Sorafenib Modulates the Gene Expression of Multi-drug Resistance Mediating ATP-Binding Cassette Proteins in Experimental Hepatocellular Carcinoma

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Abstract. *Background: High ATP-binding cassette (ABC) protein expression leads to intrinsic drug resistance of hepatocellular carcinoma (HCC). The aim of this study was to investigate the potential chemosensitizing effects of sorafenib on the multi-drug resistance (MDR) phenotype. Material and Methods: The ABC-protein gene expression and the cellular survival were determined by RT-PCR analysis and MTT assay in HUH7 cells. Results: Sorafenib inhibits MDR. The ABC-protein mRNA expression decreased by up to 51% ($p \leq 0.01$). Addition of sorafenib to conventional chemotherapy restored the chemosensitivity. Combination of gemcitabine plus sorafenib decreased the ABC-protein mRNA levels by up to 77%, compared to gemcitabine monotherapy ($p \leq 0.001$). Doxorubicin plus sorafenib decreased the ABC-protein mRNA levels up to 74% compared to doxorubicin monotherapy ($p \leq 0.001$). Conclusion: This study provides evidence that the MDR phenotype of HCC cells can be modulated by the multi-kinase inhibitor sorafenib and consequentially may lead towards personalized therapies in patients with highly resistant tumors.*

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related deaths globally and its incidence is increasing (1). Around 30-40% of HCC patients are diagnosed at an early stage of the disease and are amenable to curative treatment options such as hepatic resection and liver transplantation (2). However, the majority of patients present with advanced-stage tumors and have a median survival of approximately six months (2-4). The results of conventional systemic chemotherapy for HCC are disappointing. None of the available clinical trials reported benefits in terms of survival, and tumor response rates were well below 10% (5, 6). High intrinsic resistance against structurally and functionally unrelated cytostatic drugs has been previously demonstrated in HCC (7, 8). Multi-drug resistance (MDR) in HCC is mediated by an increased cellular efflux of cytostatic compounds *via* transmembrane ATP-binding cassette proteins (MDR proteins) (9, 10). Physiologically, these proteins modulate the absorption, distribution and excretion of endo- and xenobiotics as well as of various pharmacological compounds in the liver (11-13). However, the overexpression of MDR proteins is associated with impaired overall survival and an aggressive tumor phenotype in primary liver cancer (14-16). The interaction of intracellular signaling pathways and MDR has been discussed with regard to various tumor entities (16, 17). Previously, the involvement of the epidermal growth factor-activated tyrosine kinase pathway has been demonstrated in the regulation of multi-drug resistance in HCC. Selective inhibition of the epidermal growth factor receptor (EGFR) restored the chemosensitivity of resistant HCC cells and increased the efficacy of conventional chemotherapy (18, unpublished data). However, there is further evidence that the downstream kinases of the RAF/MEK/ERK pathway might have a direct impact on the drug resistance phenotype (15-17). The tyrosine kinase inhibitor sorafenib is the current standard of care in patients with advanced HCC and has shown significant survival benefits (19). Combination treatment strategies of sorafenib and conventional chemotherapy showed promising results and acceptable toxicity in phase I clinical trials (20, 21).

The purpose of this *in vitro* study was to investigate the effects of tyrosine kinase pathway inhibition on the MDR phenotype in experimental HCC. Sorafenib was evaluated for its potential as part of a chemosensitizing combination treatment in tailored therapies for patients with highly resistant tumors.

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Materials and Methods

HCC cell line. The human HCC cell line HuH-7 (gift from Ingrid Herr, Division of Molecular Onco-Surgery, Ruprecht-Karls-University, Heidelberg, Germany) was used for *in vitro* experiments and cultured in DMEM (PAA Laboratories GmbH, Pasching, Austria) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (Biochrom AG, Berlin, Germany) in 5% CO_2 at 37˚C (22, 23).

Chemotherapeutic treatment. Gemcitabine (Lilly, Indianapolis, USA), doxorubicin (Sandoz Pharmaceuticals GmbH, Holzkirchen, Germany) and sorafenib (Bayer Healthcare, Leverkusen, Germany) were prepared according to the manufacturer's instructions. Cells were treated as follows: untreated controls, gemcitabine (11.4 μg/ml, 114 μg/ml or 1140 μg/ml) twice weekly, doxorubicin (0.015 μg/ml, 0.15 μg/ml or 1.5 μg/ml) twice weekly, or sorafenib 5 μM twice weekly. For the evaluation of potential combinative effects, gemcitabine and doxorubicin were combined with sorafenib 5 μM twice weekly at the above-mentioned doses.

MTT assay. Cells were treated as mentioned above and MTT assay was performed by adding 10 μl of 5 mg/ml 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl-2*H*-tetrazoliumbromide. The plates were incubated for 4 hours at 37˚C, 5% CO_2 and the resulting formazan crystals dissolved in 100 μl 2-propanol (Carl Roth GmbH, Karlsruhe, Germany). The extinction was measured at 570 nm with a reference wavelength of 620 nm with the Anthos Reader 2010 (Anthos Mikrosysteme GmbH, Krefeld, Germany). A minimum of three independent experiments were performed. The survival of cells was compared to the survival of the untreated controls, which was defined as 100%.

RT-PCR. Total RNA was isolated with the RNeasy mini kit (Qiagen, Hilden, Germany) and cDNA generated with the transcriptor first strand cDNA synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. The StepOne Real-Time PCR System (Applied Biosystems, Carlsbad, USA) was used for semi-quantitative analysis with Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, USA) and QuantiTect Primer Assay (Qiagen, Hilden, Germany) for *GAPDH* (QT01192646) as endogenous control, *PGP* (QT00081928), *MRP1* (QT00061159), *MRP2* (QT00056294) and *MRP3* (QT00070602). The PCR was performed after 10 min of denaturation at 95˚C for 40 cycles with 15 s of denaturation at 95˚C and 1 min annealing and polymerization at 60˚C. The melting curve was analyzed for unspecific products or primer dimers and a 1:10 dilution series over at least five orders of magnitude revealed PCR efficiency between 89% and 109%. Data were analyzed using the comparative Ct method in the StepOne Software 2.1 (Applied Biosystems, Carlsbad, USA). All samples were analyzed in triplicate and a minimum of three independent experiments were performed.

Statistics. The one-way ANOVA test followed by a Tukey test was used to reveal significant differences between the control and treatment groups. The level of statistical significance was defined as $p \leq 0.05$. All statistical computations were performed in SigmaStat 2.03 (Jandel Scientific, San Rafael, CA, USA). The MTT assay results are presented as mean values and standard error of mean (SE), the results of PCR as mean values and 95% confidence interval.

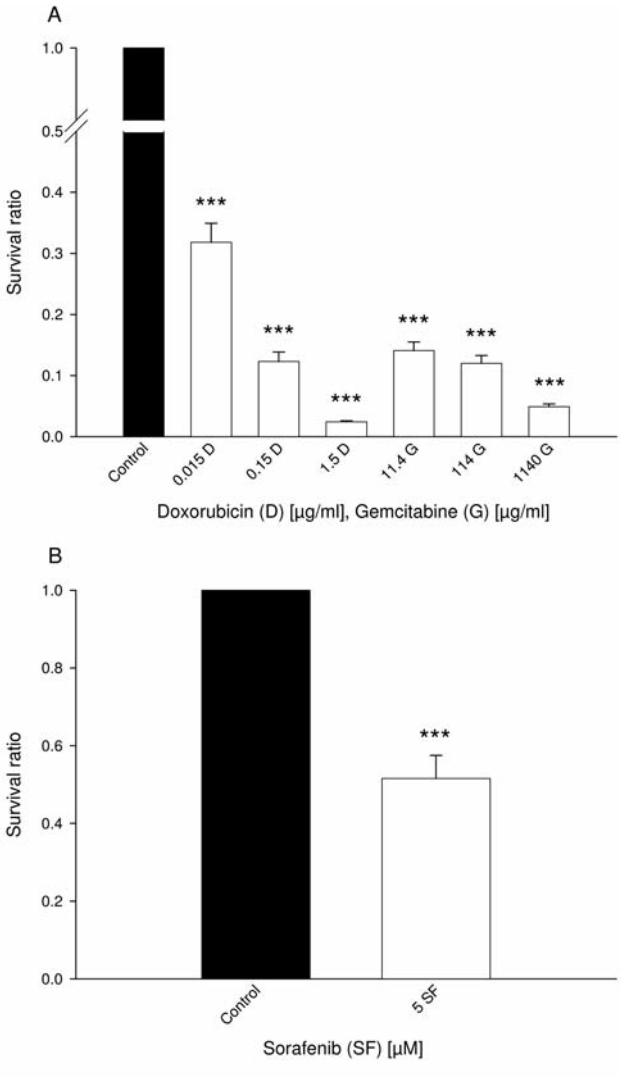
Results

Impact of conventional chemotherapeutic and molecular targeted treatment on cellular survival. Conventional chemotherapy, as well as targeted-molecular therapy with sorafenib, significantly inhibited the survival of HuH-7 compared to the untreated controls. Gemcitabine monotherapy, at concentrations of 11.4 mg/ml, 114 mg/ml and 1140 mg/ml, reduced the survival significantly by 86%, 88% and 95%, respectively, compared to the control group ($p \leq 0.001$). Doxorubicin, at concentrations of 0.015 mg/ml, 0.15 mg/ml and 1.5 mg/ml, revealed a dose-dependent survival decrease by 68%, 88% and 98%, respectively, compared to the untreated controls ($p \leq 0.001$) (Figure 1A). Sorafenib monotherapy reduced the cellular survival by 48% compared to the control group ($p \leq 0.05$) (Figure 1B). DMSO, which was used as a solvent for sorafenib, did not influence the cellular survival (data not shown).

Combinative treatment of conventional chemotherapy plus sorafenib also inhibited the survival of HuH-7 cells significantly compared to the untreated controls. The survival rate decreased by 83%, 86% and 93%, respectively, after combinative treatment with gemcitabine at 11.4 mg/ml, 114 mg/ml or 1140 mg/ml plus sorafenib compared to the controls. Furthermore, a dose-dependent survival decrease of 71%, 88% and 98%, respectively, was detectable after treatment with a combination of doxorubicin at 0.015 mg/ml, 0.15 mg/ml or 1.5 mg/ml plus sorafenib compared to the untreated controls (Figure 1C). However, the inhibitory effect on the cellular survival of the combinative treatment was not significantly different from the effects of monotherapy with gemcitabine or doxorubicin (data not shown).

Induction of MDR protein gene expression by conventional chemotherapy. As described previously (18, unpublished data), conventional chemotherapy induced *MDR* in HUH7 cells. After treatment with gemcitabine, a significant dose-dependent increase of MDR protein gene expression was detectable compared to the controls ($p \leq 0.05$). The *PGP, MRP1, MRP2* and *MRP3* mRNA expression increased two-, five-, four- and ten-fold, respectively, compared to the control group (Figure 2A). Doxorubicin led to enhanced mRNA detection compared to controls ($p \leq 0.05$). The levels of *PGP, MRP1, MRP2* and *MRP3* mRNA increased in a dose-dependent manner by up to five-, three-, four- and eight-fold, respectively, compared to the controls (Figure 2B).

Inhibition of MDR protein gene expression by sorafenib. Sorafenib monotherapy reduced the expression of *PGP* and *MRP2* mRNA significantly. The mRNA expression of *PGP* and *MRP2* was 36% ($p \leq 0.01$) and 51% lower compared to the control group ($p \leq 0.0001$) (Figure 3).



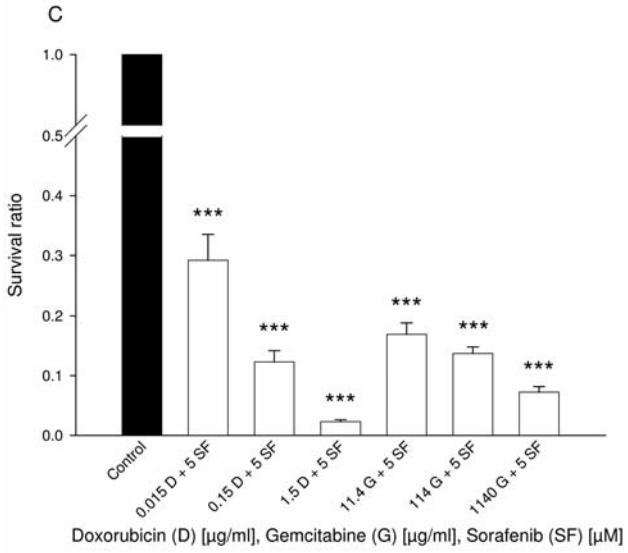


Figure 1. *Impact of conventional chemotherapeutic and molecular targeted treatment on cellular survival. A: Conventional chemotherapy reduces the survival of HuH7 cells. Gemcitabine (11.4 µg/ml, 114 µg/ml or 1140 µg/ml) or doxorubicin (0.015 mg/ml, 0.15 µg/ml or 1.5 µg/ml) were added to HuH7 cells twice weekly. B: Sorafenib inhibits the survival of HuH7 cells. Sorafenib 5 µM was added to HuH7 cells twice weekly. C: Combinative treatment of conventional chemotherapy and sorafenib reduces the survival of HuH7 cells. Gemcitabine (11.4 µg/ml, 114 µg/ml or 1140 µg/ml) or doxorubicin (0.015 mg/ml, 0.15 µg/ml or 1.15 µg/ml) plus Sorafenib 5 µM were added to HuH7 cells twice weekly. ***p≤0.001 Compared to the untreated control group.*

Effects of combined conventional and molecular targeted therapy on MDR gene expression. Simultaneous treatment with conventional chemotherapy plus sorafenib significantly reduced the MDR protein gene expression. The combination of gemcitabine (11.4 mg/ml) plus sorafenib reduced the *PGP, MRP1, MRP2* and *MRP3* mRNA levels by 75%, 67%, 59% and 77%, respectively, compared to monotherapy with gemcitabine ($p \leq 0.001$) (Figure 4A). Doxorubicin (0.15 mg/dl) plus sorafenib reduced the *PGP, MRP1, MRP2* and *MRP3* mRNA levels by 47%, 74%, 44% and 53%, respectively, compared to monotherapy with doxorubicin ($p \leq 0.001$) (Figure 4B).

Discussion

Hepatocellular carcinoma is one of the most intrinsically resistant tumors and conventional chemotherapy has shown only minor effectiveness, with response rates below 10% (5, 24). Individually designed treatment strategies to overcome MDR in HCC are being progressively investigated (25, 26). This study provides evidence that the gene expression of MDR proteins can be modulated by the multi-kinase inhibitor sorafenib and implicates the involvement of the RAF/MEK/ERK pathway in MDR regulation in HCC.

As previously described, standard chemotherapy induces MDR in HCC cell lines by increased ATP-binding cassette protein gene expression and, consequently, increased survival of drug-resistant cells (18, unpublished data). One new approach towards the abrogation of tumor-cell resistance to treatment-induced growth inhibition and cell death might be the combination of molecular-targeted and conventional systemic therapies. Several studies have implicated an involvement of tyrosine kinase-mediated signaling in the development of the chemoresistance phenotype in tumor cells (14, 27, 28). However, although reduced mRNA expression of *MRP1* has been described after ERK inhibition in HepG2 cells, data for HCC are scarce (16). To date, two potential mechanisms for the modulation of drug resistance by tyrosine kinase inhibitors have been discussed. Firstly, a direct interaction of tyrosine kinase inhibitors with the transporter and secondly, an influence of tyrosine kinase inhibitors on the expression level of MDR proteins, have been hypothesized (29). It has been reported that sorafenib itself is only a weak PGP substrate *in vitro* and *in*

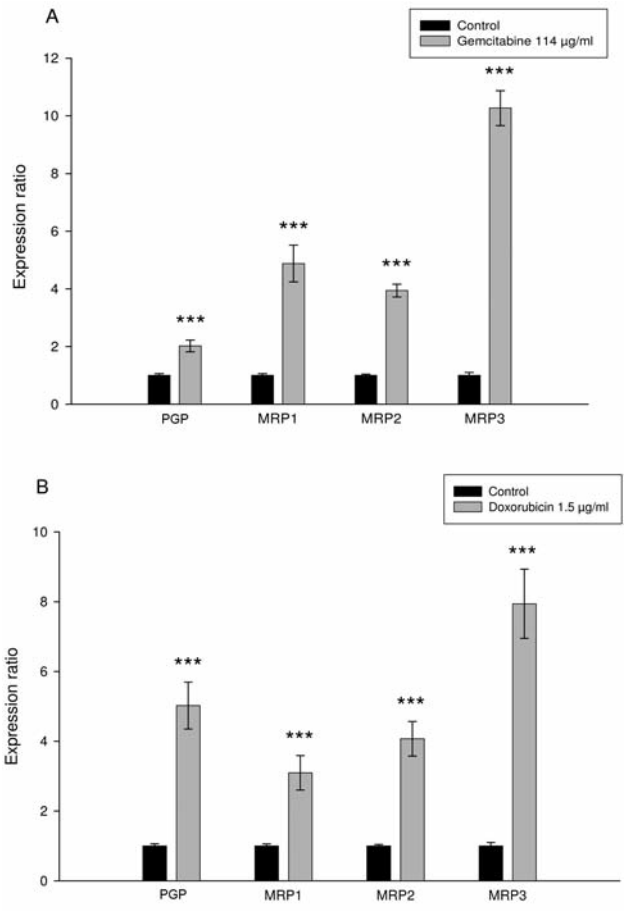


Figure 2. *Induction of MDR protein gene expression by conventional chemotherapy. A: Gemcitabine (114 µg/ml) was added to HuH7 cells twice weekly. B: Doxorubicin (1.5 µg/ml) was added to HuH7 cells twice weekly. ***p≤0.001 Compared to the untreated control group.*

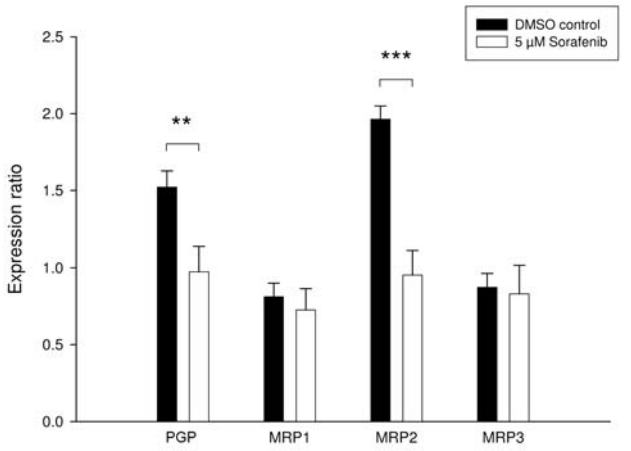


Figure 3. *Inhibition of MDR protein gene expression by sorafenib. Sorafenib 5 µM was added to HuH7 cells twice weekly. **p≤0.01, ***p≤0.001 compared to the DMSO control group.*

vivo (30). However, a reduction of the ATP-dependent efflux function of ATP-binding cassette proteins, as found after treatment with the tyrosine kinase inhibitors gefitinib and vandetanib, has not yet been described (29).

Data presented in this study clearly show that sorafenib monotherapy significantly reduced the expression of ATP-binding cassette protein mRNA in HuH7 cells. The *PGP* and *MRP2* mRNA expression decreased by 36% and 51%, respectively. The multi-kinase inhibitor sorafenib strongly inhibits VEGFR, RAF isoforms and reduces the phosphorylation of ERK and MEK1/2 (21, 31). The potential link between the inhibitory effects of sorafenib on drug resistance can therefore either be the result of receptor inhibition or modulation of the down-stream kinases. There is evidence that RAF-1 plays a role in drug resistance acquisition by prolongation of cellular survival *via* anti-apoptotic MEK/ERK-dependent mechanisms (32). Furthermore, increased resistance was found in MCF-7 breast cancer cells which overexpress active RAF-1. An effect of activated RAF-1 kinase on the *PGP* gene promoter has been described previously (33, 34). Additionally, VEGF seems to play a role in the drug-resistance phenotype. A simultaneous increase of PGP and VEGF expression was associated with a more aggressive and invasive phenotype of resistant laryngeal carcinoma cells (35).

Interestingly, in the present study, combinative treatment with conventional chemotherapy and sorafenib restored the chemosensitivity and inhibited the survival of HuH-7 cells significantly. The ABC-transport protein gene expression was found to be significantly lower after treatment with sorafenib plus gemcitabine or doxorubicin compared to monotherapy with these cytostatics. This supports the observation of the potentially chemosensitizing effects of sorafenib in phase I trials of patients with advanced refractory solid tumors. Combination treatment strategies of sorafenib and conventional chemotherapy have previously shown promising results and an acceptable toxicity (21, 36). Abou-Alfa *et al*. demonstrated in their phase II trial a significant prolongation of survival in patients treated with the combination of sorafenib and doxorubicin (37). Furthermore, Zhu *et al*. investigated the effects of conventional chemotherapy, targeting the vascular endothelial growth factor pathway with bevacizumab in a phase II trial involving patients with advanced HCC. The combination had moderate antitumor activity and lead to a median progression-free survival of 5.3 months (26).

However, despite the significant effects on ATP-binding cassette protein gene expression that were found in the present study, no additive or super-additive effect on the cellular survival was detectable in comparison to chemo-monotherapy. One reason for this might be the activation of cellular escape mechanisms and up-regulation of other

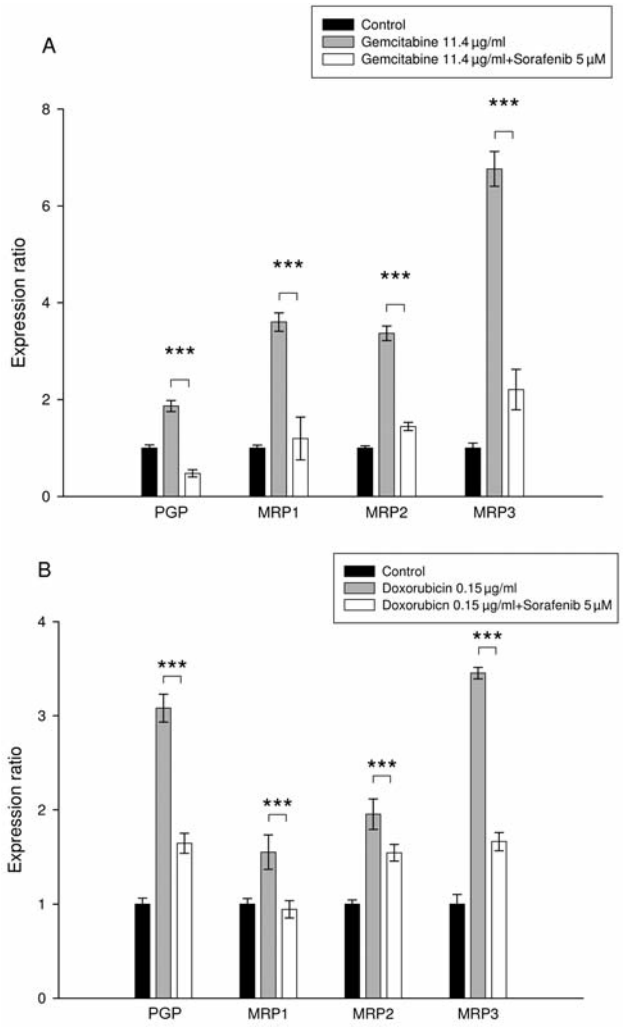


Figure 4. *Effects of combining conventional and molecular-targeted therapy on MDR gene expression. A: Gemcitabine (11.4 µg/ml) plus sorafenib 5 µM were added to HuH7 cells twice weekly and cells cultured for two weeks. ***p≤0.001 Compared to gemcitabine monotherapy. B: Doxorubicin (0.15 µg/ml) plus sorafenib 5 µM were added to HuH7 cells twice weekly and cells cultured for two weeks. ***p≤0.001 Compared to doxorubicin monotherapy.*

resistance pathways, such as alterations in the cell cycle and increased repair of DNA damage (38). Previously, it has been shown that the anti-tumoral efficiency of growth factor receptor targeting is counteracted in HCC cells due to the ability of alternative stimulation of signaling pathways (39).

The multi-kinase inhibitor sorafenib is the current gold standard for treating advanced HCC. The present study provides evidence that the gene expression of ATP-binding cassette proteins can be modulated by sorafenib and also suggests the involvement of the RAF/MEK/ERK pathway in regulating MDR in HCC. Further studies are warranted to identify the signaling pathways involved in the enduring restoration of chemosensitivity to maximize the anti-tumoral effects. Nevertheless, the combination of biologically targeted therapies and established chemotherapeutic approaches might have important implications for the development of tailored treatment strategies in patients with highly resistant hepatocellular carcinomas.

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