylation contribute to reduced expression of OCT1.⁴⁸ The role of the organic-anion-transporting polypeptide (OATP) family, particularly SLCO genes, in sorafenib uptake mechanisms was not investigated. To address these gaps in understanding, future research could focus on exploring the role of OCT1 in sorafenib chemoresistance using relevant small animal models that closely mimic human disease. By addressing these limitations, subsequent studies may provide a more comprehensive and nuanced understanding of the intricate mechanisms influencing sorafenib response in HCC.

Conclusion

The investigations involving sorafenib-resistant and sensitive cells have revealed distinct differences in the expression levels of tyrosine kinases among HCC cell lines, reflecting their responsiveness to sorafenib treatment. Importantly, our findings indicate that sorafenib resistance can occur through both OCT1-dependent and independent mechanisms. This study supports the concept that the expression of plasma membrane transporters, specifically OCT1 and OCT3, could serve as biomarkers for predicting the response to sorafenib treatment.

Our research has illuminated pivotal intrinsic mechanisms of sorafenib resistance in HCC, particularly emphasizing the role of impaired OCT1 expression at the level of drug uptake. Building upon these results, we propose that introducing tumor-targeted expression of OCT1 through gene therapy vectors may represent a promising strategy to enhance the efficacy of sorafenib in treating HCC. This approach could potentially overcome resistance mechanisms associated with impaired OCT1 expression, ultimately improving the therapeutic response to sorafenib in hepatocellular carcinoma.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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