# OPA1 downregulation is involved in sorafenib-induced apoptosis in hepatocellular carcinoma

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Sorafenib has been used to treat advanced hepatocellular carcinoma (HCC), but the underlying molecular mechanisms remain controversial and why some patients do not respond to this therapy is poorly understood. In this study, we show that sorafenib triggers cell growth inhibition and apoptosis in HCC cells by directly targeting the mitochondria. Treatment with sorafenib induces rapid mitochondrial fragmentation, which is associated with the deregulation of mitochondria fusion-related protein optic atrophy 1 (OPA1). Exposure of cells or isolated mitochondria to sorafenib substantially induces cytochrome *c* release. Our data indicate that siRNA-mediated OPA1 knockdown significantly sensitizes HCC cells to sorafenib-induced apoptosis. Furthermore, sorafenib has no apparent apoptotic toxicity to normal human primary hepatocytes. Sorafenib inhibits HCC xenograft tumor growth *in vivo* and murine xenograft tumor tissue analysis reveals mitochondria fusion protein. OPA1 expression levels are strongly downregulated by sorafenib treatment. Western blotting evaluation of patient HCC with matched non-tumor tissue samples demonstrates that OPA1 expression is decreased in up to 40% of HCC patients. Taken together, we have shown that sorafenib suppresses the tumorigenesis of HCC through the induction of mitochondrial injury via OPA1. Our results provide new insights into the pathogenesis of HCC and suggest that OPA1 is a novel therapeutic target in patients with HCC.

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Hepatocellular carcinoma (HCC) is one of the five most common cancers worldwide. The 5-year survival rate of diagnosed HCC patients is <10%.1-2 Therefore, new therapeutic strategies are urgently needed. Sorafenib was initially identified as a Raf (serine/threonine-specific protein kinase) and multikinase inhibitor, which targets Raf,<sup>3</sup> Fms-like tyrosine kinase 3,4 c-Kit (CD117),5 p38 (mitogen-activated protein kinase),<sup>6</sup> stem cell growth factor receptor 1,<sup>7</sup> vascular endothelial growth factor receptors,8 and platelet-derived growth factor receptor- $\beta^9$  to induce growth arrest or apoptosis in a variety of malignant tumor cells. It has been reported that sorafenib treatment induces apoptosis in HepG2 cells through induction of growth arrest and DNA damage 45<sup>β</sup> (GADD-45β).<sup>10</sup> Other groups have demonstrated that pretreatment with the B-cell lymphoma 2 inhibitor (Bcl-2) ABT-737<sup>11</sup> or Let-7 microRNAs,<sup>12</sup> which block B-cell lymphoma-extra

large (Bcl-xL) activity, can enhance sorafenib-induced apoptosis in HepG2 cells. Thus, the precise molecular mechanism by which sorafenib exerts its effect remains controversial. Although recent clinical studies have shown that sorafenib could inhibit HCC growth and increase the survival of patients, the efficacy of sorafenib treatment is variable and many patients do not respond well to treatment.<sup>13–16</sup> Elucidating the molecular targets of sorafenib in HCC cells that exhibit sorafenib-induced apoptosis will provide useful clues for the identification of patients who will respond well to sorafenib treatment. Using a hepatoma cell line LH86 derived from a well-differentiated HCC in combination with an *in vivo* murine model of tumorigenesis, we aim to elucidate the mechanisms by which sorafenib inhibits HCC.

Abnormalities in liver mitochondrial metabolism have been found in patients with a variety of liver diseases including

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HCC.<sup>17,18</sup> Mitochondria are involved in cancer development and progression.<sup>19,20</sup> Mitochondria are dynamic organelles undergoing continuous fission and fusion to form a reticulum structure that is considered to be an important determinant of mitochondria function.<sup>21,22</sup> In mammalian cells, mitochondrial fusion and fission rely on multiple proteins, including fusion modulating mitofusin 1 (Mfn1), mitofusin 2 (Mfn2), and optic atrophy 1 (OPA1), as well as fission-related Drp1 (dynamin-related protein Drp1) and Fis1 (fission 1).<sup>23</sup> Mitochondrial dynamics have an important role in the process of apoptosis. Many apoptotic stimuli can elicit mitochondrial fragmentation during the early stages of apoptosis, and in microscopy studies this is characterized by the formation of abundant smaller and more-rounded

mitochondria. Inhibition of mitochondrial fragmentation not only preserves the mitochondrial architecture but also prevents the release of cytochrome c and subsequent apoptotic steps. The mitochondria are an attractive target for cancer therapeutic development.<sup>24</sup>

This study aims to further clarify the mode of function by which sorafenib acts. We provide evidence to show that sorafenib induces apoptosis in HCC cells through disruption of functionally modified mitochondria rather than by inhibition of the Ras-Raf or phosphoinositide 3-kinase-protein kinase B (PI3K-Akt) signal pathways. Our data indicate that mitochondria fusion protein OPA1 expression level is critical to determining the sensitivity of HCC to sorafenib-induced apoptosis.

### MATERIALS AND METHODS Ethics Statement

With the approval of the University of Florida Gainesville Health Science Center Institutional Review Board (IRB-01), archived frozen liver cancer tissues and paired non-tumor liver tissues were used in this study. No donor organs were obtained from executed prisoners or other institutionalized persons.

#### Materials

MitoTracker®-Red CMXRos was obtained from Invitrogen (Carlsbad, CA, USA); anti-β-actin, anti-Mfn1 monoclonal antibodies, and Hoechst 33258 were obtained from Sigma (St Louis, MO, USA); anti-Mfn2 and anti-OPA1 primary antibodies were purchased from Abcam (Cambridge, MA, USA); anti-caspase 9, anti-caspase 3 (cleaved (c)), antiphosphorylated (p)-c-Raf, anti-c-Raf, anti-K-Ras (Kirsten rat sarcoma viral oncogene homolog), anti-Akt, and anti-p473-Akt primary antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA); anti-voltage-dependent anion-selective channel 1 (VDAC1) primary antibody, goat anti-rabbit, and goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); and anti-cytochrome c monoclonal antibody was obtained from BD Bioscience (San Diego, CA, USA). Sorafenib was

obtained from Selleck Chemicals (Houston, TX, USA); CellTiter<sup>®</sup>96 Aqueous Non-Radioactive Cell Proliferation Assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS)) was purchased from Promega (Madison, WI, USA).

#### **Cell Culture and Reagents**

Human HCC cell lines (Huh7 and LH86) were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (Sigma) and antibiotics (100 U/ml penicillin and 100  $\mu$ g/ml streptomycin) at 37 °C in 5% CO<sub>2</sub>. Normal primary human hepatocytes were obtained from CellzDirect (Austin, TX, USA). The cells were cultured in the DMEM/F12 (1:1) culture medium.

#### Cell Viability Assay (MTS Assay)

Cell viability assays were performed as described previously.<sup>25</sup> Briefly, cells were untreated or treated with sorafenib, and cell viability was determined using the CellTiter<sup>®</sup>96 Aqueous One Solution Cell proliferation Assay kit (Promega) according to the supplier's protocol.

#### Western Blotting Analysis

Western blotting was performed as described previously.<sup>26</sup> Cells were treated and harvested. The cell pellets were resuspended in lysis buffer containing Nonidet P-40. After centrifugation, the supernatant was transferred to a new tube and the protein concentration was determined. Equivalent amount of samples were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis on 12% gels. Proteins were transferred to nitrocellulose membranes and probed with the indicated primary antibodies, followed by the appropriate secondary antibodies. Immunoreactive bands were detected using enhanced chemiluminescence (Pierce, Rockford, IL, USA). The molecular sizes of the proteins detected were determined by comparison with prestained protein markers (Bio-Rad, Hercules, CA, USA).

#### Detection of Apoptosis by Hoechst 33258 Staining

Cells were plated at a density of  $5 \times 10^4$  cells per ml in sixwell plates and treated with sorafenib. After treatment for desired intervals, cells were stained with Hoechst 33258 for 15 min at room temperature in the dark. Cells were then analyzed using fluorescence microscope. For quantitative analysis, apoptotic cell death ratio was assessed by calculating the number of apoptotic cells with condensed nuclei in 6–8 randomly selected areas.

## Immunofluorescence Staining

To assess the modification of mitochondrial morphology, cells grown on glass coverslips were treated with sorafenib for different intervals. MitoTracker<sup>38</sup>-Red probe was directly added into the culture medium and the culture plate was incubated for 15 min at 37  $^{\circ}$ C in the dark. Cells were washed with phosphate-buffered saline (PBS), fixed with 3.7%

paraformaldehyde at 37 °C for 15 min, and then quenched with 50 mM NH<sub>4</sub>Cl. The coverslips were then extensively washed and mounted in Mowiol reagent. Fluorescent images were obtained using an Olympus fluorescence microscope.

# Isolation of Mouse Mitochondria and Measurement of Cytochrome C Release

Mouse liver mitochondria were isolated as described previously,<sup>27</sup> and the protein content of isolated mitochondria was determined by the micro-biuret method using BSA as a standard. Equal mitochondrial fractions were treated with different concentrations of sorafenib. The samples were then centrifuged and cytochrome *c* released into the supernatant was detected by western blotting.

#### SiRNA-Mediated Knockdown

The siRNA knockdown experiments were performed as described previously.<sup>28</sup> Smart-pool pre-designed siRNA duplexes targeted against human OPA1 mRNA were obtained from Cell Signaling Technology. Cells were transfected with 100 nM siRNA duplex mixtures for 24 h in the presence of Lipofectamine RNAiMax (Invitrogen) according to the manufacturer's instructions. A nonspecific random siRNA (Cell Signaling Biotechnology) was also transfected at the same concentration as the control.

#### **Detection of Apoptosis by DNA Ladder Assay**

A DNA ladder assay was performed as described previously with modification.<sup>29</sup> Briefly, cells treated under different conditions were harvested and washed with  $1 \times PBS$ . Cell pellets were resuspended with lysis buffer. The samples were centrifuged and supernatants were transferred to new tubes. SDS and RNase A were added to the supernatants, and the mixture was incubated at 56 °C for 2 h. Proteinase K was added and the samples were incubated at 37 °C for 2 h. DNA was precipitated with the addition of 10 M ammonium acetate and ethanol, washed once with 70% ethanol, and then dissolved in water and separated by electrophoresis in 1% agarose gel.

#### Subcellular Fractionation

This assay was performed as described previously.<sup>30</sup> Cells were harvested and resuspended in three volumes of hypotonic buffer. After gentle homogenization with a dounce homogenizer, cell lysates were centrifuged to remove unbroken cells and nuclei. The post-nuclear supernatant was centrifuged to pellet the mitochondriaenriched heavy membrane fraction. The supernatant was further centrifuged to obtain the cytosolic fraction. A 30- $\mu$ l aliquot cytosolic fraction was analyzed by western blotting with anti-cytochrome *c* monoclonal antibody.

#### **Tumor Growth Inhibition Assay**

The 8-week-old SCID mice managed humanely according to the criteria outlined in the 'Guide for the Care and Use of Laboratory Animals' (NIH publication 86-23, revised 1985) were divided into two groups. Each mouse (4 per group) was inoculated subcutaneously with  $5 \times 10^6$  cells resuspended in 100 µl PBS in the right flank. Treatment with sorafenib was initiated when tumors were palpable (day 7 after inoculation) by daily per oral administration of sorafenib (100 mg/kg body weight) or vehicle only for a total of 7 days. At the end point of the experiment, mice were euthanized following the University of Florida IACUC-approved protocols. Each tumor was excised, weighed, and fixed in formalin. The effect of drug treatment on tumor growth was analyzed statistically with individual group comparison and evaluated with the Student's paired *t*-test by SPSS 15.0.

#### RESULTS

#### Sorafenib Induces Apoptosis in HCC Cells

To investigate the apoptotic effects of sorafenib on HCC cells, we incubated Huh7 and LH86 cells with sorafenib. MTS assays demonstrated that sorafenib induced significant concentration-dependent cell growth inhibition in both cell lines (Figure 1a). Nuclear condensation assays showed that sorafenib treatment induced significant apoptotic nuclear condensation in both cell types (Figures 1b and c). Consistent with the results shown in Figure 1b, DNA ladder assays also demonstrated that sorafenib treatment resulted in apoptosis in a concentration-dependent manner in both HCC cell lines (Figure 1d). To further evaluate sorafenib-induced apoptosis in HCC cells, caspase activation was assessed by western blotting analysis. Our data indicated that treatment with sorafenib triggered proteolytic processing of caspase 9 and caspase 3 (Figure 1e). Taken together, these results suggest that sorafenib induces apoptosis in HCC cell lines and the proteolytic activation of caspase 9 and caspase 3 indicates that sorafenib may trigger mitochondria-mediated apoptosis.

#### Effects of Sorafenib on Ras-Raf and PI3K-Akt Antiapoptotic Signal Pathways

It has been reported that sorafenib induces apoptosis by inactivation of the Ras-Raf and Akt survival pathways in non-HCC cells.<sup>31,32</sup> To gain further insight into the downstream apoptotic effects of sorafenib on HCC, we analyzed the expression of K-Ras, c-Raf, p-c-Raf, p473-Akt, and Akt in response to sorafenib treatment. We found that treatment with sorafenib induced upregulation of p-c-Raf and p473-Akt in a time-dependent manner. Expression levels of total K-Ras, Akt, and c-Raf were not significantly affected (Figures 2a and b). More importantly, we observed that p-c-Raf and p473-Akt were increased in sorafenib-treated cells in a concentration-dependent manner, even though apoptosis occurred as mentioned in Figure 1 (Figures 2c and d). These results suggest that treatment with sorafenib increases activation of the Raf and Akt signaling pathways in the HCC cells, and that the effect of sorafenib may be cell type dependent or sorafenib targets other pathways to induce apoptosis in HCC cells.



**Figure 1** Sorafenib induces apoptosis in hepatocellular carcinoma (HCC) cells. (a) Huh7 and LH86 cells were treated with sorafenib as indicated. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) assay was performed to detect cell viability (\*P < 0.05, "P < 0.05). (b) Cells were treated with sorafenib as indicated. Nuclear condensation was assessed by Hoechst staining to show apoptotic nuclei as described in 'Materials and Methods'. (c) Cells were treated with sorafenib and nuclear condensation was assessed by 33258 staining. The apoptosis ratio was analyzed by counting cells with apoptotic nuclei under a fluorescent microscope (representative apoptotic cells were marked with white arrows, \*P < 0.05). (d) Cells were treated with sorafenib and DNA ladder assay was carried out to show DNA fragmentation (M: 1 kb plus DNA marker). (e) HCC cells were treated with sorafenib (20 µM) for different times (top panel) or were treated with various concentrations of sorafenib for 24 h (bottom panel). Cell lysates were prepared and subjected to western blotting to examine cleaved caspase 9 and caspase 3 protein levels with specific antibodies. β-Actin was used as an equal protein loading control.



**Figure 2** Effects of sorafenib on Ras-Raf and phosphoinositide 3-kinaseprotein kinase B (PI3K-Akt) signal pathways. (**a**) Huh7 and (**b**) LH86 cells were treated with sorafenib as indicated. Cell lysates were prepared and subjected to western blotting to detect phosphorylated Raf (p-c-Raf), cleaved (c)-Raf, K-Ras (Kirsten rat sarcoma viral oncogene homolog), p473-Akt, and Akt with specific antibodies, respectively.  $\beta$ -Actin was used as an equal protein loading control. (**c**) Huh7 and (**d**) LH86 cells were treated with various concentrations of sorafenib for 24 h. Cell lysates were prepared and subjected to western blotting to detect p-c-Raf, c-Raf, K-Ras, p473-Akt, and Akt with specific antibodies, respectively.  $\beta$ -Actin was used as an equal protein loading control.

## Sorafenib Induces Mitochondrial Fragmentation in HCC Cells

To examine if sorafenib can induce mitochondrial dynamic changes, cells were treated with sorafenib and then stained with membrane potential-dependent MitoTracker<sup>®</sup>-Red probes to show mitochondrial morphology and membrane potential. The mitochondrial network was severely damaged after sorafenib treatment, which was reflected by the presence of smaller, more-rounded mitochondria (from 15 min) and membrane potential loss (at 60 min) (Figures 3a and b). The number of cells with fragmented mitochondria increased upon additional incubation time (Figures 3c and d). These results suggest that sorafenib induces cellular mitochondria fragmentation in a time-dependent manner in HCC cells.

#### Sorafenib Induces Mitochondria Cytochrome C Release

To provide further evidence that sorafenib induces apoptosis through targeting mitochondria dynamics, Huh7 cells were treated with various concentrations of sorafenib. We observed that sorafenib induces cytochrome c release into the cytosol in cultured HCC cells (Figures 4a and b). Furthermore, to eliminate the possibility that sorafenib may be indirectly interacting with other cellular mechanisms or pathways, purified mitochondria were studied on incubation with sorafenib. Our data showed that sorafenib could directly induce cytochrome c release from mitochondria purified from HCC cells (Figure 4c). We found that sorafenib triggered cytochrome c release from mitochondria isolated from normal mouse liver tissues in a concentration- and timedependent manner (Figures 4d and e). These data suggest that not only does sorafenib abrogate mitochondrial remodeling in HCC cells but also that there is sufficient mitochondrial disruption to release cytochrome c into the cytoplasm to induce apoptosis. The data also provide evidence that sorafenib directly interacts with the mitochondria and does not act by an intermediate mechanism.

#### Sorafenib Deregulates Expression of Mitochondrial Fusion Protein OPA1

Dynamic control of mitochondrial structure is maintained by a number of mitochondria-shaping proteins that include both fusion (Mfn1, Mfn2, and OPA1) and fission members. OPA1 is a major organizer of the mitochondrial inner membrane, maintains mitochondria DNA stability and replication, and is involved in the sequestration of cytochrome *c*. It is reasonable to hypothesize that OPA1 may be a target for mitochondrial apoptotic effectors.<sup>33</sup> To determine the mechanisms underlying sorafenib-induced mitochondrial fragmentation, Huh7 and LH86 cells were treated with sorafenib for 0-60 min. We found that the expression of mitochondria fusion-related protein OPA1 was decreased, whereas the other two members, Mfn1 and Mfn2, were largely unchanged in both cell types (Figure 5a). Further experiments showed that, upon treatment with sorafenib over 24 h, OPA1 down regulation was more profound, with additional downregulation of Mfn1 and Mfn2 (Figure 5b). As the loss of OPA1 expression is a common consequence of sorafenib treatment in both HCC cell lines, we decided to investigate the role of OPA1 expression further to determine if OPA1 regulation by sorafenib is involved in apoptosis of HCC cells. We observed that OPA1 knockdown (Figure 5c) could significantly sensitize Huh7 cells to sorafenib-induced apoptosis (Figure 5d and Supplementary Figure S1). These data suggest that OPA1 is a target of sorafenib, and that by targeted knockdown of OPA1, HCC cells can be further sensitized to apoptosis induced by sorafenib.

## Sorafenib has no Apoptotic Toxicity to Normal Human Liver Cells

We have shown that sorafenib induced apoptosis in HCC cell lines by targeting OPA1. OPA1 has an important role in mitochondrial remodeling and stability; therefore, to determine if sorafenib treatment is toxic to normal human primary liver cells (hepatocytes), both hepatocytes and HCC cells were treated with sorafenib. We found that sorafenib had no detectable apoptotic effects on hepatocytes, whereas sorafenib treatment did induce apoptosis in HCC cells that served as a positive control (Figure 6a). To assess whether sorafenib can induce mitochondria fragmentation in hepatocytes, cells were untreated or treated with sorafenib. We found that sorafenib could not induce apparent mitochondrial fragmentation in hepatocytes, while it did in positive control HCC cells (Figure 6b). To understand why



**Figure 3** Sorafenib induces mitochondrial fragmentation in hepatocellular carcinoma (HCC) cells. (a) LH86 and (b) Huh7 cells were treated with sorafenib and were stained with mitochondria membrane-dependent MitoTracker<sup>®</sup>-Red probe. Then, mitochondria morphology was observed under fluorescence microscope (Red) (areas marked with white boxes were enlarged (zoom-in)). (c) LH86 and (d) Huh7 cells were treated with sorafenib (20  $\mu$ M) for indicated times and the number of cells with fragmented mitochondria was counted (\**P* < 0.05).

the mitochondria of hepatocytes are resistant to sorafenib, we compared mitochondrial fusion protein expression between hepatocytes and HCC cells. Hepatocytes express markedly higher levels of OPA1, Mfn1, and Mfn2 than HCC cells (Figure 6c). To further assess the roles of mitochondria fusion-related protein in hepatocytes, cells were treated with sorafenib for a short time course (0–60 min) and a longer time course for 24 h. We found that mitochondria fusion proteins OPA1, Mfn1, and Mfn2 were not significantly affected by sorafenib treatment in hepatocytes (Figures 6d and e). These results suggest that hepatocytes are resistant to

sorafenib and that the mitochondrial dynamic processes in hepatocytes are not affected by sorafenib treatment; the data also show that HCC cells with lower expression of OPA1, Mfn1, and Mfn2 are less resistant to apoptotic induction by sorafenib.

#### Sorafenib Exerts Tumor Growth Inhibition In Vivo

To investigate whether the antitumor effects of sorafenib *in vivo* are mediated by mitochondria fragmentation, HCC xenograft tumor models were established in SCID mice by injection of HCC cells. Analysis of HCC xenograft tumors



Figure 4 Sorafenib induces cytochrome c release. (a) Huh7 cells treated with different concentrations of sorafenib or  $({\bf b})$  with a various time periods were harvested and mitochondria were purified by subcellular fractionation. Cytochrome c in the supernatant was detected with anticytochrome c monoclonal antibody. Voltage-dependent anion-selective channel 1 (VDAC1) from total mitochondria was used as a control for equal protein loading. (c) Isolated mitochondria from hepatocellular carcinoma (HCC) cells were incubated with sorafenib. Cytochrome c release was assessed by western blotting with specific cytochrome c antibody. VDAC1 was set up for equal protein loading control. (d) Purified mouse liver mitochondria were incubated with different concentrations of sorafenib and cytochrome c release was measured by western blotting. VDAC1 was used as a control for equal protein loading. (e) Mitochondria were prepared from mouse liver tissue. The same amount of mitochondria was treated with sorafenib for time periods as indicated. Cytochrome c levels were detected by western blotting with cytochrome c antibody. VDAC1 was used as a control for equal protein loading.

from the control buffer- and sorafenib-treated mice revealed that sorafenib administration effectively inhibits HCC tumor growth *in vivo* (Figure 7a). There was a detectable difference in tumor mass with at least a 10-fold decrease in sorafenibtreated mice when compared with untreated control mice (Figure 7b). Moreover, tumor tissues from untreated or treated groups were analyzed to examine OPA1, Mfn1, and Mfn2 expression levels. Our results indicated that sorafenib treatment significantly reduced the expression of OPA1 as well as Mfn1 and Mfn2 in xenograft HCC tumors (Figure 7c). Similar to the *in vitro* findings, these results suggest that sorafenib inhibits HCC tumor growth *in vivo by* targeting mitochondria dynamic-related proteins.

#### OPA1 Expression in Human Normal Liver Tissues and Malignant Liver Tissues

To establish the clinical relevance of these data, paired adjacent normal liver tissue and malignant HCC liver tissue from patients were collected and the expression of mitochondria fusion-related proteins, including OPA1, Mfn1, and Mfn2, were analyzed by western blotting. Matched comparisons with adjacent normal liver tissues showed that there was consistent downregulation of OPA1 protein expression in 12 out of 30 randomly selected liver tumor



Figure 5 Sorafenib deregulates the expression of mitochondrial fusion protein optic atrophy 1 (OPA1). (a) Hepatocellular carcinoma (HCC) cells were treated with sorafenib for up to 360 min and harvested. Cell lysates were prepared and subjected to western blotting to examine protein levels of OPA1, mitofusin 1 (Mfn1), and mitofusin 2 (Mfn2) with specific antibodies, respectively. β-Actin was used as an equal protein loading control. (b) Huh7 cells were treated with various concentrations of sorafenib for 24 h and cell lysates were prepared and subjected to western blotting to detect OPA1, Mfn1, and Mfn2 expression levels with specific antibodies, respectively. B-Actin was used as an equal protein loading control. (c) Huh7 cells transfected with synthesized random control small interfering RNA (siRNA) and specific OPA1 siRNA were treated with sorafenib. Cells were harvested and subjected to western blotting analysis. OPA1 expression was detected with specific antibody. (d) Cleaved caspase 3 expression level was examined with specific polyclonal antibodies. β-Actin was served as an equal protein loading control.

tissues (only representative data were shown) (Figure 8a). However, as we know that more than 90% of HCC arise from cirrhotic background, it is likely that the comparison was made between cirrhotic liver tissues and HCC tissues. In our study, further tissue analysis revealed that the OPA1 expression levels in matched HCC tissues (HCC) are lower by 50% than those in normal (non-cirrhotic) liver tissues (NL), and the difference between matched cirrhotic tissues (Cirrhotic) and HCC tissues (HCC) is minimal (Figure 8b).

#### DISCUSSION

Approved by the FDA to treat unresectable HCC, sorafenib is currently the main drug used in the treatment of patients with advanced HCC.<sup>34–36</sup> Although oral administration of



**Figure 6** Sorafenib has no apoptotic toxicity to normal primary liver cells. (a) Normal human hepatocyte cultures or LH86 cells (positive control) were treated with sorafenib. Caspase 3 activation was measured by detecting the cleaved bands through western blotting. (b) Hepatocellular carcinoma (HCC) or normal hepatocytes were treated with sorafenib. MitoTracker<sup>®</sup>-Red probe was added into the culture medium. Mitochondria morphology was observed by fluorescence microscope (Red). White boxes demarcate areas further magnified (zoom-in). (c) Cell lysates from normal hepatocytes, LH86, and Huh7 were prepared and subjected to western blotting. Optic atrophy 1 (OPA1), mitofusin 1 (Mfn1), and mitofusin 2 (Mfn2) expression levels were assessed with specific antibodies, respectively. β-Actin was used as an equal protein loading control. Relative band intensities (RBIs) were analyzed by the Image J software. Normal hepatocytes were treated with sorafenib (d) for a short time course or (e) a longer time course. Cell lysates were prepared and subjected to western blotting. OPA1, Mfn1, and Mfn2 expression levels were assessed with specific antibodies, respectively. β-Actin was used as an equal protein loading control. Relative band intensities (RBIs) were analyzed by the Image J software. Normal hepatocytes were treated with sorafenib (d) for a short time course or (e) a longer time course. Cell lysates were prepared and subjected to western blotting. OPA1, Mfn1, and Mfn2 expression levels were assessed with specific antibodies, respectively. β-Actin was used as an equal protein loading control.

sorafenib has been shown to improve overall survival from a median of 7.9 to 10.7 months in patients with advanced liver cancer, the efficacy and adverse reactions differ in individuals. Understanding the underlying molecular mechanisms will enable the identification of HCC patients who will benefit from a sorafenib-based treatment. In this study, we find that sorafenib could induce apoptosis by targeting mitochondria dynamics rather than inhibiting the Raf and Akt survival signal pathways. In addition, deregulation of mitochondrial fusion protein OPA1 has a key role in the induction of apoptosis in HCC cells and inhibition of HCC xenograft tumor growth *in vivo*.

Sorafenib, commonly described as a Raf-1 and multikinase inhibitor, has been shown to be able to interfere with the Ras-Raf signal pathway in diverse transformed cells.<sup>3,37,38</sup> Surprisingly, here we show that sorafenib induces activation of Raf and Akt in two different HCC cell lines, suggesting that the sorafenib-induced HCC cell apoptosis may be independent of Raf and Akt pathway inhibition. Although these findings directly contrast reports that sorafenib inhibits Ras-Raf or Akt signal pathways in other cancer cell lines.<sup>39–41</sup> Other investigators have also reported that sorafenib could induce cell death independent of Raf/MEK/extracellular signal-regulated kinase (ERK) inhibition.<sup>38,42,43</sup> Our findings indicate that the effects of sorafenib on Raf/ERK1/2 activation are likely to be both cell type- and context-specific.

To determine the mechanism of sorafenib-induced apoptosis in treated HCC cells, we performed a series of experiments to examine the injury of mitochondria. We observed that sorafenib treatment induced mitochondria fragmentation within 15 min, after treatment for 60 min, mitochondria membrane potential was significantly decreased, and fragmented



**Figure 7** Sorafenib exerts tumor growth inhibition *in vivo*. (a) BALB/c severe combined immunodeficiency (SCID) mice were inoculated subcutaneously with Huh7 cells. The mice were treated with 100 mg/kg body weight sorafenib or vehicle orally for 7 days. At the end point of the experiment, mice were euthanized and tumors were excised. Pictures of excised tumors from xenograft mice, showing larger tumors from untreated (control) mice than those from sorafenib-treated mice. (b) Mass of tumors in nude mice from the control group and from the group treated with sorafenib were compared. Statistical analysis was performed to show inhibitory effects of sorafenib on the mass of implanted tumors in nude mice ( $^*P$ <0.05). (c) Xenograft HCC tumors from untreated groups and sorafenib-treated groups were collected and lysates were prepared. Western blotting was performed to detect mitochondria fusion-related proteins optic atrophy 1 (OPA1), mitofusin 1 (Mfn1), and mitofusin 2 (Mfn2) with specific antibodies, respectively.  $\beta$ -Actin protein levels were assessed as an equal protein loading control. The four pairs of tissues tested were randomly selected sample pairs from untreated or sorafenib-treated tumor mice.

mitochondria were observed in up to 90% of treated cells. We also show that sorafenib induces cytochrome c release when cells were treated with sorafenib at different concentrations. We provide critical evidence that sorafenib can directly induce cytochrome c release when mitochondria were incubated with sorafenib in a concentration- and time-dependent manner, which correlated with mitochondria fragmentation. These lines of evidence support the notion that sorafenib induces apoptosis by directly targeting mitochondria injury rather than Ras-Raf or Akt signaling pathway inhibition, at least in these evaluated HCC cells.

It is well documented that mitochondria fragmentation is regulated by mitochondria fusion- and fission-related proteins. OPA1 is a major organizer of the mitochondrial inner membrane and is important for maintaining normal mitochondria cristae structure and function and protecting cells from apoptosis by the sequestration of cytochrome c release. OPA1 mutation and knockout has been shown to cause severe mitochondria morphology collapse and subsequent cell death. Also, other studies have shown that molecules such as SLP2 and PHB1 act to induce apoptosis via an OPA1-dependent pathway, which can be blocked by overexpression or functional site mutation of OPA1.44,45 In our study, to identify the molecular target that mediates mitochondria injury induced by sorafenib, we examined OPA1 expression levels in sorafenib-treated cells. Results demonstrated that sorafenib rapidly reduced the expression of mitochondrial fusion protein OPA1 within 15 min, which coincides with mitochondria fragmentation occurrence and a delayed cytochrome c release, suggesting that sorafenib promotes mitochondria fragmentation and cytochrome c release by rapid downregulation of OPA1. More interestingly, we show that HCC cells can be sensitized to sorafenib-induced apoptosis by OPA1 siRNA knockdown. These data imply that OPA1 expression level is very important in determining cell sensitivity to sorafenib-induced cytotoxicity. These results are in agreement with previous reports that OPA1 downregulation induced mitochondria fragmentation and apoptosis in non-liver cancer cells.<sup>46</sup> Our findings contribute to the idea that OPA1 is a central regulator of mitochondrial cristae structure and is essential for the maintenance of mitochondrial structure. In addition, we also observed that Mfn1 and Mfn2 proteins are downregulated in sorafenib-treated cells and in HCC patient tissues and correlate with OPA1



**Figure 8** Optic atrophy 1 (OPA1) expression in human normal liver tissues and liver tumor (hepatocellular carcinoma (HCC)) tissues. (a) Expression of OPA1, mitofusin 1 (Mfn1), and mitofusin 2 (Mfn2) proteins in adjacent normal tissues and malignant liver tissues. Lysates from adjacent normal liver tissues and liver tumor tissues from different patients were prepared and subjected to western blotting. OPA1, Mfn1, and Mfn2 expression levels were detected with specific antibodies.  $\beta$ -Actin protein levels were assessed as an equal protein loading control (P1–12: randomly selected patient number). (b) OPA1 protein level analysis in paired normal liver (NL) tissues and liver tumor (HCC) tissues, and paired cirrhotic liver tissues and liver tumor tissues (HCC). Relative band intensities (RBIs) of OPA1 were analyzed by the Image J software to show relative OPA1 protein levels in paired tissues from patients. Statistical analysis was performed to show average OPA1 expression level in different matched tissue groups (\**P*<0.05).

downregulation. To identify the role of the mitochondrial fusion and fission pathway, as a putative target for cancer therapy, the significance of these findings will require further investigation.

Further analysis of mitochondrial morphology in sorafenib-treated hepatocytes revealed that normal hepatocyte mitochondria are resistant to sorafenib-induced fragmentation. We then hypothesized that if OPA1 downregulation has a direct correlation with sorafenib-induced mitochondria injury, OPA1 expression in hepatocytes would not be affected by sorafenib. Our data clearly indicate that normal hepatocytes had much higher concentrations of mitochondria fusion proteins, including OPA1, Mfn1, and Mfn2 than malignant HCC cells, and OPA1 was not significantly affected by sorafenib treatment. Thus, we provide further support that mitochondria fusion protein OPA1 may play a critical role in determining the sensitivity of HCC cells to sorafenib by maintaining mitochondria dynamics and function. This is consistent with a previous study that OPA1 is a key mediator for actinomycin D-induced apoptosis in cancer cells.47

Nevertheless, further investigation is needed to clarify why mitochondria fusion proteins are not downregulated by sorafenib in normal liver cells.

OPA1 protein level stability is important for maintaining normal mitochondria cristae structure and physiological function in vivo.48 One important aim of this investigation is to define the mechanism of action of sorafenib in vivo. We show that sorafenib significantly reduced xenograft HCC tumor growth. More importantly, the levels of mitochondria fusion proteins OPA1, Mfn1, and Mfn2 were significantly decreased in tumors from sorafenib-treated mice compared with untreated mice, implying that sorafenib can also impair mitochondria dynamics and function via regulation of OPA1 in vivo. In addition, we demonstrate that for HCC clinical samples from 30 patients, when compared with the paired adjacent normal tissues, 40% of malignant tissues had lower expression of OPA1, suggesting that mitochondria dysfunction in HCC is not a rare event. Moreover, a detailed analysis of tissue samples yielded a new notion that differential OPA1 expression between HCC and matched normal (non-cirrhotic)

liver tissues is much higher than that between cirrhotic and matched HCC tissues. Therefore, we believe that OPA1 downregulation may occur in premalignant stage (cirrhosis). Our data strongly indicate that mitochondria fusion protein levels, especially for OPA1, may serve as an independent prognostic marker for targeted administration of sorafenib to improve overall HCC survival. More extensive clinical studies are needed.

Taken together, our study suggests that sorafenib could induce apoptosis in HCC by directly targeting OPA1 to trigger mitochondrial injury. We believe that human HCC tissues have variable expression levels of mitochondria fusion-related proteins, which could be considered as a potential marker in determining administration of sorafenib to HCC patients.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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#### DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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