의학박사 학위논문

간암 환자의 sorafenib 내성 메커니즘에서 Raf-1 Kinase 억제 단백의 downregulation

Downregulation of Raf-1 kinase inhibitory protein as a sorafenib resistance mechanism in hepatocellular carcinoma cell lines

울산대학교 대학원

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최 광 현

Downregulation of Raf-1 kinase inhibitory protein as a sorafenib resistance mechanism in hepatocellular carcinoma cell lines

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이 논문을 의학박사 학위 논문으로 제출함

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Abstract

Introduction: Although sorafenib enhances overall survival, sorafenib resistance has been reported to be a significant limiting factor for improved prognosis in patients with hepatocellular carcinoma (HCC). Therefore, it is important to identify the mechanism of sorafenib resistance. This study aimed to identify the causative factor of sorafenib resistance and suggest methods for overcoming it.

Materials and methods: The sensitivity to sorafenib was compared in human HCC cell lines and patient-derived HCC primary cells. Based on its cytotoxicity, signaling pathways altered by sorafenib and the causative factors were examined through assays. The mechanism by which sorafenib modified the sorafenib-resistance inducer through gene or protein expression or stability was also investigated. We also designed a treatment option to overcome sorafenib resistance.

Results: Sorafenib activated the Raf/MEK/ERK pathway and caused sorafenib resistance in HCC cell lines and patient-derived HCC primary cells. Sorafenib reactivated the MAPK pathway by down-regulating RKIP at the post-translational level. Knockdown of RKIP (Raf kinase inhibitory protein) increased phosphorylated ERK and thus suppressed sorafenib-mediated cell death. We also found that sorafenib-reactivated ERK maybe an attractive target for second-line therapy for patients with sorafenib resistance. Sequential combination treatment with sorafenib and PD98059 significantly reduced the viability and proliferation of sorafenib-resistant cells, while their increasing apoptosis efficacy.

Conclusions: Our findings suggested that reactivation of the Raf/MEK/ERK pathway through aberrant expression of RKIP is one of the mechanisms behind sorafenib resistance in HCC. Sequential combination treatment with sorafenib and PD98059 could provide a new strategy to overcome sorafenib resistance in future clinical studies.

Keywords: Hepatocellular carcinoma, sorafenib, resistance, Raf-1 kinase inhibitory protein, sequential treatment

Abbreviations

CHX, cycloheximide; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; HCC, hepatocellular carcinoma; IP, immunoprecipitation; MEM, Modified Eagle Medium; PEBP, phospholipid binding protein; RKIP, Raf kinase inhibitory protein; RT, reverse transcription

Index

Figure index

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide and the third leading cause of cancer mortality (1). Sorafenib, a multikinase inhibitor, was the first approved systemic therapeutic agent showing significant survival benefit compared with placebo and is still widely used. However, its efficacy is very limited and is only based on cytostatic rather than cytotoxic effects in the majority of patients (2, 3). To improve the prognosis for patients with advanced HCC, new therapeutic agents having improved efficacy compared to sorafenib or overcoming sorafenib resistance are highly needed. Elucidation of the sorafenib resistance mechanism in HCC is a reasonable first step in the development of these new agents. Previous studies have suggested that crosstalk involving PI3K/Akt and JAK/STAT pathways (4), hypoxia-inducible pathways (5), induction of epithelial mesenchymal transition (6) and alteration of glucose metabolism (7) could be associated with sorafenib resistance, but these data are not sufficient to understand the sorafenib resistance observed in HCC. Since sorafenib resistance in HCC is a complicated multistep process involving multiple carcinogenesis pathways, additional data are required.

Raf kinase inhibitory protein (RKIP) is a highly conserved small cytosolic protein, characterized as a phospholipid binding protein (PEBP) (8, 9). RKIP is expressed in various tissues and has been identified as an inhibitor of the Raf/MEK/ERK pathway (10, 11) and a suppressor of metastasis (12). RKIP has been reported to regulate the Raf/MEK/ERK

pathway through direct interaction with both Raf-1 and MEK. It consequently disrupts the interaction of Raf-1 and MEK and inhibits the downstream MAPK signaling cascade (12). Aberrant RKIP expression may be a critical process in the development and aggressiveness of HCC (13). In 90% of HCCs, the Raf/MEK/ERK pathway is activated and on the contrary, RKIP expression is markedly reduced. Furthermore, reduction of RKIP expression is significantly correlated with vascular invasion, poor differentiation, relapse, and poor overall survival in HCC patients (14).

In this study, we aimed to identify novel sorafenib resistance mechanisms of HCC *in vitro*. We found that sorafenib regulates RKIP expression and this altered RKIP induces sorafenib resistance in HCC cell lines. This is the first report showing the involvement of RKIP in sorafenib resistance in HCC and suggests it is a potential target to overcome this resistance.

Materials and methods

HCC cell lines and patient-derived primary cultured HCC cells

The human HCC cells, Huh7, HepG2, PLC/PRF/5, SNU449, SNU398, and SNU475, were obtained from the Korean Cell Line Bank. Hep3B cells were cultured in Modified Eagle Medium (MEM; Welgene, Gyeongsan-si, South Korea) and all other cell lines were cultured

in Dulbecco's Modified Eagle Medium (DMEM; Welgene, South Korea). Culture media was supplemented with 10% fetal bovine serum (FBS; GIBCO, Gaithersburg, MD, USA) and cells were at 37°C in humidified 5% CO_2.

We performed primary cell culture from HCC specimens resected from 20 patients newly diagnosed at Asan Medical Center, South Korea. All patients' tissues were obtained after receiving written informed consent. Approval for this study was obtained from the Institutional Review Board of Asan Medical Center (Permit Number: S2014-1412-0008/2014-0897). After removal of blood, the liver sample was excised, cut into small fragments, gently dispersed, and placed in HBSS containing 0.03% pronase, 0.05% type IV collagenase, and 0.01% deoxyribonuclease (DNase, from bovine pancreas) for 20 min at 37°C. Samples were then filtered through a 100 μm nylon filter (BD Falcon, Franklin Lakes, NJ, USA) and centrifuged at 50 ×*g* for 2 min at 4°C to obtain hepatocytes. The final cell suspensions were cultured onto collagen-coated T25 flasks (BD Falcon, USA) in F12/DMEM (Lonza, Walkersville, MD, USA), supplemented with 20% FBS, 1% non-essential amino acids, 1% glutamine, and 1% penicillin/streptomycin (GIBCO, USA) at 37°C in a humidified 5% CO_2 incubator. To assess hepatocyte markers, cells were fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) and then permeabilized. Primary antibodies against α-fetoprotein (Cell Signaling, Denver, MA, USA), albumin (Cell Signaling, USA), and Hep Par-1 (Dako, Glostrup, Denmark) were used as hepatocyte markers in patient-derived HCCs. Cells were then incubated with fluorochrome-conjugated

secondary antibodies. After staining with DAPI, fluorescence was measured using the high-content screening system, Operetta (Perkin-Elmer, Waltham, MA, USA).

Chemicals

Sorafenib and PD98059 were purchased from Selleckchem (Houston, TX, USA). Cycloheximide, MG132, and DMSO were from Sigma-Aldrich. DMEM and MEM media were obtained from Welgene. Fetal bovine serum (FBS), penicillin, and streptomycin were from Life Technologies, Inc. (USA).

Cell Viability

To examine the sensitivity of HCC cell lines and patient-derived HCC primary cells to sorafenib, cytotoxicity was measured using the MTS assay (Promega, Fitchburg, WI, USA). Cells were plated in 96-well plates at 2 x 10^3/well for each cell line. Cells were exposed to different concentrations of sorafenib for 72 h. Twenty microliters of MTS solution was added to each well containing 100 μL of culture medium and the cells were then incubated for 2 h at 37°C. Absorbance at 490 nm was measured using a Sunrise^{TM} microplate reader with Magellan^{TM} software (Tecan, Seestrasse 103, Männedorf, Switzerland). Viability was expressed as a percentage of viability in untreated cells. The concentration of sorafenib resulting in 50% growth inhibition (IC_{50}) was calculated using GraphPad Prism 5 software.

Trypan blue exclusion assay

The trypan blue exclusion assay was performed as described (15). The total death rate (%) = number of dead cells / (number of living cells + number of dead cells) x 100.

siRNA transfection

RKIP siRNA and scrambled RNA were designed and synthesized by Bioneer (Daejeon, South Korea). The human HCC cell line, HepG2, was transfected using virofectamine (Promega, USA) according to the manufacturer's instructions. Briefly, the cells were seeded at 5 x 10^5 cells into 60 mm^3 dishes. Transfection complexes were prepared with different concentrations of siRNA and transfection agent. After 20 min incubation, complexes were added directly to the cells. After 24 h of transfection, cells were treated with 5 or 10 μM sorafenib. Cell death rate was examined by cell counting and alteration of signaling pathways was analyzed by western blot.

mRNA extraction and RT-PCR

Total cellular RNA was extracted using TRIzol (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The isolated RNA was resuspended in RNAse-free water and RNA concentration was measured at 260 nm using a NanoDrop 2000 UV-VIS Spectrophotometer (Thermo Scientific, USA). Reverse transcription (RT) was carried out in a 20 μL reaction mixture using a first strand cDNA synthesis kit (Invitrogen,

Waltham, MA, USA) according to the manufacturer's instructions. RT-PCR was performed using AccuPower PCR premix (Bioneer, South Korea) with addition of first-strand cDNA via thermocycling in a Perkin-Elmer9600 thermal cycler (Perkin-Elmer). The RKIP primer sequences were as follows: 5′-ATGCCGGTGGACCTCAGC-3′ (sense) and 5′-GAGAGGACTGTGCCACTG-3′ (antisense).

Immunoblotting analysis

Sorafenib-mediated alteration of signaling pathways was examined by western blot analysis. Cells were lysed in RIPA buffer (InTron, Jungwon-gu, South Korea) supplemented with protease inhibitor (Sigma-Aldrich, USA). The resultant lysate was centrifuged at 12,500 rpm for 20 min at 4°C and supernatants were collected. The protein concentration was measured by BCA assay (Promega, USA). After SDS-PAGE and transfer, membranes were blocked with 5% non-fat milk for 1 h and incubated with primary antibodies overnight at 4°C, and then incubation with secondary antibodies (Cell Signaling, USA) for 1 h at room temperature. Target protein bands were detected using ECL reagents (GE, Fairfield, CT, USA). The following antibodies were used: anti-pAkt (1:2000 dilution; Cell Signaling), anti-pERK (1:2000 dilution, Cell Signaling), pSTAT3 (1:1000 dilution, Cell Signaling), cleaved caspase 3 (1:1000 dilution, Cell Signaling), PARP (1:1000 dilution, Cell Signaling), RKIP (1:2000 dilution, Upstate, Lake Placid, NY, USA), and β-actin (1:10000 dilution, Sigma-Aldrich).

RKIP protein turnover studies

RKIP protein stability was determined in SNU449 cells in the presence or absence of the protein synthesis inhibitor, cycloheximide (CHX, Sigma-Aldrich, USA). Approximately 1 x 10^5 cells were plated into 60mm^3 plates and 24 h later, 10 μM sorafenib and/or CHX was added. After treatment with CHX, cells were harvested by centrifugation at 2,000 rpm for 5 min at indicated time intervals. Cells were lysed in RIPA buffer and the supernatant collected as described above. To assess RKIP turnover, western blot analysis was performed using anti-RKIP antibody.

Immunoprecipitation (IP)

To examine proteasomal degradation of RKIP, immunoprecipitation (IP) studies were performed. Twenty-four hours after seeding, cells were treated with 10 μM MG132 for 2 h and then treated with sorafenib for 48 h. Cells were harvested, washed with PBS, and lysed in RIPA buffer. The supernatant was obtained by centrifugation at 13,000 rpm for 20 min. After determination of protein concentration, 10 μg of antibody against ubiquitin (Santa Cruz Biotechnology, Minneapolis, MN, USA) was added to 500 μg of supernatant and the mixture was gently rotated at 4°C overnight. Protein A/G-agarose (Santa Cruz Biotechnology, USA) was then added and incubations were continued for an additional 4 h. The protein A/G agarose was collected by centrifugation and beads were washed three times with PBS.

Protein A/G-agarose beads were then suspended in 5x SDS sample buffer and heated for 5 min. Immunoprecipitated proteins were analyzed by SDS-PAGE using anti-RKIP antibody.

Results

Sorafenib mediated ERK reactivation caused sorafenib resistance to HCC cell lines

To validate the effect of sorafenib, we first examined its cytotoxic effect on multiple human HCC cell lines as a single agent. The inhibitory effect of sorafenib on cell proliferation was measured by MTS assay. Different sensitivities to sorafenib were observed in the HCC cells we examined. With the exception of two cell lines, HepG2 and Huh7, all cells exhibited higher IC_{50} values for sorafenib than would be clinically applicable. HepG2 and Huh7 cells were sensitive to sorafenib (Fig. 1A), exhibiting IC_{50} values of 2.4μM and 4.8μM, respectively. We determined the sorafenib sensitivity cut-off value based on IC_{50} values. Sorafenib inhibits various receptor tyrosine kinases (RTKs) and as a result, inhibits the MAPK pathway, where activated RTK signaling mostly converges. Based on this, the sensitivity of HCC cell lines to sorafenib can be examined through inhibition of the MAPK pathway, especially ERK. According to Fig. 1B, most HCC cell lines we examined showed pERK inhibition by sorafenib. SNU449 cells, which has a higher sorafenib IC_{50} (12.8μM), exhibited ineffective inhibition of pERK. Based on these results, we used HepG2 and

SNU449 as representative HCC cell lines for sorafenib sensitivity and resistance, respectively. The sorafenib-mediated cell death rate in HepG2 and SNU449 cells was confirmed by trypan blue exclusion assay. Cell death increased in a dose- and time-dependent manner in sorafenib-sensitive HepG2 cells, but not in sorafenib-resistant SNU449 cells (Fig. 1C). Sorafenib caused apoptosis by induction of cleavage of caspase 3 and PARP1 in HepG2 cells, but not in SNU449 cells (Fig. 1D). To examine the altered signaling pathways responsible for differences in sorafenib sensitivity in HCC cell lines, we performed western blot analysis after sorafenib treatment (Fig. 1C). Phospho-STAT3, a known target for sorafenib, decreased in a dose- and time-dependent manner in both cell lines. The PI3K/Akt signaling pathway, which is activated by various RTKs, was suppressed by sorafenib in both cell lines, but to a different extent in each cell line. In contrast to the effect on the PI3K/Akt pathway, sorafenib influences the MAPK pathway in a different manner in HepG2 and SNU449 cells. Sorafenib decreased pERK levels in HepG2 cells in a dose- and time-dependent manner, but this inhibitory effect was not observed in SNU449 cells. Rather, pERK levels increased with increasing sorafenib concentration in SNU449 cells. Our data suggest that activation of pERK by sorafenib maybe one of the causative factors in sorafenib resistance.

Figure 1.

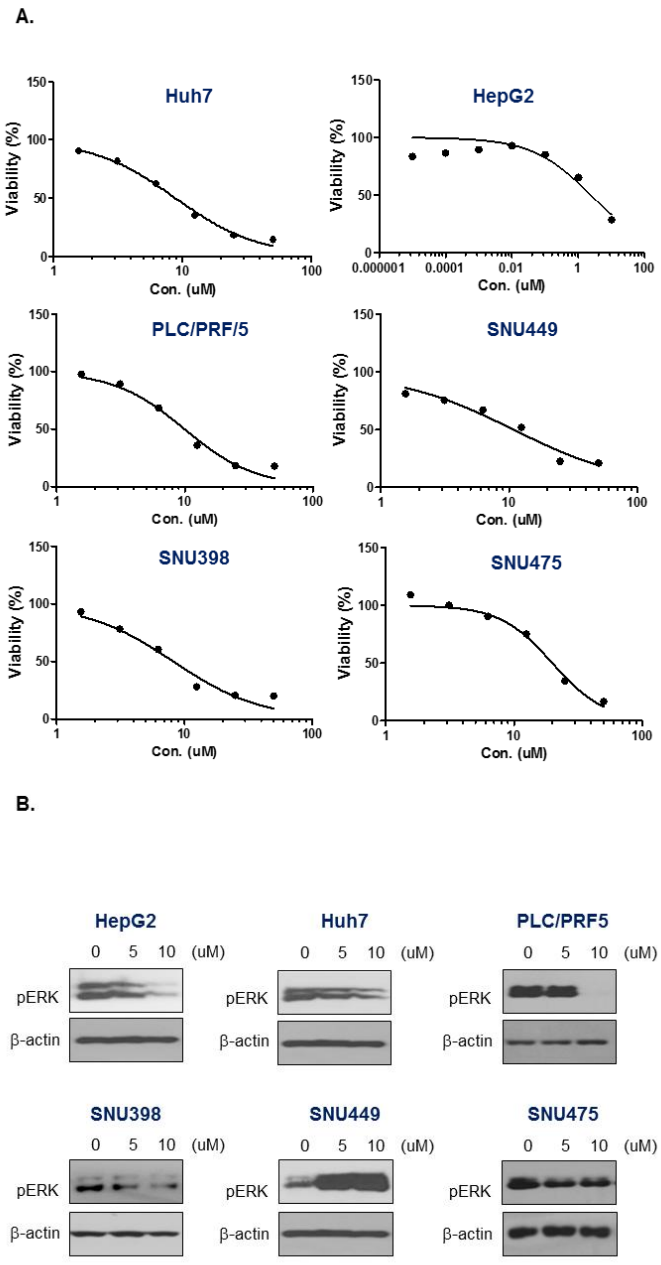
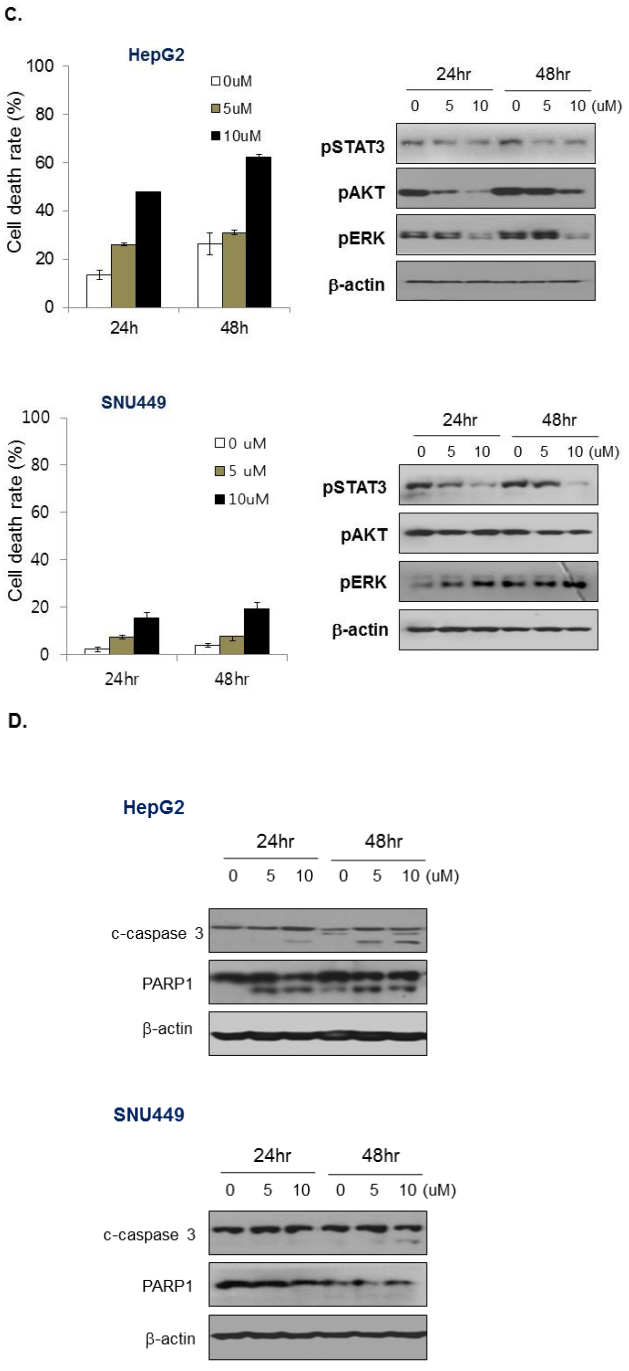


Figure 1. pERK reactivation is responsible for sorafenib resistance in HCC cell lines

(A) Sorafenib cytotoxicity was measured by MTS assay using various human HCC cell lines. (B) The change in pERK expression with sorafenib treatment was measured by western blot analysis using human HCC cell lines.



(C) Sorafenib-mediated cell death rate and altered signaling pathways were examined in HepG2 and SNU449 cells after treatment with sorafenib (5 or 10 μM) for the indicated time. Cell death rate was measured by trypan blue exclusion assay and alteration of signaling pathway was examined by western blot analysis. (D) Sorafenib induced apoptosis in HepG2 cells, but not in SNU449 cells. Sorafenib-induced apoptosis was measured by western blot analysis using cleaved caspase 3 and PARP1.

Sorafenib-mediated ERK reactivation is associated with down-regulation of RKIP expression

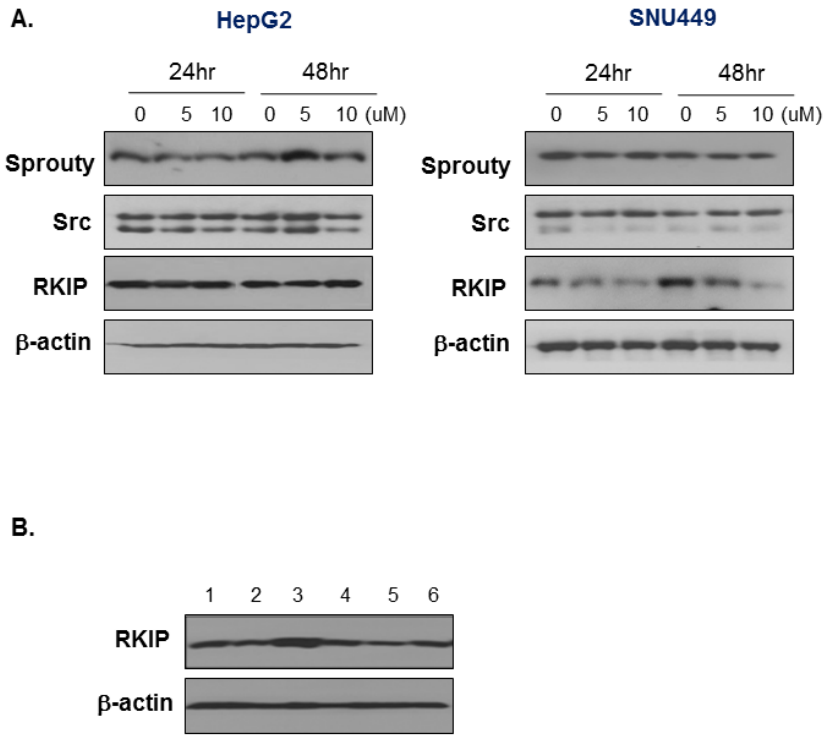
Sorafenib targets various RTKs including VEGFR, PDGFR, and IGFR, and also inhibits Raf kinase. As shown in Fig. 1C, sorafenib inhibited the PI3K/Akt pathway irrespective of sorafenib sensitivity, but the inhibitory effect of sorafenib distinctively influenced MAPK pathways in the two cell lines. This suggests that inefficient inhibition of the MAPK pathway in sorafenib-resistant HCC cell line may be the reason for the resistance. Thus, we hypothesized that sorafenib-mediated inhibition or reactivation of ERK may be due to aberrant regulation of Raf kinase. To examine how sorafenib influences the regulation of the Raf kinase-mediated MAPK signaling pathway, we examined molecules involved in the regulation of Raf kinase activity. As observed in Fig.2A, sorafenib did not affect the expression level of sprouty in either cell line (16). This suggests that the negative feedback between Raf kinase and ERK is independent of the ERK reactivation observed in sorafenib-treated SNU449 cells. We also examined the expression levels of Src, which directly phosphorylates Raf-1. However, the expression level of Src did not change with sorafenib treatment in either cell line. In contrast to Raf kinase and Src, sorafenib modified the expression of RKIP in both HCC cell lines differently. Surprisingly, RKIP expression did not change or slightly increased in HepG2 cells, despite a high concentration of sorafenib. By contrast, sorafenib decreased RKIP expression in SNU449 cells. Since RKIP is an important inhibitor of Raf-1 activity via direct interaction, down-regulated RKIP may be one of the

factors responsible for ERK activation. We also examined endogenous RKIP expression level to determine whether it can influence sorafenib sensitivity. However, there was no significant difference in the level of endogenously expressed RKIP between HCC cell lines (Fig. 2B). To determine whether RKIP plays a substantial role in sorafenib resistance through ERK reactivation, we constructed an RKIP siRNA and transfected it into the sorafenib-sensitive HepG2 cell line (Fig. 2C). RKIP knockdown by RKIP siRNA was confirmed by western blot analysis. RKIP-down-regulated HepG2 cells were less sensitive to sorafenib than HepG2 cells transfected with a scrambled siRNA. Forty-eight hours after sorafenib treatment, RKIP down-regulation in HepG2 cells greatly decreased sorafenib-mediated cell death. Similar to results in Fig. 2A, no change in RKIP expression or phosphorylation of ERK were observed with increasing sorafenib doses in scrambled siRNA-transfected HepG2 cells. On the other hand, knockdown of RKIP in HepG2 cells increased phosphorylation of ERK with sorafenib treatment, as seen in SNU449 cells. This suggests that alteration of RKIP expression is a key factor in inducing ERK reactivation and the resultant sorafenib resistance.

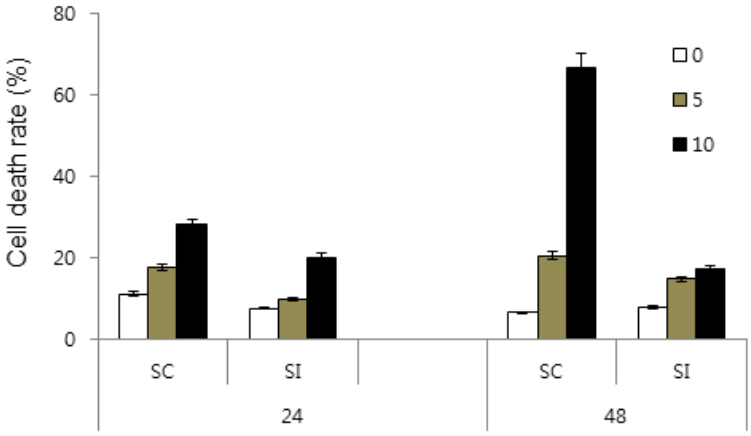
To determine whether the sorafenib-mediated alteration of RKIP expression could explain sorafenib resistance in HCC patients, we examined patient-derived primary cultured HCC cells. Cell death rate, ERK reactivation, and altered expression of RKIP were measured after sorafenib treatment. We obtained primary cultured HCC cells from patients and treated them with sorafenib. Figure 2D shows a representative data. Similar to results in HepG2 and

SNU449 cells, cells in which sorafenib had little or effect concurrently showed increased ERK phosphorylation and decreased RKIP expression. In contrast, cells that were more sensitive to sorafenib showed inhibition of pERK and up-regulated or unchanged RKIP expression. Consistent with data from established HCC cell lines, patient-derived primary cultured HCC cells that are less sensitive to sorafenib have reactivation of pERK, which may be mediated by RKIP down-regulation. According to our data, we propose that RKIP is related to sorafenib resistance through an increase in ERK activity.

Figure 2.



C.


Cell death rate (%)
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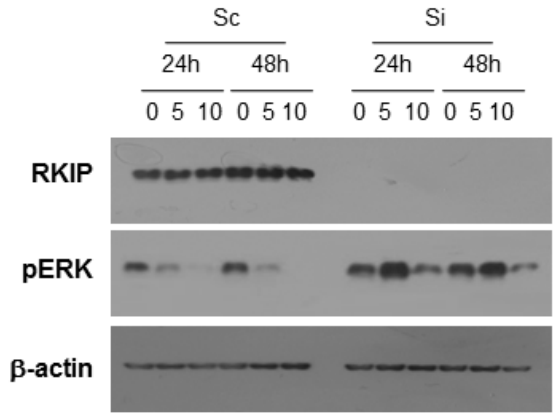
60
40
20
0
0
5
10
SC
SI
SC
SI
24
48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Si
24h
48h
24h
48h
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0 5 10 0 5 10
RKIP
pERK
β-actin

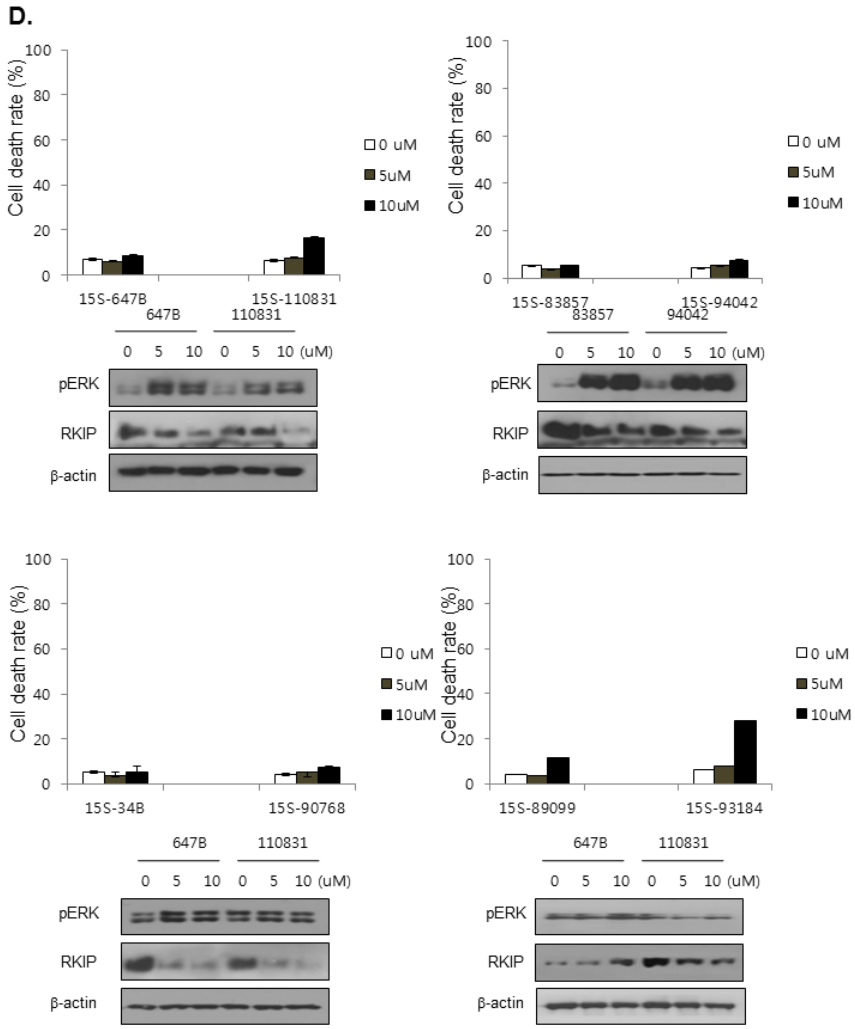


Figure 2. The modulation of RKIP expression determined sorafenib sensitivity in HCC

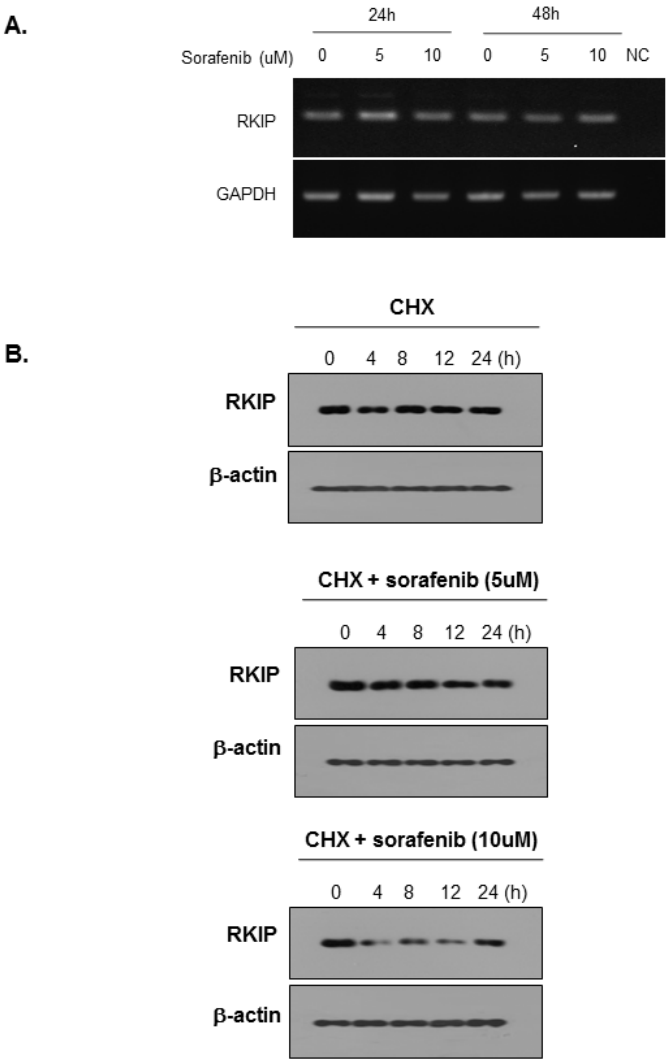
(A) HepG2 and SNU449 cells were treated with sorafenib and expression of sprout, src, and RKIP were examined by western blot analysis. (B) Endogenously expressed RKIP in human HCC cell lines was measured by western blot analysis. Lane 1; HepG2, lane 2; Huh7, lane 3; PLC/PRF/5, lane 4; SNU398, lane 5; SNU449 and lane 6; SNU475. (C) RKIP siRNA was transfected into HepG2 cells and then sorafenib-mediated cell death rate was measured by trypan blue exclusion assay. RKIP down-regulation and phosphorylation of ERK were measured by western blot analysis. (D) Patient-derived HCC primary cells were treated with sorafenib. Cell death rate was measured by trypan blue exclusion assay. pERK and RKIP expression were examined by western blot analysis.

Sorafenib regulates RKIP expression by modification of protein stability

We found that sorafenib-mediated ERK activation via decreased RKIP expression is responsible for sorafenib resistance in both established HCC cell lines and patient-derived primary HCC cells. Next, we studied the mechanisms sorafenib regulates the expression of RKIP, thus causing sorafenib resistance in HCC. To determine whether sorafenib down-regulates RKIP expression at the transcriptional level, the corresponding mRNA levels were measured by RT-PCR after sorafenib treatment (5 and 10 μM for 24h) of SNU449 cells. RKIP mRNA levels did not change with sorafenib treatment (Fig. 3A). Therefore, we next explored if sorafenib regulates RKIP expression at the translational level. To do this, we treated cells with cycloheximide (CHX), an inhibitor of *de novo* protein synthesis, and then with sorafenib for different times. RKIP protein levels were analyzed by western blot. As seen in Fig. 3B, CHX itself did not affect RKIP protein level at the indicated time intervals. However, addition of 10 μM sorafenib significantly abolished RKIP induction. This result indicates that sorafenib affects RKIP protein stability rather than its translation. To investigate whether sorafenib decreased RKIP expression by reducing stability, we examined proteasomal degradation of RKIP. To do this, we blocked proteasome-mediated protein degradation by treating cells with the proteasome inhibitor, MG132, and then followed with sorafenib treatment. RKIP was slightly increased with MG132 treatment, which suggests that RKIP is continuously turned over quickly, even without sorafenib. We also found that treatment with both MG132 and sorafenib increased RKIP expression with time and the

increased RKIP level was higher than with MG132 treatment only (Fig. 3C). After 6 h of treatment with MG132 and sorafenib, RKIP protein level increased until 48 h. This suggests that the decline in RKIP protein levels could be attributable to accelerated proteasomal degradation of RKIP protein after sorafenib treatment. We tested if sorafenib promotes the proteasomal degradation of RKIP using IP. According to IP results (Fig. 3D), endogenous turnover of RKIP occurred in the MG132-treated group and poly-ubiquitinated RKIP was significantly increased in cells treated with MG132 and sorafenib. This suggests that sorafenib increased poly-ubiquitinated RKIP and subsequently, reduced its expression, via proteasomal degradation. Taken together, these results indicate that sorafenib accelerates proteasomal degradation of RKIP and consequently a decline in RKIP protein level and associated increase in ERK activity.

Figure 3.


A.
24h
48h
Sorafenib (uM)
0
5
10
0
5
10
NC
RKIP
GAPDH
B.
CHX
0 4 8 12 24 (h)
RKIP
β-actin
CHX + sorafenib (5uM)
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β-actin

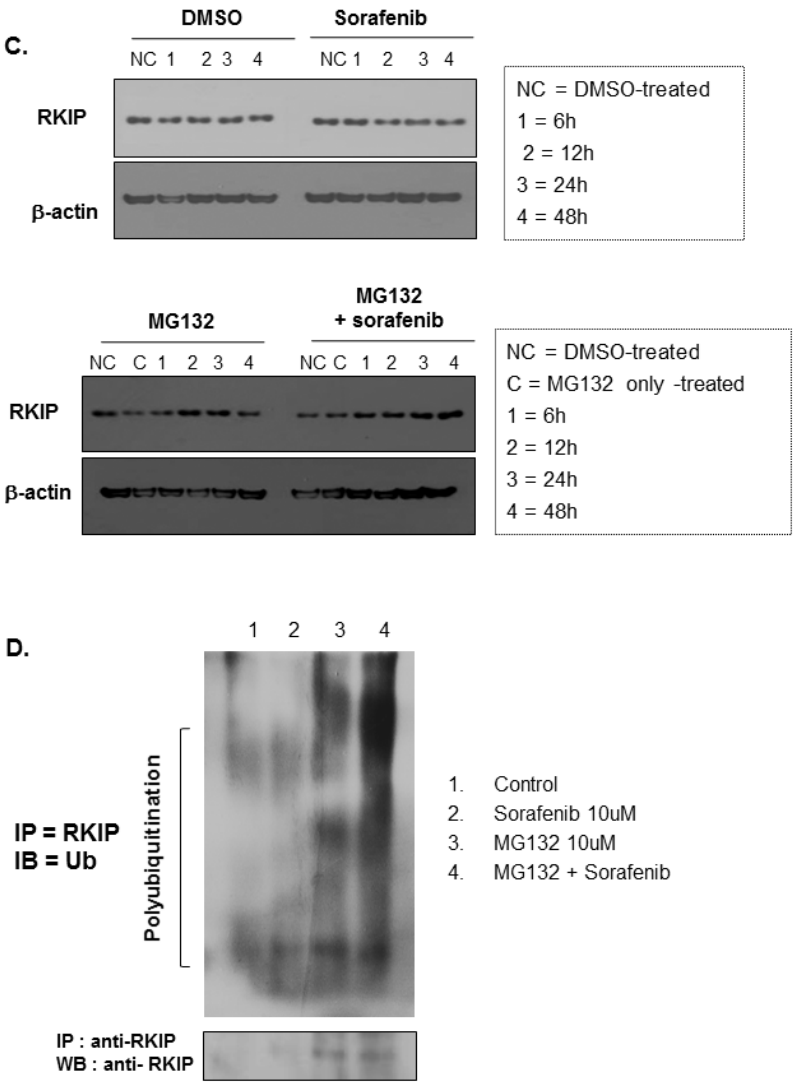


Figure 3. Sorafenib decreases protein stability of RKIP and down-regulates its expression by enhancing proteasomal degradation

(A) RKIP mRNA expression level was measured by RT-PCR after treatment with sorafenib for indicated times. (B) RKIP protein expression was examined after treatment with cycloheximide (CHX) at 10 μg/mL for 1h and then treated with sorafenib for indicated times. RKIP expression level was measured by western blot analysis. (C) Proteasomal degradation of RKIP was detected after treatment with10 μM MG132 for 2 h prior to treatment with sorafenib. (D) Poly-ubiquitinated RKIP was examined by immunoprecipitation using anti-Ubiquitin and anti-RKIP antibodies.

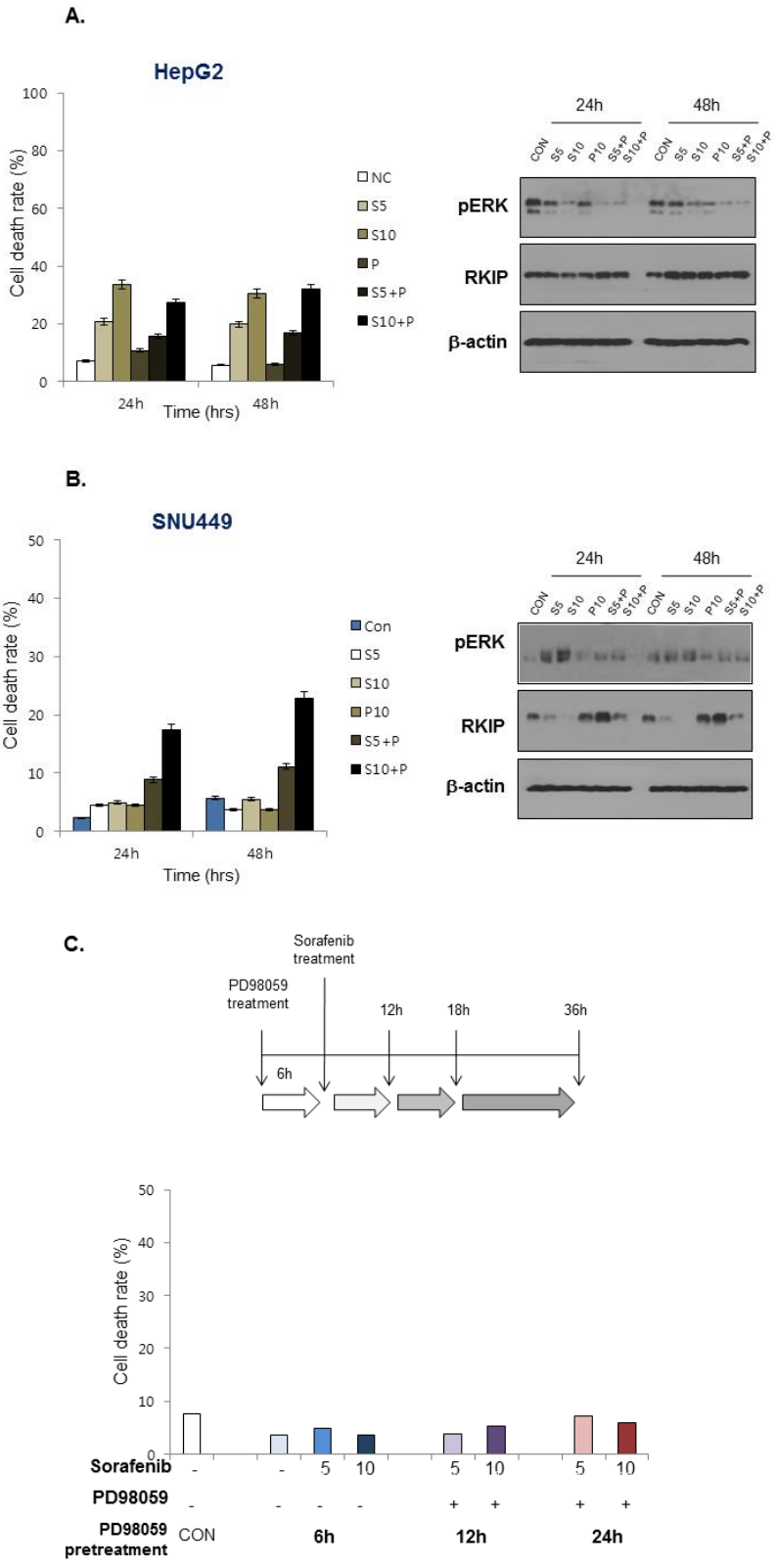
Pharmacological inhibition of reactivated ERK pathway overcomes sorafenib resistance in HCC

Our results showed that sorafenib reactivates ERK, resulting in resistance to sorafenib in HCC. Based on these results, we tested if the inhibition of reactivated ERK can overcome sorafenib resistance and consequently, enhance the therapeutic efficiency of sorafenib. To do this, we used the MEK/ERK inhibitor, PD98059, as a combination drug. Sorafenib and PD98059 were first used to concurrently treat HepG2 and SNU449 cells. In the sorafenib-sensitive HepG2 cells, the effect of sorafenib and PD98059 in combination was not different from that of sorafenib alone. After 48 h of treatment with sorafenib alone or combined with PD98059, cell death rate was 27% and 31%, respectively (Fig. 4A). In addition, inhibition of pERK was not different between HepG2 cells treated with sorafenib alone and cells undergoing the combined treatment. Sorafenib alone exerts a significant cytotoxic effect on HepG2 cells and thus, co-treatment with PD98059 did not increase the cytotoxicity of sorafenib. On the other hand, the combination effect of both drugs in sorafenib-resistant SNU449 cells differed from that of HepG2 cells (Fig. 4B). Treatment with PD98059 alone was not more effective than sorafenib alone. This can be explained by the fact that endogenous ERK activity in SNU449 cells is not high enough for the inhibitory effect of PD98059 to be evident. In contrast, treatment with sorafenib and PD98059 increased cell death of SNU449 cells to a greater extent than sorafenib or PD98059 alone. ERK reactivation induced by sorafenib was decreased by combined treatment with sorafenib and

PD98059 for 48 h. However, after 48 h of treatment, the inhibitory effect of the combined treatment was not maintained. Cell death rate decreased and down-regulated pERK was recovered to a similar level as cells treated with sorafenib alone (data not shown). This suggests that simultaneous treatment with sorafenib and PD98059 is not effective enough to overcome sorafenib resistance.

Based on these results, we hypothesized that the timing of ERK activity inhibition during sorafenib treatment could play a pivotal role in the growth inhibition of sorafenib-resistant HCC cell lines. Thus, we examined the effect of sequential treatment of sorafenib and PD98059 in SNU449 cells. When cells were treated first with PD98059 and then with sorafenib, we found no differences compared to cells treated with sorafenib alone (Fig. 4C). Since the Raf/MEK/ERK pathway is already inhibited by PD98059, this may preclude further inhibition of the MAPK pathway by sorafenib. In contrast, sorafenib treatment first, followed by PD98059 after 24 h, overcame sorafenib resistance significantly in SNU449 cells and the inhibitory effect was maintained for 96 h of treatment, in contrast to results of co-treatment with sorafenib and PD98059. Strikingly, cell death rate induced by sequential treatment of sorafenib then PD98059 reached almost40% after 96 h of treatment (Fig. 4D). As observed in our previous results, sorafenib-mediated ERK activation via RKIP degradation could be an Important mechanism of sorafenib resistance. Therefore, adding sequential treatment of a MEK/ERK inhibitor after sorafenib treatment maybe an effective therapeutic strategy to overcome sorafenib resistance in HCC patients.

Figure 4.


A.
HepG2
Cell death rate (%)
Time (hrs)
24h
48h
NC
S5
S10
P
S5+P
S10+P
CON
pERK
RKIP
β-actin
B.
SNU449
Con
P10
C.
Sorafenib treatment
PD98059 treatment
6h
12h
18h
36h
Sorafenib
PD98059
PD98059 pretreatment
CON
6h
12h
24h

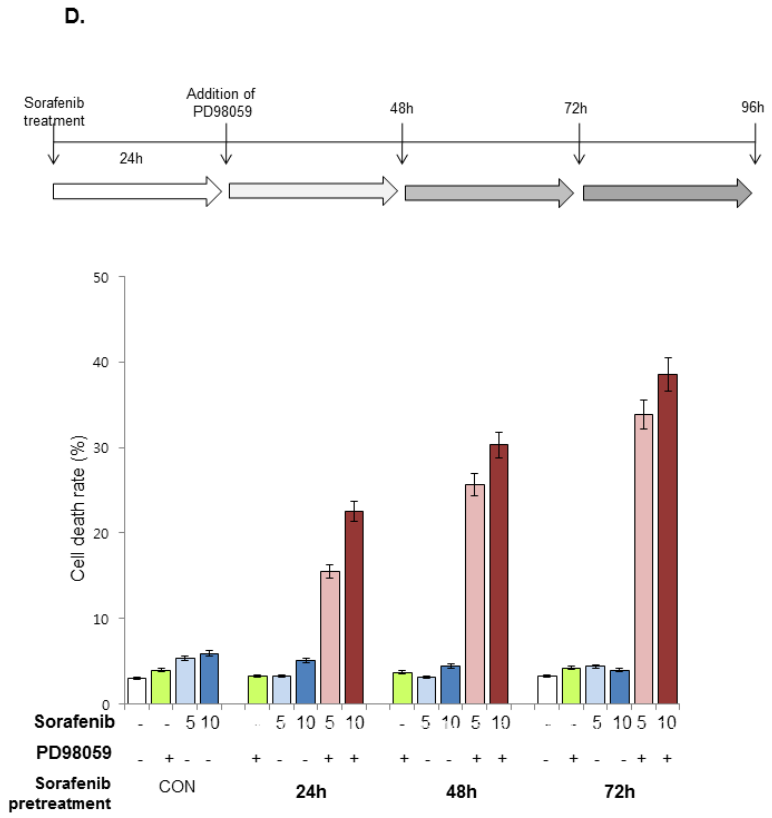


Figure 4. Sequential combination treatment with sorafenib and PD98059 can overcome sorafenib resistance

(A) Co-treatment of HepG2 cells (B) SNU449 cells with sorafenib and PD98059. Cell death rate was determined by trypan blue exclusion assay. CON, control; S5,5 μM sorafenib; S10, 10 μM sorafenib; P10, 10 μM PD98059; S5+P,co-treatment with 5 μM sorafenib and PD98059; and S10+P,co-treatment with10 μM sorafenib and PD98059. (C) Sequential treatment with PD98059 prior to sorafenib. After 24 h of treatment with PD98059, cells were treated with sorafenib for an additional 72 h. (D) Sequential treatment with sorafenib followed by PD98059. After 24 h of sorafenib treatment, cells were treated with PD98059 for an additional 72 h. Cell death rate after co-treatment and sequential treatment was measured by the Operetta system.

Discussion

HCC is one of the most common primary tumors with a high incidence and dismal outcome (17). Furthermore, HCC is frequently diagnosed at advanced stages and there are few curative therapeutic options for these patients. Many studies have aimed to develop effective HCC therapies based on alteration of signaling pathways or molecules known to have an important role in hepatocarcinogenesis. Sorafenib, a multikinase inhibitor, was the first FDA approved targeted therapy for advanced HCC (3) and enhanced overall survival in randomized phase III clinical trials (3, 18). Despite significant therapeutic improvement, the response rate of patients is low and a significant proportion of HCC patients show sorafenib resistance. Several mechanisms have been reported to be involved in sorafenib resistance, but the exact mechanism remains unclear (4). Therefore, there is a great need to identify the mechanisms of sorafenib resistance and develop ways to overcome this resistance. Previous studies demonstrated that endogenous pERK expression in HCC can be used as a predictor of the therapeutic response to sorafenib, with a higher activity of the Raf/MEK/ERK pathway being associated with greater sorafenib efficiency, despite contradictory results (19, 20). It has also been shown that a sorafenib-resistant cell line exposed to sorafenib for a long period has a simultaneous increase in PI3K/Akt and ERK pathway activity (21). There is no report stating that ERK activation by early sorafenib treatment can be the reason of sorafenib resistance in HCC. Our study is the first to demonstrate the involvement of ERK reactivation

in the mechanism of sorafenib resistance in HCC. In that process, decreased expression of RKIP acts as a key factor inducing ERK reactivation. Furthermore, we suggest that reactivated ERK maybe a potential target for sequential treatment after sorafenib treatment as second-line therapy in sorafenib-resistant HCC patients.

In this study, the inhibitory effect of sorafenib was examined in multiple human HCC cell lines. We observed that various HCC cell lines have different sensitivities to sorafenib. As shown in Figure 1, down-regulation of pERK was observed in HCC cells showing sensitivity to sorafenib, but not in sorafenib-resistant HCC cells. However, the inhibitory effect of sorafenib on other targets, such as the PI3K/Akt pathway and STAT3, was not different among HCC cell lines having different sorafenib sensitivities. These data show that the inhibitory effect of sorafenib on ERK activity is important for the efficacy of sorafenib.

There are several factors that influence ERK activity through the Raf/MEK/ERK signaling cascade. Among them, we examined factors that regulate Raf-1 activity, such as sprouty and Src (22-24) and found that RKIP expression was affected by sorafenib, but others were not. The decrease in RKIP expression was associated with an increase in ERK phosphorylation. The involvement of RKIP in sorafenib-mediated ERK reactivation was confirmed by modulation of RKIP expression using RKIP siRNA. In addition to human HCC cell lines, we used patient-derived HCC primary cultured cells to examine the function of RKIP in sorafenib resistance, since it could reflect HCC patients' genetic and physiological complexity. Similar to results in HCC cell lines, sorafenib increased ERK

activity in HCC cells derived from sorafenib-resistant patients. These results suggest that RKIP may be a key factor in modulating sorafenib sensitivity in HCC. The aberration of RKIP expression changed the level of phosphorylated ERK and, consequently altered sorafenib sensitivity in HCC cell lines.

There have been no previous reports of RKIP expression being involved in sorafenib resistance or of how sorafenib might regulate RKIP expression. As far as we know, this is the first report which shows the mechanism by which sorafenib regulates RKIP expression. Sorafenib did not regulate RKIP at the transcriptional level, but decreased RKIP stability in a sorafenib-resistant HCC cell line (Fig. 3). Our findings suggest that sorafenib-mediated alteration of RKIP expression is a factor responsible for resistance to sorafenib.

To confirm that sorafenib-reactivated ERK is the causative factor for sorafenib resistance, we treated cells with a combination of the MEK/ERK inhibitor, PD98059, and sorafenib. In contrast to sorafenib-sensitive HepG2 cells, the sorafenib-mediated increase of pERK was inhibited and cell death rate was somewhat increased in SNU449 cells after co-treatment with PD98059. Based on our data, we hypothesized that ERK reactivated by sorafenib could be a target as a combination therapy in HCC patients with sorafenib resistance. However, the inhibitory effect of the sorafenib and PD98059 co-treatment was short-lived and a relapsed into resistant status occurred after 48 h of simultaneous co-treatment. To optimize the inhibitory effect of PD98059, the drug treatment scheme was changed from simultaneous co-treatment to sequential treatment. As shown in Figure 4, the time sequence of each drug

treatment was important to enhance the anti-proliferative effect. Treatment with PD98059 first or simultaneous co-treatment with sorafenib had no additional inhibitory effect compared with sorafenib alone. This may be due to the fact that sorafenib is a Raf inhibitor as well as multikinase inhibitor and thus, sorafenib exerts its cytotoxic effect more effectively when the Raf/MEK/ERM signaling pathway is activated. Therefore, pre-treatment with PD98059 or simultaneous co-treatment with sorafenib down-regulates the activity of the Raf/MEK/ERK pathway, which acts as the target for sorafenib cytotoxicity. The anti-proliferative effect of sorafenib and PD98059 co-treatment was greatest when we treated cells with PD98059 24 h after sorafenib treatment. Simultaneous co-treatment of sorafenib and PD98059 had an anti-proliferative effect for a shorter time. In comparison, a 24 h pre-treatment with sorafenib increased the length of time of the inhibitory effect of sequential treatment with PD98059. This suggests that sorafenib-mediated ERK reactivation may be an attractive target for overcoming sorafenib resistance in HCC patients and sequential treatment with a MEK/ERK inhibitor after sorafenib may be a better strategy than simultaneous co-treatment in future clinical trials.

Conclusions

In conclusion, our study revealed that sorafenib resistance in HCC can be mediated by ERK reactivation and the responsible factor for ERK reactivation is RKIP. Other groups

have reported that long-term exposure to sorafenib can cause sorafenib resistance via PI3K/Akt and MAPK activation and have suggested endogenous pERK activity as a predictor of therapeutic response to sorafenib in HCC. However, there is no report that sorafenib-mediated RKIP down-regulation is the reason for the ERK reactivation that causes sorafenib resistance in HCC. Furthermore, we suggest a MEK/ERK inhibitor as a potential therapy to overcome sorafenib resistance using a sequential combination protocol of sorafenib and the MEK/ERK inhibitor. Although simultaneous combination treatment was less effective than we expected, sequential treatment of sorafenib and PD98059 was considerably effective in a sorafenib-resistant HCC cell line. Our innovative approach employing sequential treatment of sorafenib and a MEK/ERK inhibitor may provide a strategy for effective therapy in HCC patients with sorafenib resistance.

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국문 요약

서론: 간세포암종 (Hepatocellular carcinoma, HCC) 환자에서 sorafenib 이 생존률을 향상시켰지만, sorafenib 저항성은 예후 향상에 주요한 제한 요소로 보고되어 왔다. 그러므로 sorafenib 저항성의 메커니즘을 규명하는 것은 중요하다. 본 연구는 sorafenib 저항성의 원인 인자를 규명하고, 극복방안을 제시하기 위한 목적으로 시행하였다.

재료 및 연구방법: Sorafenib 에 대한 감수성은 인간 간세포암종 세포주와 환자 유래-간세포암종 일차 세포들을 비교하였다. 이것들의 세포독성에 근거하여, sorafenib 에 의해 변화되는 신호 경로 (signaling pathway)와 원인 인자를 분석을 통해 검사하였다. Sorafenib 이 sorafenib-저항성 유발자를 유전자 또는 단백 발현 또는 안정성을 변화시키는 지에 대한 매커니즘도 역시 연구하였다. 저자들은 sorafenib 저항성을 극복하기 위한 치료 대안 역시 구상하였다.

결과: Sorafenib 은 Raf/MEK/ERK 경로를 활성화시키고, 간세포암종 세포주와 환자 유래-간세포암종 일차 세포들에서 sorafenib 저항을 야기했다. Sorafenib 은 전사후 단계에서 RKIP (Raf kinase inhibitory protein)을 하향 조절(downregulation) 함으로써 MAPK 경로를 재활성화시켰다. 녹다운된 RKIP 은 인산화된 ERK 를 증가시켰고, 이로써 sorafenib 과 연관된 세포사를 억제했다. 또한 sorafenib 에 의해 재활성화된 ERK 는 sorafenib 저항성을 가진 환자에서 이차 치료 목표로 생각되었다. Sorafenib 후에 PD98059 를 순차 병합 치료했을 때, sorafenib-저항 세포들의 세포자멸 효능을 증가시키는 반면, 생존 및 증식이 유의하게 감소하였다.

결론: 간세포 암종의 sorafenib 저항성에 있어서 RKIP 발현의 변형으로 인한 Raf/MEK/ERK 경로의 재활성화가 하나의 메커니즘으로 생각된다. 또한 Sorafenib 과 PD98059 의 순차 병합 치료가 미래의 임상 연구를 통해 sorafenib 저항성 극복을 위한 새로운 치료 대안이 될 수 있겠다.