

Sorafenib with doxorubicin augments cytotoxicity to renal cell cancer through PERK inhibition

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Abstract. Although cytokine therapy involving interleukin-2 or interferon- α has been employed for metastatic renal cell cancer (RCC) treatment, these therapies yielded limited response and benefit. Recently, several molecular-targeted agents have become available, and one newly developed anti-RCC agent, sorafenib (BAY 43-9006), is known to target multiple kinases. In this study, sorafenib was found to inhibit phosphorylation of the eukaryotic initiation factor-2 α (eIF2 α) and induce cell cycle arrest at G2/M phase and increase cell death. One of eIF2 α kinases, PERK was responsible for eIF2 α phosphorylation in RCC cells and PERK knockdown induced cell death similar to sorafenib treatment. The efficiency of sorafenib treatment correlated with phosphorylation level of eIF2 α and nuclear Nrf2 expression level in eight RCC cell lines. Furthermore, sorafenib made Caki-1 and 786-O cells, but not ACHN cells sensitive to oxidative stress exerted by both hydrogen peroxide and doxorubicin. In addition, PERK knockdown sensitized Caki-1 and 786-O cells, but not ACHN cells to oxidative stress. In conclusion, levels of phospho-eIF2 α and nuclear Nrf2 expression level in RCC might be a predictor of outcome in sorafenib treatment. In addition, PERK inhibition as well as sorafenib plus doxorubicin might be a promising therapeutic approach for RCC characterized by high levels of phosphorylated-eIF2 α and nuclear Nrf2.

Introduction

Renal cell cancer (RCC) is characterized by the loss of von Hippel-Lindau tumor suppressor protein, resulting in dys-

regulation of growth factor signaling, including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and transforming growth factor- α (TGF- α). These factors play key roles in angiogenesis and lymphangiogenesis as well as in dysregulation of Raf pathways that regulate tumor growth and survival (1-6). Patients with metastatic RCC (mRCC) face a poor prognosis, with a historical median survival of 6-10 months and a 2-year survival of 10-20% (7). Traditional chemotherapy or radiotherapy has been relatively ineffective, with only 10% of the patients showing response to these treatments (8). Cytokine therapy involving interleukin-2 (IL-2) or interferon- α (IFN- α) has also yielded limited benefit, with objective response rates of only 15% for both (7,9,10). Combination therapies with IFN- α in RCC have involved using it together with IL-2, 5-fluorouracil, retinoic acid, and/or thalidomide; none of these studies has shown any consistent improvement in outcome over IFN- α monotherapy (7,11-13). Thus, there are many requirements for new effective therapies for mRCC. Sorafenib (BAY 43-9006) is a novel bi-aryl urea that has been previously shown to inhibit Raf-1 and tumor cell line proliferation and tumor xenograft models (14,15). In addition to Raf-1 inhibition, sorafenib is a multikinase inhibitor, which targets vascular endothelial growth factor receptor (VEGFR) 2 and 3; platelet-derived growth factor receptor- β (PDGFR- β); FMS-like tyrosine kinase 3 (FLT3); c-Kit; and RET-receptor tyrosine kinase (16,17). The clinical benefit of sorafenib was initially found in a phase II randomized trial, which indicated a lengthened progression-free survival with sorafenib compared with placebo in patients with mRCC refractory to cytokine therapy (18). A subsequent phase III randomized controlled trial [Treatment Approaches in Renal cancer Global Evaluation Trial (TARGET)] confirmed the findings of the phase II trial (19). We have previously conducted a phase II study to investigate the efficacy, safety, and pharmacokinetics of sorafenib in Japanese patients with advanced renal cell carcinoma, and revealed that sorafenib exerted encouraging efficacy and was well tolerated in Japanese patients with mRCC (20).

Phosphorylation of the eukaryotic initiation factor-2 α (eIF2 α) is an important mechanism regulating protein synthesis in response to a diverse range of environmental stresses

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(21-23). Four eIF2 α kinases have been identified in mammals, each responding to different stresses through their unique regulatory regions. For example, phosphorylation of eIF2 α by PKR-like endoplasmic reticulum kinase (PERK, also known as EIF2AK3) is induced by accumulation of misfolded proteins in the endoplasmic reticulum (ER), a phenomenon designated as unfolded protein reaction (UPR) or ER stress (24-26). Phosphorylation of eIF2 α during ER stress inhibits global translation by lessening eIF2-GTP (21-23). Together with reduced protein synthesis, eIF2 α phosphorylation increases the preferential translation of ATF4 mRNA, encoding a basic zipper transcription activator that is important for directing the expression of genes involved in metabolism, the redox status of cells and apoptosis (27-29). Furthermore, eIF2 α phosphorylation has been shown to regulate an expression of NF-E2-related factor 2 (Nrf2), which is the Cap 'n' Collar transcription factor closely involved in oxidative stress response through expression of phase II antioxidative stress proteins (30). Decreased protein synthesis conserves energy and provides sufficient time for ATF4, Nrf2 and other stress-responsive transcription factors, to reconfigure gene expression that would block or ameliorate damage elicited by the underlying stress. Other members of the eIF2 α kinase family include GCN2 (EIF2AK4), whose activity is enhanced by amino acid depletion, UV irradiation or proteasome inhibition; PKR (EIF2AK2), which function in an antiviral pathway; and HRI (EIF2AK1), which is regulated by heme deficiency and oxidative stress (22,23,26,31). Aberrations in these eIF2 α kinase pathways are associated with a number of diseases, including diabetes, viral infection, anemia, neurological disorders and various cancers.

In this study, we investigated whether the multikinase inhibitor, sorafenib could inhibit the eIF2 α kinase family. Then, we attempted to elucidate a mechanism that sorafenib inhibited RCC-cell growth through an inhibition of eIF2 α kinase pathway and provide a possible molecular marker of RCC-cell sensitivity to sorafenib. Furthermore, to improve treatment of mRCC patients, we investigated the effects of combined therapy of conventional chemotherapy and sorafenib based on a pharmacological effect of sorafenib toward eIF2 α kinase pathway.

Materials and methods

Cell culture and chemical. Human renal cell cancer KG2, KPK13, SKR1, KPK1 and SN12C cells were established and maintained as described previously (32-35). KG2 cells were a kind gift of Dr Kazuo Gohji (Kobe University, Kobe, Japan). Human renal cell cancer Caki-1 cells were cultured in Eagle's minimal essential medium (Invitrogen, San Diego, CA, USA). Human renal cell cancer ACHN and 786-O cells were cultured in Eagle's minimal essential medium (Sigma, St. Louis, MO, USA) and RPMI1640 (Sigma), respectively. These media were contained 10% fetal bovine serum. Cell lines were maintained in a 5% CO₂ atmosphere at 37°C. Sorafenib was obtained from Bayer Pharmaceuticals Corp. (West Haven, CT, USA). For assay, stock solution was prepared in dimethyl sulfoxide (DMSO) (10 mM), and stored at -20°C until use. Stock solution was diluted to appropriate concentration in culture medium before addition to the cells.

Antibodies. Antibodies against GCN2 (#3302), p-PERK (#3191), p-eIF2 α (#9721), eIF2 α (#2103) and p-ERK1/2 (#9106) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibody against ERK1 (sc-94) and PERK (sc-13073) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Nrf2 (#2178-1), anti-p-GCN2 (#2313-1), anti-PKR (#1511-1) and anti-p-PKR (#2283-1) antibodies were purchased from Epitomics (Burlingame, CA, USA). Anti-Lamin B1 and anti- β -actin antibodies were purchased from Sigma.

Western blotting. Whole-cell and nuclear extracts were prepared with phosphatase inhibitor Cocktail (Nacalai tesque, Kyoto, Japan) as previously described (36-40). The protein concentration was determined using a Protein Assay Kit (Bio-Rad, Hercules, CA), based on the Bradford method. Whole-cell and nuclear extracts (30 μ g) were separated by SDS-PAGE and transferred to polyvinylidene difluoride microporous membranes (GE Healthcare Bio-Science, Piscataway, NJ, USA) using a semi-dry blotter. The blotted membranes were incubated for 1 h at room temperature with a primary antibody. Membranes were then incubated for 40 min at room temperature with a peroxidase-conjugated secondary antibody. The bound antibody was visualized using an ECL kit (GE Healthcare Bio-Science) and membranes were exposed to Kodak X-OMAT film. For the correlation assay, the intensity of each signal was quantified using the NIH Imaging program, version 1.62 (NIH, Bethesda, MD, USA).

Flow cytometry analysis. Flow cytometry analysis was performed as described previously (36). Briefly, Caki-1, 786-O and ACHN cells (2.0x10⁵) were seeded in 6-well plates, applied to 10 μ M of sorafenib and cultured for 24 h, or transfected with 40 nM of the indicated siRNA and cultured for 72 h. The cells were harvested, washed twice with ice-cold PBS containing 0.1% bovine serum albumin (BSA) and resuspended in 70% ethanol. After washing twice with ice-cold PBS, cells were resuspended in PBS containing 0.1% BSA, incubated with RNase (Roche Applied Science, Indianapolis, IN, USA) and stained with propidium iodide (Sigma). Cells were analyzed using a FACSCalibur (BD Biosciences, San Jose, CA, USA).

Knockdown analysis using siRNAs. Knockdown analysis using siRNAs was performed as described previously (36-40). Briefly, the following double-stranded RNA oligonucleotides were commercially generated: 5'-GGUAAAAGCAGUGG GAUUTT-3' (sense) and 5'-AAUCCACUGCUUUUAC CTT-3' (antisense) for PERK #1 (B-Bridge International, Mountain View, CA, USA); 5'-GGGAAAAGGUAAGCG AGATT-3' (sense) and 5'-UCUCGCAUUAACCUUUUCC CTT-3' (antisense) for PERK #2 (B-Bridge International); 5'-CCACAUGAUAGGAGGUUUATT-3' (sense) and 5'-UAAACCUCCUAUCAUGUGGTT-3' (antisense) for PKR (B-Bridge International); 5'-AUUAGAUGCACUCAGGAC CUUAUGC-3' (sense) and 5'-GCAUAAGGUCCUGAGUG CAUCUAAU-3' (antisense) for GCN2 (Invitrogen). Caki-1, 786-O and ACHN cells were transfected with 40 nM of the indicated siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

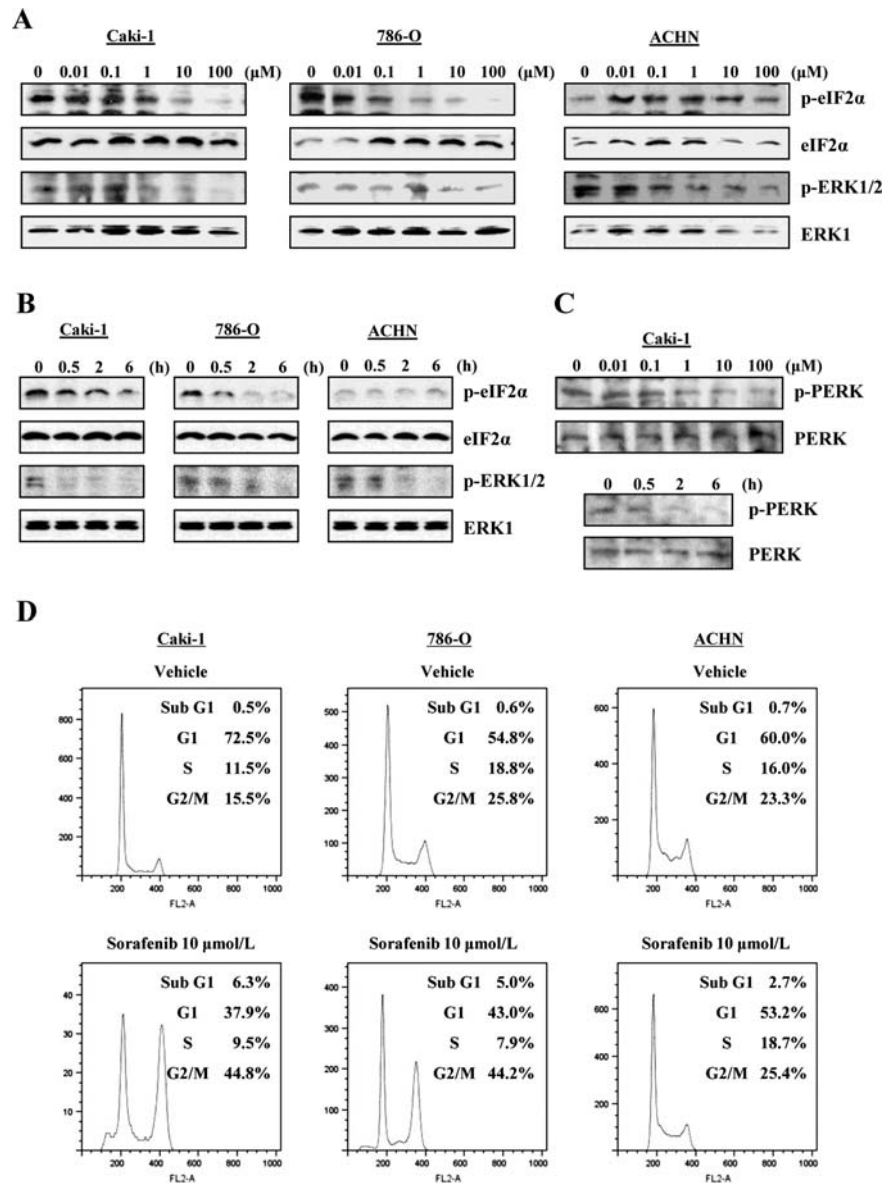


Figure 1. Sorafenib reduces phosphorylated form of eIF2 α and ERK1/2, and induces cell-cycle arrest at G2 phase and cellular apoptosis in Caki-1 and 786-O cells. (A) Caki-1, 786-O and ACHN cells were cultured with various concentrations of sorafenib for 6 h, and the cells were harvested. Whole-cell extracts were subjected to SDS-PAGE, and Western blotting was performed using the indicated antibodies. (B) Caki-1, 786-O and ACHN cells were cultured with 10 μ M of sorafenib for various durations, and the cells were harvested. Whole-cell extracts were subjected to SDS-PAGE, and Western blotting was performed using the indicated antibodies. (C) Caki-1 cells were cultured with various concentrations of sorafenib for 6 h or 10 μ M of sorafenib for various durations, and the cells were harvested followed by 0.5 μ g/ml of tunicamycin treatment for 6 h. Whole-cell extracts were subjected to SDS-PAGE, and Western blotting was performed using the indicated antibodies. (D) Caki-1, 786-O and ACHN cells were applied to vehicle or 10 μ M of sorafenib for 24 h, and the cells were stained with propidium iodide and analyzed by flow cytometry. The cell cycle fractions are shown at the top right of each graph.

Cytotoxicity analysis. Cytotoxicity analysis was performed as described previously (36,38,40). Briefly, Caki-1, 786-O and ACHN cells (2.5×10^3) applied to 0.5 μ M of sorafenib or transfected with 40 nM of the indicated siRNA were seeded into 96-well plates. The following day, the indicated concentrations of hydrogen peroxide or doxorubicin and/or N-acetyl-L-cysteine (NAC) were applied. After 48 h, surviving cells were stained with alamarBlue Assay (TREK Diagnostic systems, Cleveland, OH, USA) for 180 min at 37°C. The absorbance was then measured using a plate reader (ARVO™ MX, Perkin-Elmer Inc., Waltham, MA, USA).

Determination of inhibitory concentration 50 (IC₅₀) to sorafenib in RCC cells. Eight RCC cell types (2.5×10^3) were

seeded into 96-well plates. The following day, the various concentrations of sorafenib were applied. After 48 h, cytotoxicity assay was performed as described above. Then, IC₅₀ was defined as sorafenib concentration when 50% of cells survived.

Results

Sorafenib reduces eIF2 α phosphorylation levels and induces cell cycle arrest at G2/M phase and cell death. To investigate whether multikinase inhibitor, sorafenib could inhibit the eIF2 α kinase family, Western blot analysis was performed after RCC cells were treated with increasing concentrations of sorafenib. As shown in Fig. 1A, various concentrations of

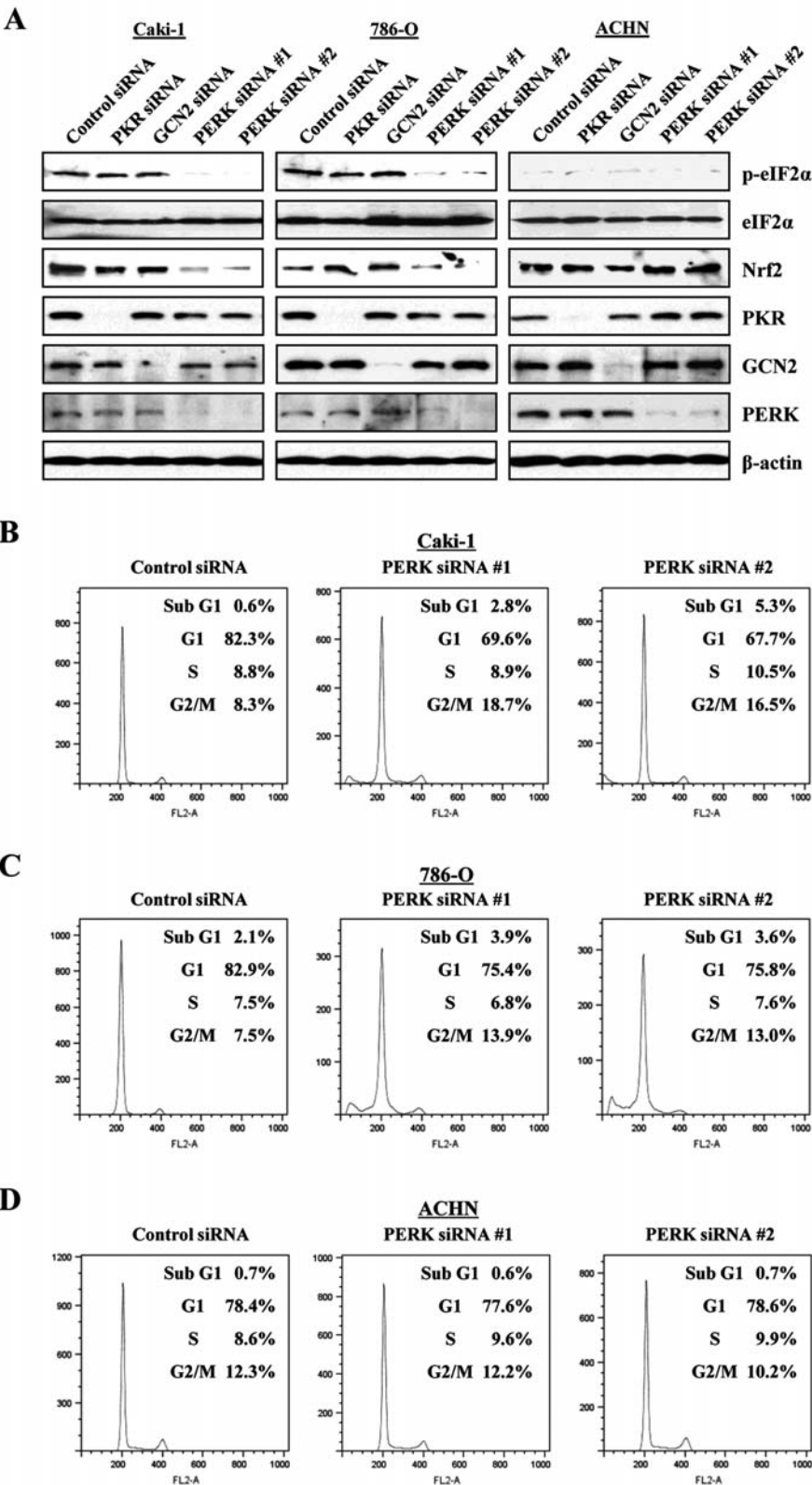


Figure 2. Silencing of PERK reduces phosphorylation form of eIF2α, and induces cell cycle arrest at G2 phase and cell death in Caki-1 and 786-O cells. (A) Caki-1, 786-O and ACHN cells were transfected with 40 nM of control siRNA, PKR siRNA, GCN2 siRNA, PERK siRNA #1 or PERK siRNA #2. At 72 h after transfection, whole-cell extracts were subjected to SDS-PAGE, and Western blotting was performed using the indicated antibodies. (B-D) Caki-1 (B), 786-O (C) and ACHN (D) cells were transfected with 40 nM of control siRNA, PERK siRNA #1 or PERK siRNA #2. At 72 h after transfection, cells were stained with propidium iodide and analyzed by flow cytometry. The cell cycle fractions are shown at the top right of each graph.

sorafenib treatment for 6 h reduced phosphorylation of ERK1/2 (p-ERK1/2) as well as phosphorylation of eIF2α (p-eIF2α) in Caki-1 and 786-O cells. On the contrary, p-eIF2α level was

increased in ACHN cells although p-ERK1/2 level declined. About 1 μM of sorafenib inhibited phosphorylation of eIF2α to 50% of vehicle treatment in Caki-1 and 786-O cells. When

RCC cells were treated with 10 μ M of sorafenib for various durations, p-ERK1/2 as well as p-eIF2 α level declined in Caki-1 and 786-O cells whereas p-eIF2 α did not in ACHN cells (Fig. 1B). There are four members of eIF2 α kinase family: PKR, GCN2, PERK, and HRI, the former three are expressed in RCC cells; HRI expression is restricted to erythroblast cells. To determine which p-eIF2 α kinase was responsible for dephosphorylation of eIF2 α by sorafenib treatment in Caki-1 cells, immunoblotting for phosphorylated forms of these eIF2 α kinases was performed. The results showed sorafenib treatment decreased p-PERK level in concentration- and duration-dependent manner (Fig. 1C). On the other hand, p-PKR and p-GCN2 levels were constant during sorafenib treatment (data not shown). Because sorafenib is known to suppress cell proliferation in RCC cells, we performed cell cycle analysis using flow cytometry analysis to investigate a mechanism of tumor growth suppression by sorafenib. First, Caki-1 cells were subjected to cell cycle analysis. Application of 10 μ M sorafenib induced cell cycle arrest at G2 phase and cellular death represented by sub-G1 fraction. Similar results were obtained when 786-O cells were investigated. Of note, both Caki-1 and 786-O cells have high levels of p-eIF2 α . On the other hand, ACHN cells possessing low eIF2 α phosphorylation status exhibited only a slight increase in the G2-phase fraction and little effect of cellular apoptosis as compared with either Caki-1 or 786-O cells (Fig. 1D).

PERK knockdown reduces eIF2 α phosphorylation level and nuclear Nrf2 expression, and induces cell cycle arrest at G2 phase and cellular death. To confirm that PERK is the responsible eIF2 α kinase for eIF2 α phosphorylation in RCC cells, PKR, GCN2 and PERK eIF2 α kinases were silenced by the corresponding specific-siRNAs, then eIF2 α phosphorylation status was investigated by Western blot analysis. As shown in Fig. 2A, PERK knockdown using two kinds of PERK-specific siRNA decreased eIF2 α phosphorylation level in Caki-1 and 786-O cells, whereas PKR or GCN2 knockdown had no effect. This result also indicated that in Caki-1 and 786-O cells, PERK is mainly responsible for eIF2 α phosphorylation. In addition, Nrf2, whose expression is known to be regulated by PERK (30), decreased when only PERK was silenced. On the other hand, eIF2 α phosphorylation was hardly detected in ACHN cells, and Nrf2 expression level was slightly affected. We performed cell cycle analysis using flow cytometry to investigate a functional correlation between sorafenib and PERK inhibition. First, Caki-1 cells were subjected to cell cycle analysis and PERK knockdown induced cell cycle arrest at G2/M phase and cell death although at a lesser degree than sorafenib treatment (Fig. 2B). Similar results were obtained when 786-O cells which possess high eIF2 α phosphorylation level similar to Caki-1 cells were investigated (Fig. 2C). On the other hand, ACHN cells possessing low eIF2 α phosphorylation level exhibited little effect on cell cycle and cell death (Fig. 2D).

Phosphorylation level of eIF2 α and nuclear Nrf2 expression level are correlated with efficiency of sorafenib treatment. Because the finding that sorafenib inhibits eIF2 α phosphorylation was revealed, whether eIF2 α phosphorylation level

Table I. IC₅₀ to sorafenib, phosphorylated eIF2 α /total eIF2 α and nuclear Nrf2 expression in RCC cell lines.

| RCC cell line | IC ₅₀ to sorafenib (μ M) | Phosphorylated eIF2 α /total eIF2 α | Nuclear Nrf2 expression |
|---------------|--|---|-------------------------|
| Caki-1 | 7.8 | 0.99 | 0.31 |
| KG2 | 8.8 | 0.79 | 0.36 |
| KPK13 | 18 | 0.12 | 0.13 |
| SKR1 | 11 | 0.17 | 0.54 |
| KPK1 | 12 | 0.05 | 0.28 |
| 786-O | 6.5 | 1 | 1 |
| ACHN | 10 | 0.12 | 0.51 |
| SN12 | 14 | 0.08 | 0.13 |

and nuclear Nrf2 expression level could predict an efficiency of sorafenib treatment in RCC cells was examined. First, eight RCC cell lines were subjected to Western blot analysis. Because sorafenib is well-known to inhibit ERK phosphorylation, p-ERK1/2 and ERK1 in addition to p-eIF2 α , eIF2 α and Nrf2 were investigated in untreated cells. Although ERK1/2 phosphorylation status was high in ACHN cells, basal eIF2 α phosphorylation status and nuclear Nrf2 expression level were high in 786-O cells (Fig. 3A). As speculated from previous finding (30), correlation between eIF2 α phosphorylation status and nuclear Nrf2 expression level was suggested. In RCC cells, coefficient of correlation between basal eIF2 α phosphorylation status and nuclear Nrf2 expression level was 0.50 although not significant statistically (Fig. 3B). Next, IC₅₀ for cell-growth inhibitory effects of sorafenib was determined in these eight RCC cell lines. As shown in Table I, 786-O cells were most sensitive to sorafenib whereas KPK13 cells were most resistant and ACHN cells were relatively resistant. To reveal a possible predictor of sensitivity to sorafenib treatment, correlations between either eIF2 α phosphorylation status or nuclear Nrf2 expression level and sorafenib IC₅₀s were examined. The results showed that both basal eIF2 α phosphorylation status and nuclear Nrf2 expression level were inversely correlated with IC₅₀ value of RCC cells to sorafenib. Coefficients of correlation between eIF2 α phosphorylation status and IC₅₀ value, and nuclear Nrf2 expression level and IC₅₀ value were -0.75 (p=0.033) and -0.73 (p=0.040), respectively (Fig. 3C and D).

Sorafenib sensitizes both Caki-1 and 786-O cells to oxidative stress exerted by hydrogen peroxide and doxorubicin. Although mRCC was believed to be resistant to conventional chemotherapy (8), conventional chemotherapy combined with sorafenib may be useful to improve treatment outcome. Sorafenib was shown to suppress nuclear Nrf2 expression, which regulates several antioxidative stress proteins. First, cytotoxicity of hydrogen peroxide to RCC cells treated with sorafenib was investigated. Hydrogen peroxide combined with sorafenib suppressed Caki-1 and 786-O cell viability more effectively compared with hydrogen peroxide alone, although

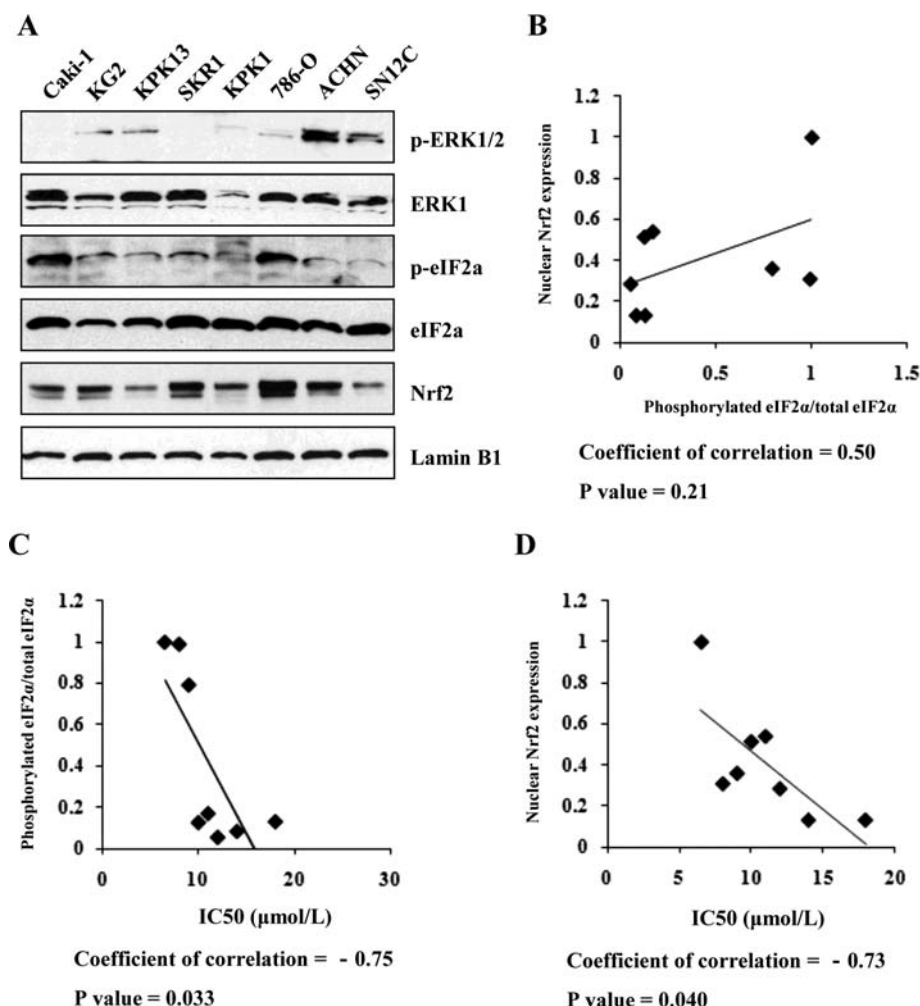


Figure 3. Phosphorylation level of eIF2 α and nuclear Nrf2 expression level are correlated with efficiency of sorafenib treatment. (A) Western blot analysis was performed with whole-cell extracts (p-ERK1/2, ERK1, p-eIF2 α and eIF2 α) and nuclear extract (Nrf2 and Lamin B1) from eight RCC cell lines using the indicated antibodies. (B) Correlation between phosphorylation ratio to total eIF2 α and nuclear Nrf2 expression level were analyzed. The maximum levels of phosphorylation ratio to total eIF2 α and nuclear Nrf2 expression in 786-O cells were set to 1. (C) Correlation between IC₅₀ to cytotoxicity of sorafenib and phosphorylated eIF2 α ratio to total eIF2 α in untreated cells was analyzed. (D) Correlation between IC₅₀ to cytotoxicity of sorafenib and nuclear Nrf2 expression level was analyzed.

ACHN cells were not affected by an addition of sorafenib (Fig. 4A). To investigate a mechanism of an additional effect of sorafenib to hydrogen peroxide, we assayed cytotoxicity of hydrogen peroxide combined with sorafenib and with NAC antioxidant added. When NAC was added, the enhancement of hydrogen peroxide on sorafenib activity was abolished in Caki-1 and 786-O cells (Fig. 4B).

Doxorubicin is well known to exert oxidative stress to cells (41). The cytotoxicity of RCC cells to doxorubicin combined with sorafenib was investigated. As shown in Fig. 4C, sorafenib could increase the sensitivity of Caki-1 and 786-O cells, but not of ACHN cells, to doxorubicin. In addition, NAC abolished this favorable effect of sorafenib similar to hydrogen peroxide combined with sorafenib (Fig. 4D).

PERK knockdown sensitizes both Caki-1 and 786-O cells to oxidative stress exerted by hydrogen peroxide and doxorubicin. Because sorafenib was suggested to inhibit PERK-kinase activity, the effects of PERK knockdown using PERK-specific siRNAs on RCC viability was determined. First, cytotoxicity

of hydrogen peroxide to RCC cells transfected with PERK-specific siRNAs was examined. Hydrogen peroxide with PERK knockdown suppressed survival of Caki-1 and 786-O cells more effectively compared with hydrogen peroxide alone, although survival of ACHN cells were not affected by silencing of PERK (Fig. 5A). To confirm a mechanism of an additional effect of PERK knockdown to hydrogen peroxide, we assayed cytotoxicity of hydrogen peroxide with PERK knockdown when further NAC was added. When NAC was added to hydrogen peroxide combined with PERK knockdown, the enhanced cytotoxicity was abolished in Caki-1 and 786-O cells (Fig. 5B).

Similarly, doxorubicin plus PERK knockdown increased cytotoxicity to both Caki-1 and 786-O cells, but not to ACHN cells (Fig. 5C). In addition, NAC abolished the enhancement of doxorubicin plus PERK knockdown similar to hydrogen peroxide with PERK knockdown (Fig. 5D).

Doxorubicin sensitizes both Caki-1 and 786-O cells to sorafenib. Because sorafenib sensitized doxorubicin in RCC cells possessing high levels of eIF2 α phosphorylation status

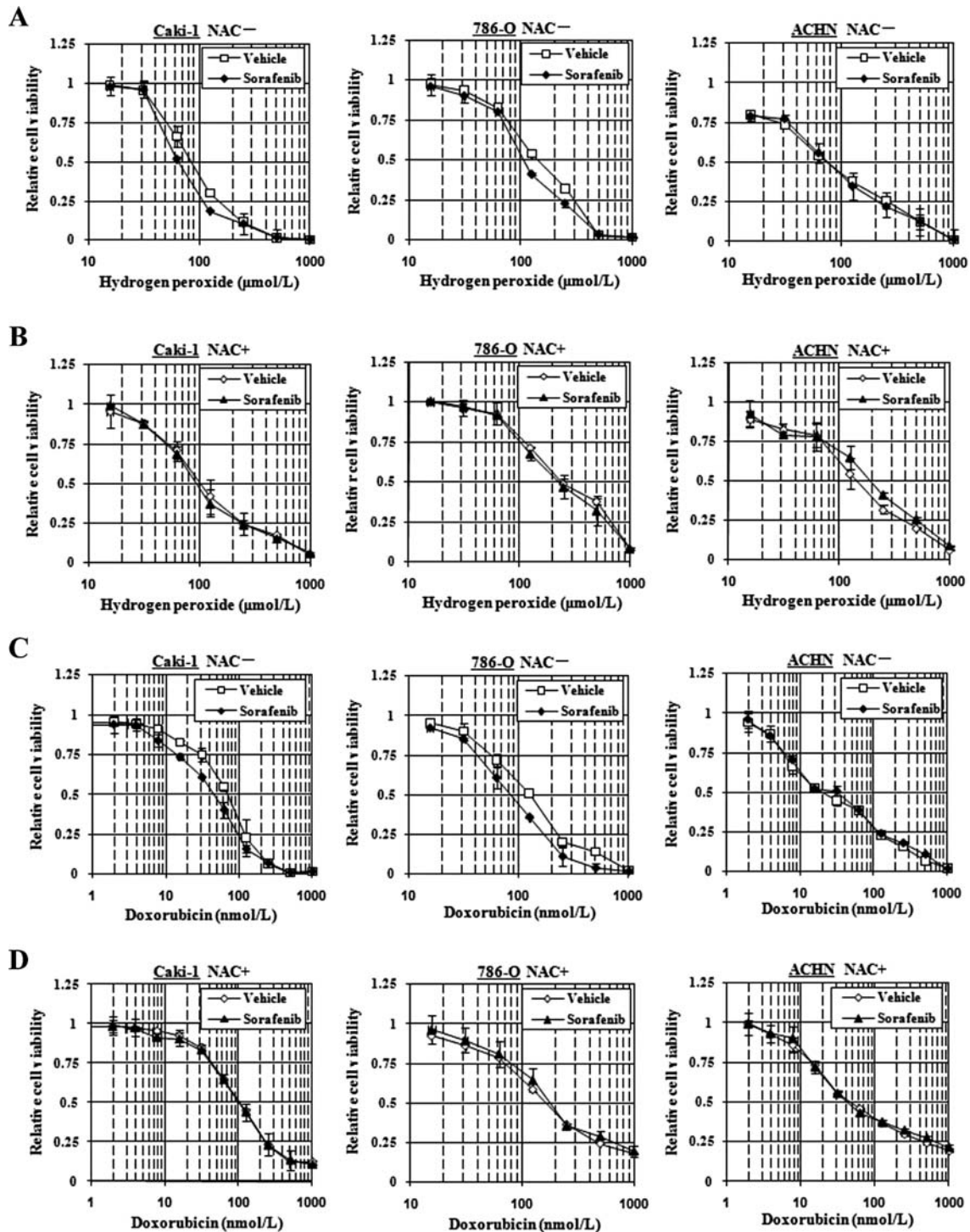


Figure 4. Sorafenib sensitizes both Caki-1 and 786-O cells to oxidative stress exerted by hydrogen peroxide and doxorubicin. (A and C) Caki-1, 786-O and ACHN cells were seeded into 96-well plates and treated with 0.5 μ M of sorafenib. The following day, various concentrations of hydrogen peroxide (A) or doxorubicin (C) were applied. After 48 h, cell survival was analyzed by cytotoxicity assay. Cell survival in the absence of hydrogen peroxide (A) or doxorubicin (C) was set as 1. All values are representative of at least three independent experiments. Boxes, mean; bars \pm SD (B and D) Caki-1, 786-O and ACHN cells treated with 0.5 μ M of sorafenib and 5 mM of NAC were seeded into 96-well plates. The following day, various concentrations of hydrogen peroxide (B) or doxorubicin (D) were applied. Cytotoxicity assay was performed as described in (A) and (C). Boxes, mean; bars \pm SD.

and nuclear Nrf2 expression, whether doxorubicin could sensitize RCC cells to sorafenib was investigated. As shown in Fig. 6A and B, doxorubicin sensitized both Caki-1 and 786-O cells to sorafenib about 2-fold. As expected, ACHN cells could not be sensitized to sorafenib by an addition of doxorubicin (Fig. 6C).

Discussion

Sorafenib has been shown to exert an anti-tumor effect through inhibition of multiple kinases, such as Raf-1, VEGFR 2 and 3, PDGFR- β , FLT3, c-Kit and RET-receptor tyrosine kinase (16,17). A possible involvement of ER stress and generation

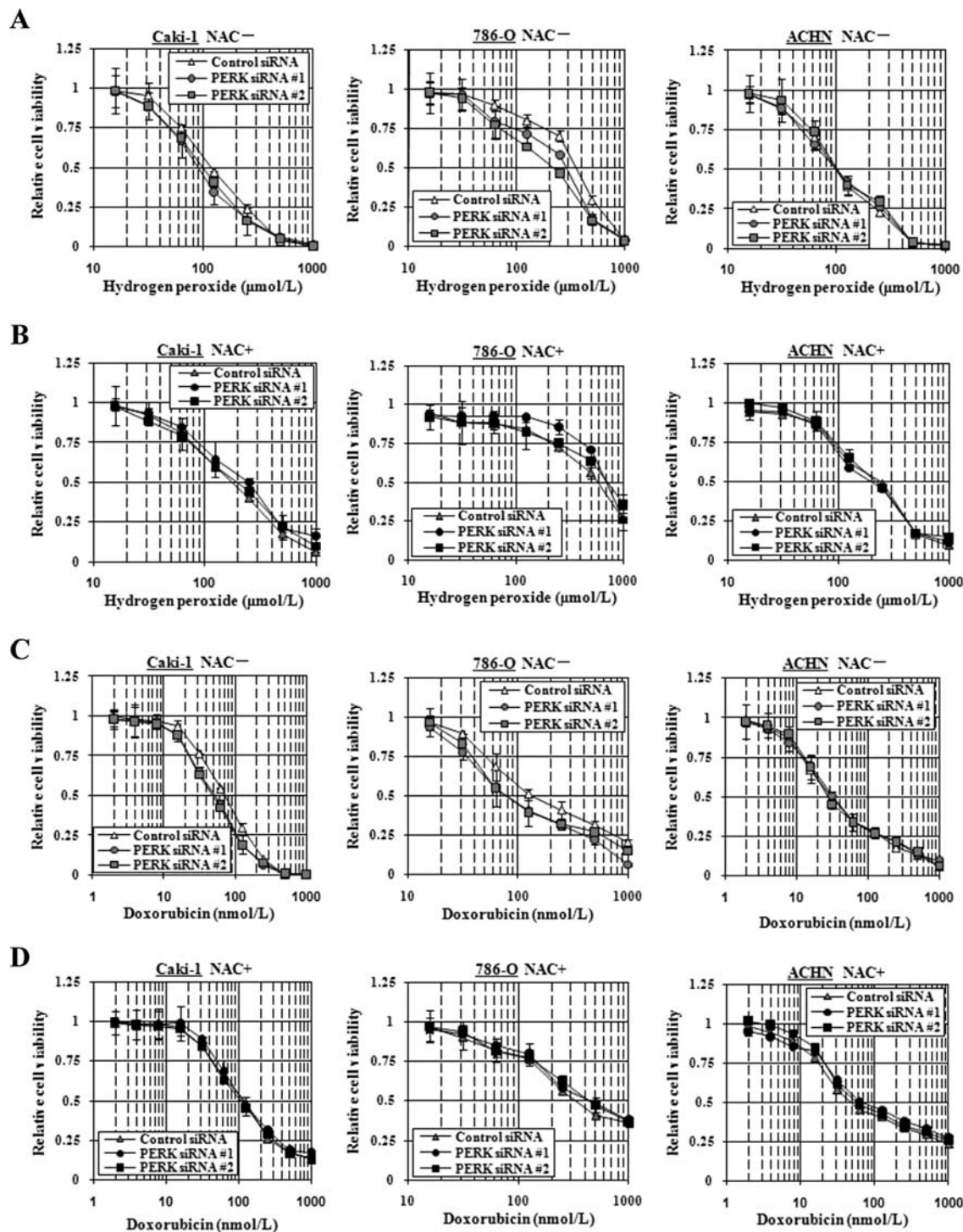


Figure 5. PERK knockdown sensitizes both Caki-1 and 786-O cells to oxidative stress exerted by hydrogen peroxide and doxorubicin. (A and C) Caki-1, 786-O and ACHN cells transfected with 40 nM of control siRNA, PERK siRNA #1 or PERK siRNA #2 were seeded into 96-well plates. The following day, various concentrations of hydrogen peroxide (A) or doxorubicin (C) were applied. After 48 h, cell survival was analyzed by cytotoxicity assay. Cell survival in the absence of hydrogen peroxide (A) or doxorubicin (C) corresponds to 1. All values are representative of at least three independent experiments. Boxes, mean; bars \pm SD (B and D) Caki-1, 786-O and ACHN cells transfected with 40 nM of control siRNA, PERK siRNA #1 or PERK siRNA #2, and treated with 5 mM of NAC were seeded into 96-well plates. The following day, various concentrations of hydrogen peroxide (B) or doxorubicin (D) were applied. Cytotoxicity assay was performed as described in (A and C). Boxes, mean; bars \pm SD.

of reactive oxygen species in the cytotoxicity of sorafenib against leukemia cells has been reported (42). The role of ER stress in pancreatic function has been well characterized. Pancreatic β -islet cells produce large amounts of pro-insulin proteins and are continuously exposed to ER stress and must control the protein-synthesis process to protect themselves

from over-dosing ER stress. Therefore, pancreatic β -islet cells regulate protein synthesis at the translational level. In this process of translation control, PERK plays a critical role to phosphorylate eIF2 α and repress translation; with the exception of ATF4. Mutations in PERK were shown to induce diabetes mellitus through apoptosis of pancreatic β -islet cells,

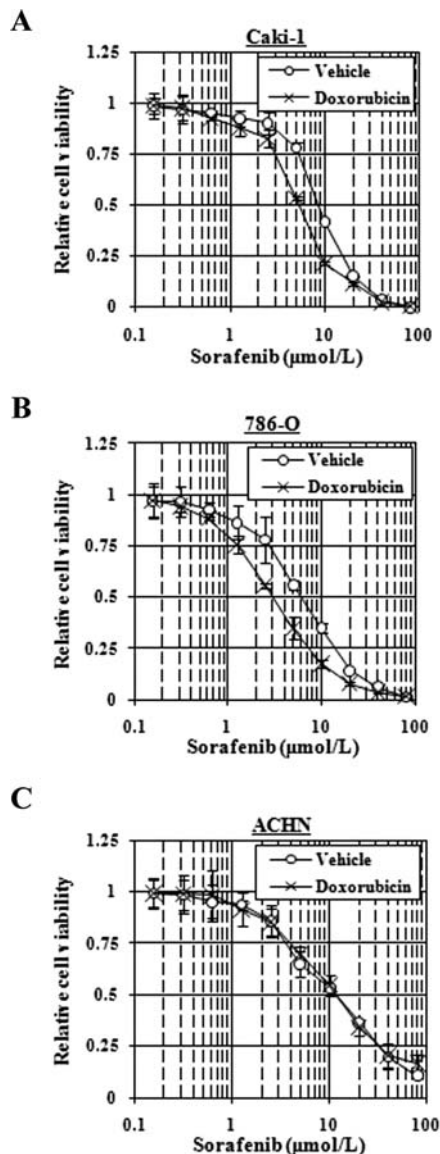


Figure 6. Doxorubicin sensitizes both Caki-1 and 786-O cells to sorafenib. (A-C) Caki-1 (A), 786-O (B) and ACHN (C) cells were seeded into 96-well plates and treated with 1 nM of doxorubicin. The following day, various concentrations of sorafenib were applied. After 48 h, cell survival was analyzed by cytotoxicity assay. Cell survival in the absence of doxorubicin corresponds to 1. All values are representative of at least three independent experiments. Boxes, mean; bars \pm SD.

probably due to disorder of a mechanism of UPR (43). Elevation of serum lipase, pancreatic disorder and drug-induced pancreatitis could occasionally occur during sorafenib administration, suggesting that multikinase inhibitor; sorafenib inhibits PERK activation (44). As expected, results clearly showed that phosphorylation level of eIF2 α was reduced by sorafenib treatment in Caki-1 and 786-O cells (Fig. 1A). There are four eIF2 α kinases, that is, HRI, PRK, PERK and GCN2. In RCC cells, the latter three eIF2 α kinases are expressed. Sorafenib was able to suppress PERK phosphorylation level. Therefore, sorafenib is suggested to reduce phosphorylation of eIF2 α through an inhibition of PERK kinase activity in Caki-1 and 786-O cells. However, eIF2 α phosphorylation level was inversely increased by sorafenib treatment in ACHN cells. Although exact mechanism could

not be revealed, PERK mutation may affect inhibitory effect by sorafenib similar to FLT3 (45). This paradoxical response was shown in another study reporting that phosphorylated form of MEK, sorafenib target kinase, was increased in response to sorafenib in cells containing high level of B-Raf and low Ras activity (46). In addition, this opposite response of p-eIF2 α to sorafenib treatment may determine cell fate after sorafenib administration because increases of p-PERK and p-eIF2 α can rescue cell death induced by ER stress (27,30). Furthermore, a newly revealed finding in this study that sorafenib could inhibit a kinase other than the previously known targets suggests that multikinase inhibitor may indeed inhibit kinases more than thought and that comprehensive assay to global kinases should be performed, which is supported by the previous finding that sorafenib interacted with relatively broad spectrum of kinases (47). Discrepancy in degree of cell cycle arrest at G2 phase and cell death between sorafenib treatment and PERK knockdown may suggest that sorafenib prevents activation of other kinases.

Imatinib is a multikinase inhibitor suppressing BCR-ABL, c-kit and PDGFR. The mechanism of imatinib resistance in chronic myeloid leukemia appears to result from the emergence of mutations in the kinase domain of BCR-ABL (48). In addition, a product of *MDR* gene, P-glycoprotein and human organic cation transporter family member 1 (hOCT1) are suggested to be a molecular predictor of imatinib treatment in chronic myeloid leukemia (49,50). Furthermore, imatinib resistance in gastrointestinal stromal tumors by second mutations of imatinib target, c-kit was reported (51,52). Although sorafenib is used widely in many countries, predictive molecular markers of therapeutic efficiency have not been elucidated. Naturally, sorafenib exhibits therapeutic effects and adverse events differentially on individuals. It is very important for ideal treatment of mRCC to distinguish patients who are likely to benefit from sorafenib administration. In this study, phosphorylation ratio of eIF2 α and its associated molecule, Nrf2 was shown to be possibly useful in predicting efficiency of sorafenib administration, although this finding should be confirmed in *in vivo* model and in clinical setting.

It is important to improve the outcome of mRCC patients. Cytokine therapy using IL-2 or IFN- α has been employed for the treatment of mRCC, however, outcomes have been unsatisfactory (7,9,10). Recently, new small molecule agents were started to be administered for mRCC patients, and one of them is sorafenib. Although sorafenib improved progression-free survival and overall survival, its benefit is limited (18,19). Therefore, more beneficial therapeutics is needed for RCC patients. Recently, sorafenib in combination with doxorubicin has been reported to double the median overall survival from 6.5 months in the doxorubicin-mono therapy group to 13.7 months in the combination group in a randomized, double-blind, placebo-controlled phase II study of patients with hepatocellular carcinoma, indicating the possibility of better sorafenib effect in combination with doxorubicin (53). However, sorafenib in combination with conventional chemotherapeutic agents, paclitaxel plus carboplatin for melanoma patients failed to show improvement in overall survival (54). In our study, sorafenib *in vitro* sensitized RCC cells possessing high eIF2 α phosphorylation

status and nuclear Nrf2 expression level to oxidative stress exerted by hydrogen peroxide and doxorubicin, indicating that combination therapy of anticancer agents exerting oxidative stress and sorafenib may be a beneficial in the RCC dependent on PERK pathway. This finding is supported by the previous report that doxorubicin with sorafenib have an over-additive growth-inhibitory effect (55). In addition, some anticancer agents combined with anti-angiogenic drugs could improve therapeutic index through their anti-angiogenic effects (56). As it was shown that in mouse model, doxorubicin combined with anti-VEGFR2 antibody could exert an excellent therapeutic effect (57), sorafenib plus doxorubicin might be a novel method in terms of anti-angiogenic therapeutics. Metronomic therapy has also been focused on in anti-angiogenic therapy. Sorafenib plus doxorubicin might be an ideal combination because non-toxic dose of doxorubicin could enhance cytotoxic effect of sorafenib, which prevent neovascularization and re-vascularization after maximum-tolerated dose chemotherapy (58). However, further investigation of the effect of the sorafenib and doxorubicin combination using *in vivo* models and in clinical trials are necessary to confirm the promising *in vitro* results reported here.

In conclusion, the multikinase inhibitor sorafenib was found to inhibit the PERK pathway regulating protein translation in the cells possessing high p-eIF2 α level and high nuclear Nrf2 expression. Furthermore, its pharmacological effects on the phosphorylation ratio of eIF2 α and its associated molecule, Nrf2 might be useful to predict efficiency of sorafenib administration. Silencing of PERK sensitized RCC cells to hydrogen peroxide and doxorubicin, probably due to oxidative stress. In addition, PERK inhibitor, sorafenib also sensitized to hydrogen peroxide and doxorubicin. Conversely, doxorubicin could sensitize to sorafenib. These findings indicate that doxorubicin might be a useful agent for treatment of mRCC patients in combination with sorafenib.

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