Activation of Phosphatidylinositol 3-Kinase/Akt Signaling Pathway Mediates Acquired Resistance to Sorafenib in Hepatocellular Carcinoma Cells

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ABSTRACT

Hepatocellular carcinoma (HCC) is one of the most common potentially lethal human malignancies worldwide. Sorafenib, a tyrosine kinase inhibitor, was recently approved by the United States Food and Drug Administration for HCC. In this study, we established two sorafenib-resistant HCC cell lines from Huh7, a human HCC cell line, by long-term exposure of cells to sorafenib. Sorafenib induced significant apoptosis in Huh7 cells; however, Huh7-R1 and Huh7-R2 showed significant resistance to sorafenib-induced apoptosis at the clinical relevant concentrations (up to 10 

Whereas tumor suppressor phosphatase and tensin homolog were down-regulated in these resistant cells. In addition, ectopic expression of constitutive Akt in Huh7 demonstrated similar resistance to sorafenib. The knockdown of Akt by RNA interference reversed resistance to sorafenib in Huh7-R1 cells, indicating the importance of Akt in drug sensitivity. Furthermore, the combination of 8-[4-(1-aminocyclobutyl)phenyl]-9-phenyl-1,2,4-triazolo[3,4-f][1,6]naphthyridin-3(2H)-one dihydrochloride (MK-2206), a novel allosteric Akt inhibitor, and sorafenib restored the sensitivity of resistant cells to sorafenib-induced apoptosis. In conclusion, activation of PI3K/Akt signaling pathway mediates acquired resistance to sorafenib in HCC, and the combination of sorafenib and MK-2206, an Akt inhibitor, overcomes the resistance at clinical achievable concentrations.

Introduction

Human hepatocellular carcinoma (HCC) is one of the most prevalent malignant tumors worldwide (Bruix and Llovet, 2003; Huo et al., 2005). Surgical resection and traditional chemotherapy are typical forms of treatment for patients with HCC (Bioulac-Sage et al., 2009; Di Maio et al., 2009; Fransvea et al., 2009); however, targeted anticancer therapies using small molecules provide significant benefits in patients with HCC who do not respond well to traditional treatment (Thomas, 2009; Huynh, 2010; Midorikawa et al., 2010; Villanueva et al., 2010; Vitale et al., 2010).

Sorafenib (Nexavar), a multiple kinase inhibitor, is the first and only drug that is clinically approved for patients with advanced HCC (Palmer, 2008; Di Maio et al., 2009; Johnson and Billingham, 2009; Liu et al., 2009; Scanga and Kowdley, 2009). The major target of sorafenib is the serine-threonine kinase Raf-1, which is involved in the Ras/Raf/MEK/mitogen-activated protein kinase signaling cascade (Wilhelm et al., 2004; Panka et al., 2006). In in vitro kinase assay, sorafenib could efficiently inhibit the activity of Raf-1 at a very low dose (IC50 of 6 nM) (Adnane et al., 2006; Wilhelm et al., 2006). Other receptor tyrosine kinases are...
also suppressed by sorafenib, including vascular endothelial growth factor receptors 1, 2, and 3, platelet-derived growth factor receptor, and fibroblast growth factor receptor (Wilhelm et al., 2006; Zhang et al., 2008). Although sorafenib showed survival benefits in large randomized phase III studies, the response rate of sorafenib is actually quite low (2–3%) (Llovet et al., 2008; Cheng et al., 2009). In addition, therapeutic biomarkers that may predict the response to sorafenib are not currently available. Therefore, to improve the treatment response in HCC, it is important to identify the molecular mechanism of sorafenib resistance.

PI3K/Akt signaling is an important survival/proliferative pathway involving various growth factors, cytokines, and activation of receptors (Liu et al., 2009). Akt is up-regulated in many human cancer types and links to oncogenesis to alter cellular functions (Liu et al., 2009). Akt-related molecules

**Fig. 1.** Establishment and verification of sorafenib-resistant Huh7 cell lines. A, the apoptotic effect of sorafenib in wide-type and resistant Huh7 HCC cells. Cells were exposed to sorafenib at the indicated concentrations for 48 h, and the apoptotic assay was analyzed by sub-G1 percentage. Columns, mean (n = 6); bars, mean ± S.D. *P < 0.05. B, dose-dependent apoptotic analysis of sorafenib in Hep3B and PLC5. Apoptotic cells were determined by flow cytometry. C, analysis of caspase activation and PARP cleavage. Wild-type and resistant Huh7 cells were exposed to sorafenib at the indicated doses (micromolar) in 5% FBS-containing medium for 48 or 72 h. Cell lysates were prepared and assayed for the activation of caspase-9, caspase-3, and PARP by Western blot. CF, cleaved form (activated form). D, the cytotoxic effects of sorafenib in Huh7 cells. Cells were exposed to sorafenib at the indicated concentrations for 24 h, and cell viability was analyzed by MTT assay. Columns, mean (n = 6); bars, mean ± S.D. *P < 0.05.
A. Raf-1 activity assay in sensitive and resistant Huh7 cells. Cells were treated with sorafenib at 10 μM, and cell lysate was incubated with recombinant MEK protein for 30 min. Quantitative Raf-1 activity was determined by immunoblot analysis of pMEK (Ser218/222)/MEK2 (Ser222/226) expression. Results are representative of three independent experiments. Columns, mean (n = 3); bars, mean ± S.D. * P < 0.05.

B. Analysis of phospho-Akt (Ser473 and Thr308), Akt1, caspase-9, and PARP in sensitive and resistant Huh7 cell lines. Cells were exposed to sorafenib at 10 μM for 48 h. Apoptotic-related proteins were blotted with anti-caspase-9 and PARP antibody. CF, cleaved form (activated form).

C. Protein levels of PI3K/Akt pathway and other sorafenib-related molecules. Cells were exposed to sorafenib at 10 μM for 48 h, and cell lysates were prepared for the analysis of protein expression by Western blotting.

Fig. 2. Activation of PI3K/Akt pathway is associated with resistance to sorafenib in HCC cells. A, Raf-1 activity assay in sensitive and resistant Huh7 cells. Cells were treated with sorafenib at 10 μM, and cell lysate was incubated with recombinant MEK protein for 30 min. Quantitative Raf-1 activity was determined by immunoblot analysis of pMEK (Ser218/222)/MEK2 (Ser222/226) expression. Results are representative of three independent experiments. Columns, mean (n = 3); bars, mean ± S.D. * P < 0.05.

B, analysis of phospho-Akt (Ser473 and Thr308), Akt1, caspase-9, and PARP in sensitive and resistant Huh7 cell lines. Cells were exposed to sorafenib at 10 μM for 48 h. Apoptotic-related proteins were blotted with anti-caspase-9 and PARP antibody. CF, cleaved form (activated form).

C, protein levels of PI3K/Akt pathway and other sorafenib-related molecules. Cells were exposed to sorafenib at 10 μM for 48 h, and cell lysates were prepared for the analysis of protein expression by Western blotting.
2,5-diphenyl-2H-tetrazolium bromide (MTT) assay in six replicates. Cells (5000/well) were seeded and incubated in 96-well, flat-bottomed plates in DMEM with 10% FBS for 24 h and exposed to various concentrations of test agents in 5% FBS-supplemented DMEM for 24 h. Controls received DMSO vehicle at a concentration equal to that of drug-treated cells. The medium was removed and replaced by 200 μl of 0.5 mg/ml MTT in 10% FBS-containing DMEM, and cells were incubated in the CO2 incubator at 37°C for 2 h. Supernatants were removed from the wells, and the reduced MTT dye was dissolved in 200 μl/well DMSO. Absorbance at 570 nm was determined on a plate reader.

**Apoptosis Analysis.** The following three methods were used to assess drug-induced apoptotic cell death: the measurement of apoptotic cells by flow cytometry (sub-G1), Western blot analysis of caspase activation, and PARP cleavage.

**Raf-1 Activity.** The Raf-1 kinase assay kit was obtained from Millipore Corporation (Billerica, MA) and used according to the protocol provided by manufacturer. In brief, wide-type or resistant Huh7 cells were treated with 10 μM sorafenib for 24 h, and the Raf-1-containing extract was collected. The Huh7 lysate, including Raf-1 kinase protein, was incubated with recombinant MEK at 30°C for 30 min. Phospho-MEK1/2 antibody was used to evaluate the kinase activity of Raf-1.

**Statistical Analysis.** Comparisons of mean values were performed using the independent sample Student’s t test in SPSS for Windows 11.5 software (SPSS, Inc., Chicago, IL) (Chen et al., 2008).

### Results

**Establishment and Verification of Sorafenib-Resistant Huh7 Cell Lines.** To investigate the molecular mechanism of the acquired resistance to sorafenib, we established several HCC cell lines by long-term exposure to sorafenib at low doses escalating to higher doses for a long period of time. We obtained two sorafenib-resistant cell lines. We examined the apoptotic effects of sorafenib by analyzing the sub-G1 percentage with flow cytometry. As shown in Fig. 1A, wild-type Huh7 cells showed apoptosis in the presence of sorafenib in a dose-dependent manner. In contrast, sorafenib-resistant Huh7 cells (Huh7-R1 and Huh7-R2) showed resistance to sorafenib-induced apoptosis, even at 10 μM (the highest clinical achievable concentration). We also examined the apoptotic effect of sorafenib in other HCC cell lines. Sorafenib exhibited apoptotic effects in Hep3B and PLC5 cells in a dose-dependent manner, giving results similar to those obtained with wild-type Huh7 (Fig. 1B). Furthermore, our data demonstrated that sorafenib induced the activation of caspase-9 and caspase-3 and the cleavage of PARP in a dose- and time-dependent manner in wild-type Huh7 cells, but not in Huh7-R1 or -R2 cells (Fig. 1C), indicating that our resistant cell lines did not respond to sorafenib treatment at the clinical relevant dose (10 μM). In addition, we examined the effect of sorafenib on cell viability in all Huh7
cells. Sorafenib had significant cytotoxic effects in wild-type Huh7 cells in a dose-dependent manner, whereas resistant Huh7 cells (Huh7-R1 and -R2) were less sensitive to sorafenib. These data indicate that Huh7-R1 and -R2 are resistant to the cytotoxic effect of sorafenib (Fig. 1D).

Activation of PI3K/Akt Is Associated with Resistance to Sorafenib. To identify the molecule mechanism of the resistance to sorafenib, we first examined the effect of Raf-1 activity on wild-type or resistant Huh7 cells in the presence of sorafenib treatment. We unexpectedly found that there was no significant difference between Huh7 and Huh7-R1 cells in sorafenib-induced reduction of Raf-1 activity (Fig. 2A). The Raf-1 kinase activity on phospho-MEK1/2 was down-regulated significantly by sorafenib treatment in both Huh7 and Huh7-R cells, suggesting that Raf-1 may not play a role in mediating the drug resistance in resistant cells. It is noteworthy that we found that resistant cells showed up-regulation of Akt signaling in comparison with sensitive cells. As shown in Fig. 2B, phospho-Akt (Ser473 and Thr308) and Akt were overexpressed in resistant Huh7 cells. It is noteworthy that sorafenib induced apoptosis as shown by the activation of caspase-9 and PARP cleavage in wild-type Huh7, whereas no apoptosis was noted in resistant cells (Fig. 2B). In light of the significant role of the PI3K/Akt pathway in tumorigenesis, we examined the molecules related to the PI3K/Akt pathway to clarify its role in mediating the sorafenib resistance (Fig. 2C). PI3K is composed of a p85-regulatory subunit as well as a p110-catalytic subunit and mediates receptor binding, cellular localization, and enzyme activation by the cooperation of the two subunits. Our data showed that up-regulation of PI3K (p85) was found in Huh7-R1 and -R2. In contrast, PTEN, a PI3K suppressor, was down-regulated in resistant cells. We further assayed expression levels of downstream PI3K/Akt signaling proteins, which might also be affected in sorafenib-resistant cells. As shown in Fig. 2C, significant increases of mammalian target of rapamycin and S6 kinase were demonstrated in Huh7-R cells. In addition, we examined several known targets of sorafenib, including Erk, Mcl-1, and cyclin D1 in our HCC cells, and found that sorafenib down-regulated phospho-Erk, Mcl-1, and cyclin D1 in both sensitive and resistant cells, suggesting that these molecules may not play a role in mediating the resistance to sorafenib in our HCC cells (Fig. 2C).

Target Validation of Akt in Sorafenib-Resistant Cells. According to our previous findings, we hypothesized that the activation of Akt played a role in mediating the drug resistance; thus, next we validated the role of Akt in the effects of sorafenib. Our data showed that the percentage of apoptotic cells (Fig. 3A, left), along with the activation of caspases and the cleavage of PARP (Fig. 3A, right), increased in wild-type Huh7 cells under sorafenib treatment. However, Huh7 cells with ectopic expression of Akt were significantly protected from sorafenib-induced apoptosis. MK-2206, an Akt inhibitor, sensitized resistant cells to sorafenib-induced apoptosis. A, resistant cells were exposed to sorafenib and/or MK-2206 at the indicated concentrations for 48 h, and apoptotic assay was analyzed by flow cytometry. Columns, mean (n = 6); bars, mean ± S.D. *, P < 0.05. B, the combination of sorafenib and MK-2206 induced significant cell death in resistant cells. Huh7-R1 and -R2 cells were treated with sorafenib at 10 μM and/or MK-2206 at 10 μM for 48 h. Cell lysates were analyzed for phospho-Akt(Ser473), Akt, caspase-9, caspase-3, and PARP by Western blots. CF, cleaved form (activated form). C, the Akt-related proteins were assayed in the presence of 10 μM sorafenib and 10 μM MK-2206. Cell lysates were prepared for analysis of protein expression by Western blotting.
apoptosis, indicating that the activity of Akt could determine the drug sensitivity in HCC cells (Fig. 3A). We next employed small interference RNA (siRNA) to knock down Akt in HCC cells. As shown in Fig. 3B, down-regulation of Akt sensitized the HuH7-R1 cells to sorafenib-induced cell death, as shown by caspase activation and PARP cleavage (Fig. 3B). These data suggest that Akt, a vital survival factor, mediated resistance to sorafenib in HCC cell lines.

**MK-2206, an Akt Inhibitor, Sensitized Resistant Cells to Sorafenib-Induced Apoptosis.** We further explored the possibility of combining sorafenib with a novel drug that is potentially feasible for use in the clinic to overcome sorafenib-induced resistance. MK-2206, a highly selective non—ATP-competitive allosteric inhibitor of Akt, is currently undergoing clinical investigation for use with several types of a solid tumor. Our data showed that cotreatment with sorafenib and MK-2206 overcame the resistance to sorafenib in both HuH7-R1 and -R2 cells. The combination of these two drugs significantly increased the percentage of apoptotic cells in sorafenib-resistant HuH7 cells (Fig. 4A). In addition, down-regulation of phospho-Akt by MK-2206 sensitized resistant cells to sorafenib-induced cell death, as shown by the activation of caspases and PARP cleavage (Fig. 4B). It is noteworthy that MK-2206 alone did not show any significant effects on apoptosis in our resistant cells, suggesting that the inhibition of Akt is not sufficient to cause cell death in HCC cells. Moreover, we examined the molecules related to the PI3K/Akt signaling pathways and found that the 40-kDa proline-rich Akt substrate (PRAS40) was down-regulated in MK-2206-treated HuH7-R1 cells, indicating that MK-2206 inhibited Akt and its downstream signals in our cells. Other proteins, including p85, p110, PTEN, phosphatidylinositol 3-kinase-dependent 1, and mammalian target of rapamycin, were not altered significantly in the presence of combinational treatment for 48 h (Fig. 4C). Down-regulation of phospho-Erk was noted in sorafenib-treated cells, which is consistent with our previous finding (Fig. 2C). Therefore, our results indicated that the combination of sorafenib and Akt inhibitor may be a novel therapeutic strategy to overcome the sorafenib resistance in HCC.

**Sorafenib-Resistant HepG2 and Sk-Hep1 Cell Lines.** Besides HuH-7, we obtained two sorafenib-resistant cell lines from HepG2 and Sk-Hep1. We examined the cytotoxic effects of sorafenib in these cells. Sorafenib showed differential effects on cell viability in wild-type and resistant cells, indicating that HepG2-R and Sk-Hep1-R cells are resistant to the cytotoxic effect of sorafenib (Fig. 5, A and B). In addition, wild-type HepG2 and Sk-Hep1 cells showed apoptosis in the presence of sorafenib in a dose-dependent manner. However, sorafenib-resistant cells (HepG2-R and Sk-Hep1-R) showed resistance to sorafenib-induced apoptosis (Fig. 5, C and D). Moreover, resistant cells (HepG2-R and Sk-Hep1-R) had higher expressions of phospho-Akt (Ser473 and Thr308) and Akt. These data indicate that activation of Akt signaling pathway is present in resistant HepG2 and Sk-Hep1 cells (Fig. 5E).

**Discussion**

The recent clinical approval of sorafenib has marked the start of a new era in the pharmacological treatment of ad-

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**Fig. 5.** Sorafenib-resistant HepG2 and Sk-Hep1 cell lines. A and B, cell viability of sorafenib in wide-type and resistant HCC cells. A, HepG2. B, Sk-Hep1. Cells were exposed to sorafenib at the indicated concentrations for 24 h, and cell viability was analyzed by MTT assay. Columns, mean (n = 6); bars, mean ± S.D. *, P < 0.05. C and D, dose-dependent apoptotic analysis of sorafenib in HepG2 and Sk-Hep1. Apoptotic cells were determined by flow cytometry. E, analysis of phospho-Akt (Ser473 and Thr308) and Akt1 in HepG2 and Sk-Hep1 cells. WT, wild type; R, resistant.
advanced HCC (Palmer, 2008). Despite significantly prolonging the survival of patients with advanced HCC in large clinical trials (Llovet et al., 2008; Cheng et al., 2009), the tumor-response rate of sorafenib in HCC is very low (Escudier et al., 2007; Palmer, 2008; Liu et al., 2009). Therefore, we need to identify patients who might respond to drug treatment to improve the response rate. This study explored a new mechanism that may be important in mediating sorafenib resistance in HCC. We first selected resistant HCC cell lines from high-dose sorafenib-treated cells. Although sorafenib was designed as a Raf kinase inhibitor, there was no significant difference in Raf-1 kinase activity between the wild-type and resistant cell line. It is surprising that the activation of the PI3K/Akt signaling pathway was common in both resistant cell lines. Our data further validated the role of Akt in mediating the drug resistance. We found that siRNA knockdown did sensitize resistant cells to sorafenib. In contrast, Huh7 cells with overexpressed Akt were insensitive to sorafenib treatment. These data indicated that Akt plays a vital role in sorafenib resistance and provides a novel strategy through which sorafenib-based treatment may be improved. To test this new approach, we applied MK-2206, an Akt-specific inhibitor, in combination with sorafenib to our resistant cells. Our data showed that MK-2206 sensitized both Huh7-R1 and Huh7-R2 to sorafenib-induced cell death, indicating that combinational therapy of sorafenib and a PI3K/Akt inhibitor may be a good therapeutic approach to enhance current treatment.

PI3K/Akt signaling is aberrant pathways in HCC, and this pathway may be the critical target for therapeutic design (Liu et al., 2009; Lindsley, 2010). In addition to MK-2206, perifosine was tested in phase II clinical trials as an oral Akt inhibitor that targets the pleckstrin homology domain of Akt, thus preventing its translocation from the cytosol to the plasma membrane (Gills and Dennis, 2009). These Akt inhibitors could be promising agents to sensitize cells to sorafenib and improve the response rates in clinic.

Although we found that high levels of phospho-Akt expression were responsible for mediating the acquired resistance to sorafenib, the mechanism by which sorafenib regulates Akt and other PI3K molecules is still unclear. In our previous study, we showed that PP2A played an important role in regulating Akt activity and affected the response of HCC cells to molecular targeted drugs (Chen et al., 2008, 2009). Our recent study also showed that sorafenib might affect PP2A-Akt signals in HCC (Chen et al., 2010). Therefore, PP2A or other Akt-related phosphatase might be important in identifying the molecular mechanism of up-regulated Akt in sorafenib resistance. In conclusion, we have found that activation of PI3K/Akt may play a key role in mediating acquired resistance to sorafenib in HCC cells. The combination of sorafenib and an Akt-specific inhibitor is a feasible way to improve drug response. This study provides a new strategy to improve current therapy, and further clinical investigation is warranted.

Authorship Contributions
Participated in research design: K.-F. Chen, Hsu, P.-J. Chen, and Cheng.
Performed data analysis: K.-F. Chen and H.-L. Chen.
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