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ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

Glucose-Regulated Protein 78 Is a Novel Contributor to Acquisition of Resistance to Sorafenib in Hepatocellular Carcinoma

Jeng-Fong Chiou, MD[1,2], Cheng-Jeng Tai, MD[1,2], Ming-Te Huang, MD[4,5], Po-Li Wei, MD[2,5], Yu-Huei Wang, MS[2], Jane An, BS[3], Chih-Hsiung Wu[4,5], Tsan-Zon Liu, PhD[2], and Yu-Jia Chang, PhD[2,5]

[1]Department of Radiation Oncology, Taipei Medical University and Hospital, Taipei, Taiwan; [2]Cancer Center, Taipei Medical University and Hospital, Taipei, Taiwan; [3]New York Medical College, Valhalla, NY; [4]Department of Surgery, Taipei Medical University-Shuang Ho Hospital, Taipei, Taiwan; [5]Department of Surgery, Taipei Medical University and Hospital, Taipei City, Taiwan

ABSTRACT

Background. Sorafenib is a newly established cancer drug found to be an effective systemic treatment for advanced hepatocellular carcinoma (HCC). However, little is known about any potential effectors that modify tumor cell sensitivity towards sorafenib. Here, we present the first evidence that glucose-regulated protein 78 (GRP78) is intimately associated with acquisition of resistance towards sorafenib.

Methods. The role of GRP78 in acquisition of resistance towards sorafenib was determined using HepJ5 (a GRP78-overexpressing subline) and HepG2 as its pair-matched control. RNA interference in cancer cells was applied to determine the influence of GRP78 expression on sensitivity to sorafenib treatment.

Results. We found that HepG2 cells exhibited higher sensitivity toward sorafenib, with 50% inhibition concentration (IC_{50}) >20 μM for HepJ5 and 4.8 μM for HepG2. Specifically, when HepG2 cells received 20 μM sorafenib treatment for 24 h, over 80% of cells underwent apoptosis compared with only 32% of HepJ5 cells under similar experimental conditions. Similarly, GRP78 knockdown in HepJ5 cells by small interfering RNA (siRNA) technique enhanced the efficacy of sorafenib-mediated cell death. This was reflected by a shift of IC_{50} values from >20 μM to 4.8 μM.

Conclusions. GRP78 is a positive modifier for sorafenib resistance acquisition in HCC and represents a prime target for overcoming sorafenib resistance.

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the third leading cause of cancer-related death worldwide, with an especially high incidence in Asia.[1] Prognosis of HCC patients remains poor after surgical or regional therapies because of its high tumor recurrence and metastasis rates.[2,3] Current therapies have been largely ineffective, and for this reason new treatment strategies are needed.[4]

It has been established that diverse signaling pathways are involved in HCC. Thus, molecular targeting agents that have multiple functional attributes should be the drugs of choice for the treatment of HCC. Recently, several agents targeting multiple signaling pathways have entered clinical trials for HCC patients.[5] Amongst these, sorafenib is a multikinase inhibitor that blocks Raf serine/threonine kinases and receptors for tyrosine kinase. Sorafenib has been established as an effective cancer drug for various kinds of tumors and was recently approved by the Food and Drug Administration as a standard therapy for renal cell carcinoma.[6] In addition, sorafenib has been shown to result in improved survival in patients with advanced HCC.[7]

Being a recently approved cancer drug for the treatment of HCC, there are few experimental studies pertaining to potential effectors that modulate tumor cell sensitivity towards sorafenib. Thus, physicians will be faced with the

Jeng-Fong Chiou, Cheng-Jeng Tai, Ming-Te Huang contributed equally to this study.

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Y.-J. Chang, PhD
e-mail: r5424012@tmu.edu.tw

challenge of acquisition of resistance to sorafenib. This problem emphasizes the importance of research aiming to identify potential effectors that may be linked to acquisition of resistance to this drug.

Glucose-regulated protein 78 (GRP78) belongs to the HSP 70 protein family and is an endoplasmic reticulum (ER)-resident protein responsible for protein folding and assembly. GRP78 targets misfolded protein for degradation, ER Ca^{2+} binding, and controls the activation of transmembrane ER stress sensor.[8,9] Its expression has been shown to be stimulated by a variety of environmental and physiological stress conditions that perturb ER function and homeostasis. Stress induction of GRP78 represents an important prosurvival component of the unfolded protein response, an adaptive process that blocks protein translation and allows cells to compensate for protein accumulation and misfolding in the ER. In a variety of cancers, elevated levels of GRP78 were correlated with malignancy.[10–12] Several studies also showed that pre-induction of GRP78 in a variety of human cancer cell lines could confer resistance to topoisomerase II inhibitors (e.g., etoposide), but increased sensitivity to DNA cross-linking agent such as cisplatin.[13,14] Some studies indicated that arsenic trioxide, cadmium chloride, and volatile organic compounds have the ability to induce a significant number of stress genes such as *GRP78*, in human liver carcinoma cells, HepG2.[15–17] A recent report showed that GRP78 functions as an endogenous anti-hepatitis B virus (HBV) factor.[18]

Thus, the possibility exists that increased GRP78 expression or function in cancer cells may also confer resistance to other types of cancer drugs, but there are few published studies regarding this area of research.

Recently, we discovered that HepJ5 was capable of expressing increased amounts of GRP78 protein, which provides us with a unique opportunity to explore if GRP78 per se can be a positive contributor involved in the modulation of tumor cell sensitivity towards sorafenib. In this study, we present that GRP78 expression can confer HCC cell resistance to sorafenib. GRP78 appears to act by alleviating sorafenib-induced apoptotic cell death through the suppression of reactive oxygen species (ROS) production and stabilization of mitochondrial function.

MATERIALS AND METHODS

Compounds

Sorafenib [*N*-(3-trifluoromethyl-4-chlorophenyl)-*N*-(4-(2-methylcarbamoyl pyridine-4-yl)oxyphenyl)urea] was synthesized by Bayer Corporation (West Haven, CT). Compounds were dissolved in 100% dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO) and diluted with Dulbecco's modified Eagle's medium (DMEM) to the desired concentration with a final DMSO concentration of 0.1% for in vitro studies.

Cell Preparations

Two hepatocellular carcinoma cell lines, HepJ5 and HepG2, were used in this study. All cells were grown in medium consisting of Dulbecco's modified Eagle's medium (Life Technologies, NY) supplemented with 10% (v/v) fetal bovine serum in 5% CO_2 in 37°C humidified incubator. The cells were plated onto glass coverslips coated with poly-L-lysine for fluorescence measurement (VWR Scientific, CA).

Chemicals, Antibodies, and Fluorescent Dyes

Fluorescent dyes were purchased from Molecular Probes Inc. (Eugene, OR), antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Loading concentrations of fluorescent probes were as follows: mitochondrial fluorescent dyes: carboxy-2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), 0.5 μM; nucleic acid dyes: propidium iodide, 1 μM; Hoechst 33342 (0.5 μg/ml). Fluorescent probes were loaded at room temperature for 30–60 min. After the dyes were loaded, cells were rinsed three times with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered saline (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 M glucose, 5 mM HEPES, pH 7.4).

MTT Assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to assess cell viability. Cells were plated at 3×10^5 per well in six-well plates. The following day, sorafenib up to 20 μM (1, 2, 5, 10, 20 μM) in 0.1% DMSO was added to wells and incubated for 24 h. Tetrazolium salt (1 ml, 0.5 mg/ml) was added per well and incubated for 3 h. The precipitate was dissolved in 500 μl DMSO, and read with a microplate reader at 550 nm.

Small Interfering RNA (siRNA) Preparation

We ablated GRP78 expression in HepJ5 cells with small interfering RNA (siRNA), which was modified as previously described.[19] The target sequences for the human GRP78 messenger RNA (mRNA) was 5′-AAGGTTACCCATGCAGTTGTT-3′. The luciferase gene was used as a negative control target, and the sequence of scrambled siRNA was 5′-AAGGTGGTTGTTTTGTTCACT-3′. After basic local alignment search tool (BLAST) analysis

verified no significant sequence homology with other human genes, the selected sequence was inserted into a pSUPERIOR vector to generate the pSUPERIOR-GRP78-siRNA and pSUPERIOR-scramble-siRNA plasmid. All constructs were confirmed by DNA sequence analysis. The transfection protocol has been described previously.[20,21]

Western Blot Analysis

Cells were harvested and lysed by radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitor. Protein was quantified using a protein assay kit (Bio-Rad Laboratories, Hercules, CA). Equal amount of protein (50 μg per lane) were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories) which were blotted using anti-GRP78 and anti-actin, and horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5,000), and visualized with enhanced chemoluminescence reagent (Amersham, Piscataway, NJ) on Amersham Hyperfilm.

Confocal Imaging Microscopy

Confocal fluorescence images were obtained using a Leica SP2 MP (Leica-Microsystems; Mannheim, Germany). Wavelength of 800 nm with average laser power of 600 mW was selected for illumination. All images were processed and analyzed using Leica QWin software (Clifton Road, Cambridge, UK). Intensity levels were analyzed from the original images and graphed using SigmaPlot software and PhotoImpact.

Investigation of Mitochondrial Functions

Mitochondrial functions were studied by imaging mitochondrial morphology, ROS formation, and membrane potential. Cells were loaded with mitochondrial targeted fluorescent probes, Mito-Tracker Red at a final concentration of 100 nM. Intracellular and mitochondrial ROS were detected by monitoring caboxy-2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) at concentration of 500 nM, the fluorescent product of intracellular H_2DC oxidation.[22–24]

JC-1 is a nontoxic fluorescent probe used to monitor membrane potential.[25] The electrochemical gradient is responsible for this JC-1 aggregation.[26] At low transmembrane potentials, JC-1 maintains its monomeric form, thus the cells emit green fluorescence. In contrast, at high transmembrane potentials, JC-1 forms more aggregates and red fluorescence. The green-to-red (G/R) ratio is an indicator of $\Delta\psi$. Green-to-red fluorescence ratios of cells stained by JC-1 were detected in merged images by a confocal imaging technique as described elsewhere.[27,28] After loading, the cells were rinsed with HEPES-buffered saline and then loaded with H_2DCFDA, requiring an additional 30 min.

Detection of Membrane Phosphatidyl Serine Extraversion

Cells were treated with sorafenib (20 μM) for 8 h. Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit from Strong Biotech Corporation (Paisley, UK) was used according to the manufacturer's instructions. Confocal imaging analysis was applied as stated above.

Analysis of Caspase-3/7

Caspase-3/7 were measured using the Apo-ONE Homogeneous Caspase-3/7 assays according to the manufacturer's instructions (Promega Corporation, Madison, WI). A confocal microscopy system was used to measure the relative fluorescent units at excitation wavelength of 485 nm and emission wavelength of 535 nm.

TUNEL Assay

Cells were treated with 20 μM sorafenib for 24 h. Cellular DNA fragmentation was detected by terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) using an Apo-BrdU in situ DNA Fragmentation Assay Kit (BioVision, Mountain View, CA) according to the manufacturer's instructions. TUNEL-positive cells were then visualized and analyzed using a confocal microscopy system.

Cell Cycle Analysis

Cells were plated in six-well plates at 3×10^5 per well. After treatment with sorafenib, cells were harvested and washed with phosphate-buffered saline (PBS). Cells were fixed in pure methanol, washed and resuspended in 1 ml PBS, treated with RNase A with a final concentration of 40 μg/ml, and stained with propidium iodide at 40 μg/ml for 30 min at room temperature. The stained cells were analyzed by flow cytometry (BD Biosciences, San Jose, CA) and DNA content was quantified using Modfit software. The percentage of hypodiploid cells (sub-G1) was used to quantify dead cells.

Statistical Analysis

Data are presented as mean ± standard deviation from at least three independent experiments and analyzed using Student's t test. A P value of less than 0.05 was considered as statistically significant.[29]

RESULTS

The Antiproliferation Effect of Sorafenib in HCC Cells

Sorafenib has exhibited antiproliferation ability in several cancers.[30–32] To quantify this activity in HCC cells, different concentrations of sorafenib were incubated with HepG2 and HepJ5 cells and cell viability was determined by MTT assay. Dose-dependent cytotoxic effects for sorafenib were demonstrated (Fig. 1a). HepG2 cells were more sensitive to sorafenib treatment than HepJ5 cells. The half maximal inhibitory concentration (IC_{50}) of a 24 h sorafenib treatment upon the proliferation of HepG2 and HepJ5 cells was approximately 4.8 μM and >20 μM (Fig. 1a). We further checked the growth inhibitory effect of sorafenib by TUNEL assay. Apoptotic cells numbers were increased dramatically by 20 μM sorafenib treatment (Fig. 1b, c), indicating that apoptotic mechanisms are involved in the antiproliferative effects of sorafenib. Interestingly, more positive cell signals were found in HepG2 treated with sorafenib compared with HepJ5 (80% versus 37%). These results indicate that HepJ5 is more resistant to sorafenib treatment compared with HepG2 and that the antiproliferative effects of sorafenib are due to induction of cell apoptosis.

Effect of Sorafenib on Intracellular ROS Production in HCC Cells

Detection of ROS was performed to determine if sorafenib-induced apoptosis is mediated by ROS accumulation. Elevated levels of intracellular ROS were found following 20 μM sorafenib treatment (Fig. 2). Accumulation of ROS was significantly induced after 1 h sorafenib treatment, indicating that the increased apoptosis was due to ROS accumulation. Interestingly, ROS accumulated signals were higher in HepG2 cells after sorafenib treatment than that in HepJ5 cells (Fig. 2). The quantitative results are shown in Fig. 2b. The fluorescence intensity in HepG2 cells treated with sorafenib was approximately 1.5-fold higher than in HepJ5 cells. This demonstrates that ROS formation in HepG2 cells is faster than that in HepJ5 cells and that HepG2 cells were more sensitive to sorafenib treatment. This is consistent with the results of proliferation and TUNEL assays (Fig. 1).

Changes in Mitochondrial Membrane Potential Assessed by JC-1 Staining

Collapse of mitochondrial membrane potential is one of the earliest events in apoptosis. We proceeded to measure mitochondrial membrane potential ($\Delta\psi_m$) using JC-1 as a probe. In comparison, $\Delta\psi_m$ [as reflected by the G/R ratio

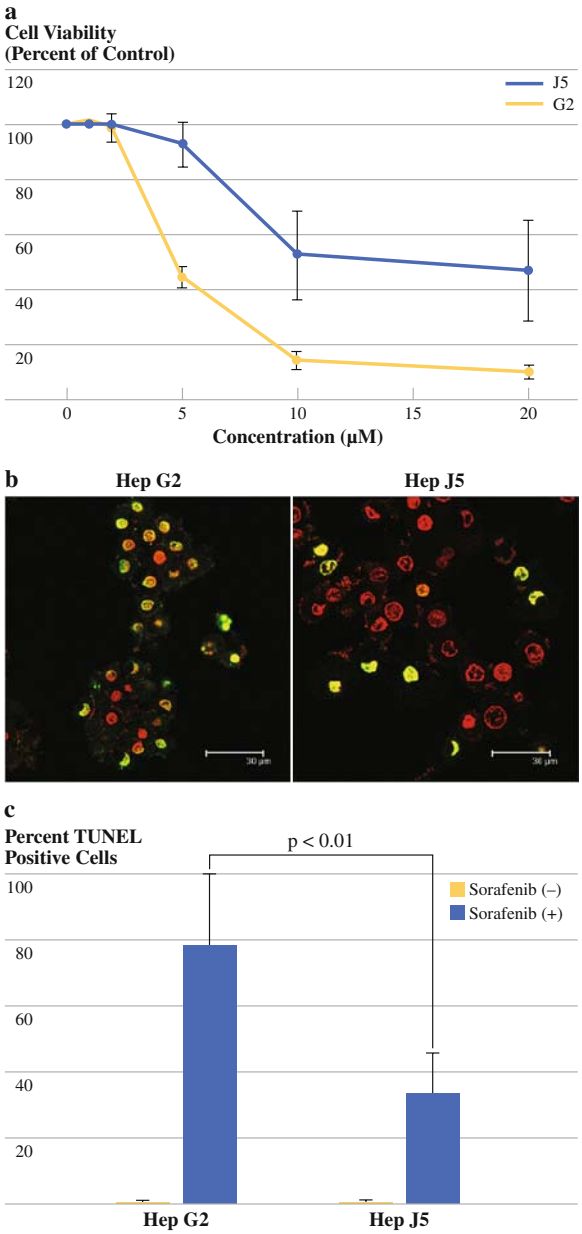

FIG. 1 Apoptotic effect of sorafenib on HCC cells. **a** Cells were treated with various concentrations of sorafenib (0–20 μM) for 24 h. Cell viability was determined by MTT assays. Sorafenib treatment caused the reduction of cell viability in both HepG2 and HepJ5 cells in dose-dependent manners. HepG2 cells were more sensitive to sorafenib treatment than were HepJ5 cells. **b** Apoptosis signals determined by TUNEL assay were increased dramatically after exposure to 20 μM sorafenib in HepG2 and HepJ5 cells. **c** The positive cell numbers in TUNEL assay were measured by confocal microscopy. More positive signals were shown in HepG2 than in HepJ5 cells after treatment ($P < 0.01$)

of both low (monomer green fluorescence) and high (J-aggregated red fluorescence)] of JC-1was severely depolarized in HepG2 cells as compared with that in GRP78-expressing HepJ5 cells (Fig. 3). This data demonstrated that GRP78 is indeed capable of stabilizing

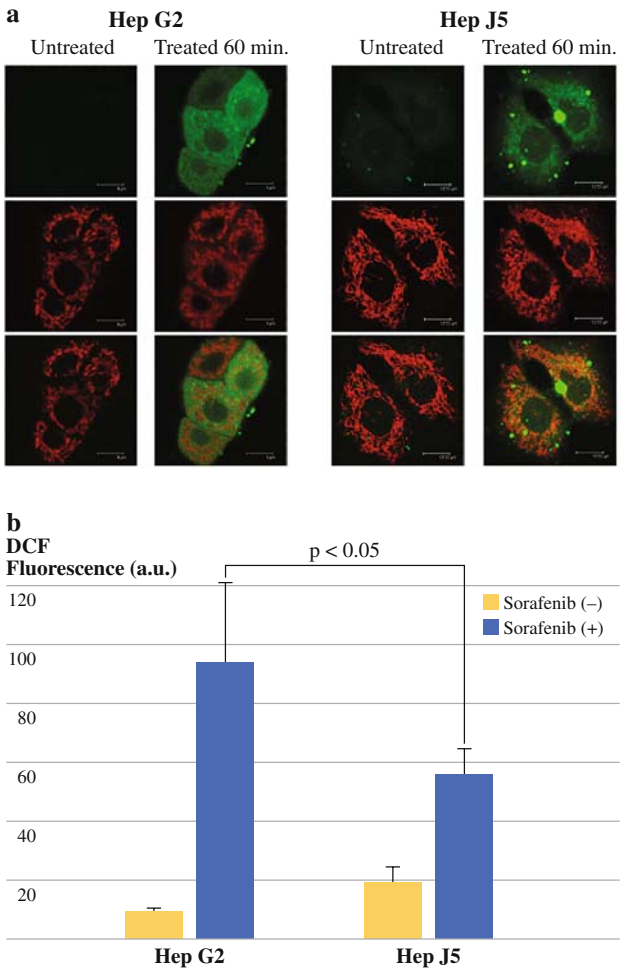

FIG. 2 Effect of sorafenib on intracellular ROS production in HCC cells. **a** HepG2 and HepJ5 cells were treated with 20 μM sorafenib for 1 h. Cells were then incubated with DCFDA which would emit green fluorescence after reacting with ROS. Mitochondria were labeled by Mito-Tracker Red (*red*). Intracellular DCF (*green*) was detected using confocal microscopy. The intracellular ROS levels were significantly increased by 20 μM sorafenib in HepG2 and HepJ5 cells. **b** The florescence intensity was quantified by Leica Q-WIN. ROS formation in HepG2 and HepJ5 cells was dramatically increased by sorafenib treatment. The fluorescence intensity of ROS is stronger in HepG2 than HepJ5 ($P < 0.05$)

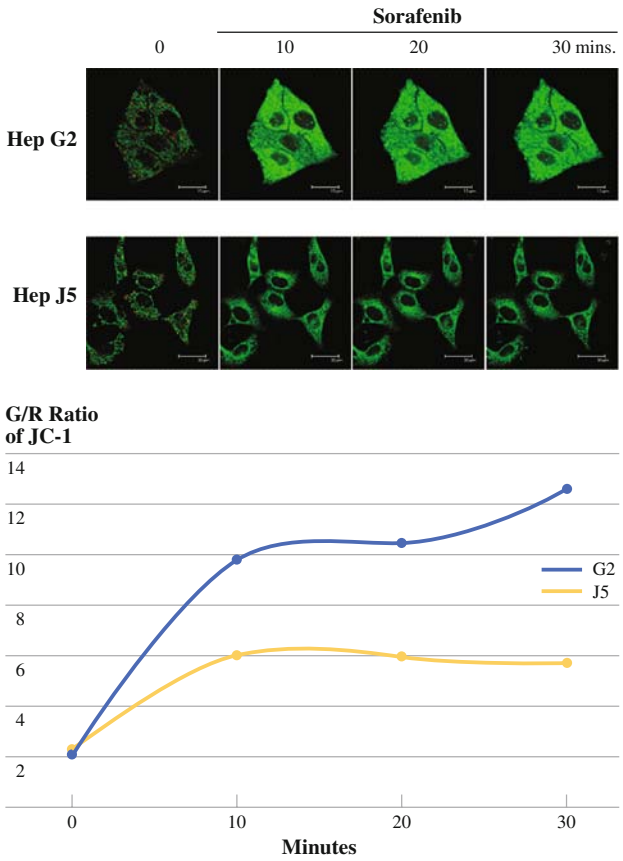

FIG. 3 Sorafenib caused severe depolarization of mitochondrial membrane potential in HepG2 cells. Cells were stained by a potential dye JC-1 before treatment with 20 μM sorafenib (referred to as control). Regions of high mitochondrial polarization are indicated by *red* fluorescence due to J-aggregate formation by the concentrated dye. Depolarized regions are indicated by the *green* fluorescence of the JC-1 monomers. Sorafenib may cause mitochondria membrane potential collapse on administration because J-aggregate forms in red shifted to green fluorescent monomeric forms. The brighter green fluorescence of HepG2 cells indicated greater loss of mitochondrial membrane potential. The ratio of green and red fluorescence of JC-1 is an indicator to assess mitochondrial membrane potential. The images were quantified to a curve chart using Leica Q-Win, a built-in image processing software in the Leica confocal scanning system

mitochondrial membrane potential and thus maintaining stable calcium homeostasis.

Annexin V Binding for Determining Early Apoptosis

In order to appreciate the effect of sorafenib treatment in HCC cells, we used annexin V staining to visualize the early apoptosis signal. As shown in Fig. 4a, the annexin V signal was increased in HepG2 and HepJ5 cells after sorafenib treatment. More annexin V staining was found in HepG2 cells than in HepJ5 cells after exposure of sorafenib, which is consistent with the results of the TUNEL assay (Fig. 1). This data indicates that sorafenib caused cell apoptosis in both HepG2 cells and HepJ5 cells. HepG2 cells were more sensitive than were HepJ5 cells to sorafenib.

Activation of Caspase-3/7 Signal

As executor caspases, caspase-7 and caspase-3 could act in concert to facilitate the apoptotic process. As shown in Fig. 4, the signal of green fluorescence was weak in the untreated group, indicating few apoptotic cells. After 20 μM sorafenib treatment, the signal of activated caspase3/7 was dramatically increased in HepG2 cells but not in HepJ5 cells (Fig. 4b, c). This suggested that HepJ5 cells were more resistant to sorafenib through the suppression of the mitochondrial caspase-mediated cell death pathway.

The Expression Status of GRP78 in HCC Cells

In order to delineate the key molecular-mediated resistance feature to sorafenib in HepG2 and HepJ5 cells, the expression level of GRP78 was determined by Western blotting. As shown in Fig. 5a, the transcriptional and translational levels of GRP78 were higher in HepJ5 than in HepG2. The increased expression level of GRP78 in HepJ5 cells may contribute to the resistance feature of sorafenib treatment.

GRP78 Knockdown in HepJ5 Cells by siRNA

To confirm if GRP78 may be related with drug resistance in HCC cells, GRP78 knockdown in HepJ5 cells by siRNA technique was performed and the stably transfected cells were selected by neomycin. Expression levels of GRP78 were studied in knockdown or control cells by Western blotting, real-time polymerase chain reaction (PCR), and immunofluorescence staining (Fig. 5b–d). Both the translational and transcriptional levels of GRP78 were reduced over 80% in GRP78 knockdown cells compared with control cells. In addition, immunofluorescence staining was performed and recognized by anti-GRP78 antibody using confocal microscopy to visualize the GRP78 signal. As shown in Fig. 5d, the control cells were highly stained by anti-GRP78 antibody, but not in the GRP78 knockdown cells. These results indicated that GRP78-siRNA can efficiently reduce the expression of GRP78 in HepJ5 cells.

The Growth Inhibitory Effect of Sorafenib in GRP78 Knockdown Cells

To further evaluate the antiproliferation effect of sorafenib in GRP78 control and knockdown cells, GRP78 knockdown cells were treated with different dosages of sorafenib (0–20 μM) and cell viability was determined by MTT assay. Figure 6a shows that the antiproliferation effect of sorafenib in HepJ5 cells was similar to that in scramble-siRNA cells indicating that the transfected and selected processes might not change the cells' features. In contrast, we found that GRP78 knockdown cells showed more sensitivity to sorafenib treatment. The IC_{50} was shifted from >20 μM to 4.8 μM after GRP78 knockdown in HepJ5 cells (Fig. 6a). This may imply that reduction of GRP78 expression will sensitize HepJ5 cells to sorafenib.

Increased Apoptotic Signals After GRP78 Knockdown Toward Sorafenib Treatment

We studied cell cycle distributions in these cells after 20 μM sorafenib treatment for 24 h. GRP78 knockdown cells

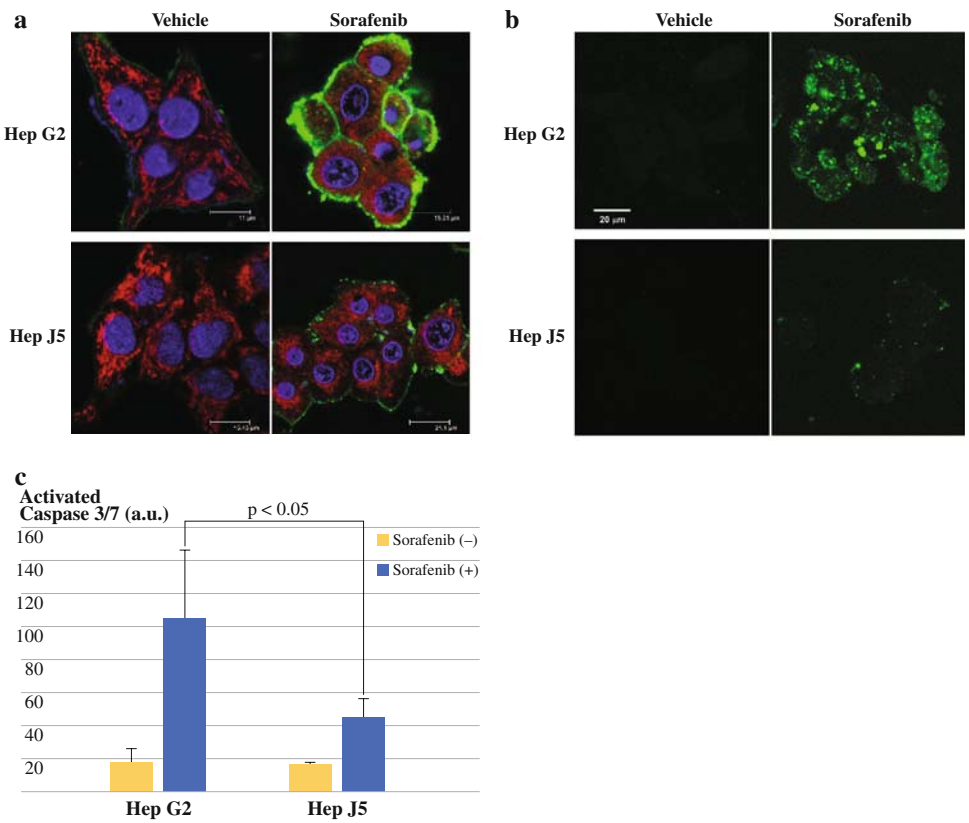

FIG. 4 Hallmarks of apoptosis induced by sorafenib. **a** Cells were fixed then stained with annexin V staining kit. Annexin V was labeled with FITC-specific antibody, mitochondria were labeled with Mito-Tracker Red, and DNA was stained by 4,6-diamidino-2-phenylinode (DAPI). **b** Cells were stained with Promega™ Caspase-3/7 Activation Detection kit. The bright *green* fluorescence indicated the level of activated caspase-3/7 in HepG2 and HepJ5 cells. **c** The intensity of fluorescence was quantified by Leica Q-Win

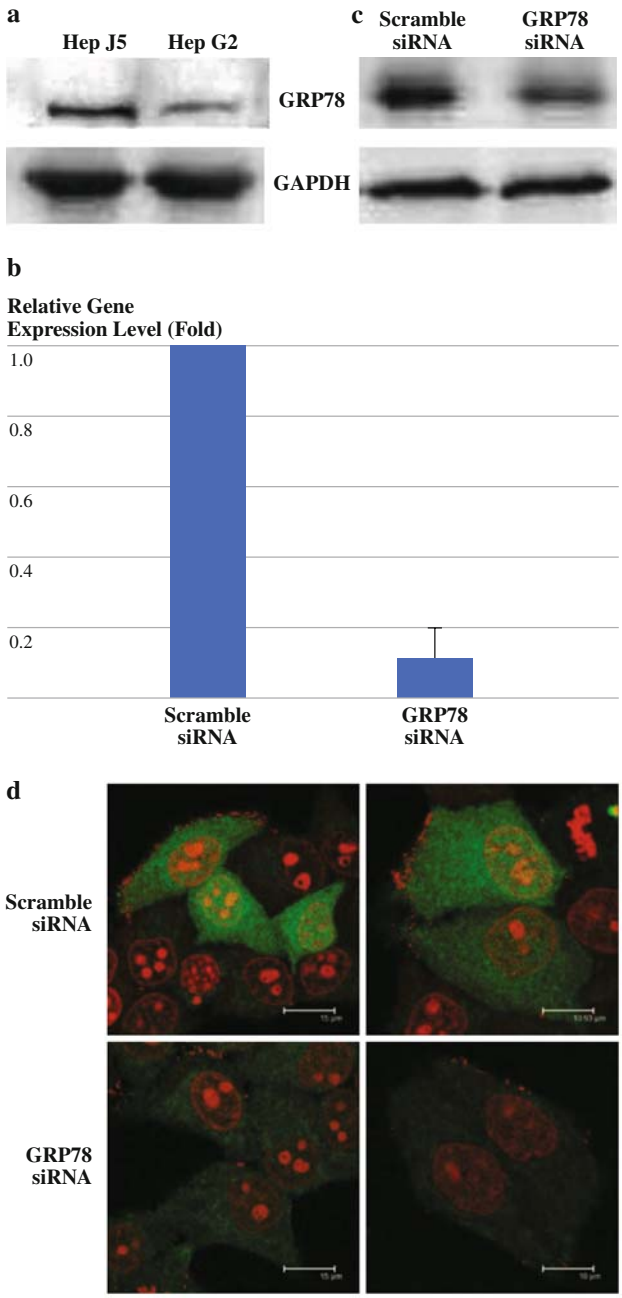

FIG. 5 Silence of GRP 78 expression in HCC cells. **a** Highly expressed GRP78 in HepJ5 cells. The expression levels of GRP78 in HCC cells were determined by Western blotting. **b–d** Knockdown of GRP78 expression by siRNA in HepJ5 cells. The GRP78 siRNA and scramble siRNA were transfected into HepJ5 cells. The expression of GRP78 was determined by real-time PCR (**b**), Western blotting (**c**), and immunofluorescence staining (**d**). The GRP78 was reduced over 85% in GRP78 siRNA HepJ5 cells

increased their cell population at the sub-G1 phase (6.44% versus 20.19%)—one indicator in the cell apoptotic process (Fig. 6b)—but there were no changes in other phases of cell cycle. Further, we confirmed the sorafenib-induced apoptotic effects by TUNEL assay. As seen in Fig. 7, the positive signal in control cells was 30% after 24 h sorafenib treatment. After GRP78 expression knockdown, the signal increased significantly to 47%. This indicated that GRP78 knockdown may reduce the protective ability of HCC cells to sorafenib treatment. Together, we demonstrated that GRP78 might play an essential role in mediating drug resistance in HCC cells.

DISCUSSION

A number of studies considering the potential role of GRP78 expression in the modulation and development of drug resistance, mainly focused on topoisomerase (etoposide) and cyclooxygenase-2 (COX-2) (celecoxib), have been reported.[8,33–35] Most of these studies were associated with inherent problems because the induction of GRP78 was based on the use of stress inducers or deficiencies in certain cell functions. As a consequence, these kinds of inducing conditions can exert other unknown pleiotropic effects that possibly affect multiple cellular pathways.[36] In addition, because cancer cells are inherently heterogeneous, different types can use diverse signaling and defense mechanisms to acquire resistance to specific drugs.[37] Thus, for precise and definite elucidation of the role of GRP78 on the resistance acquisition of a newly developed drug such as sorafenib, there must be an experimental cancer cell line that can express GRP78 ectopically and normoxically. In this regard, we screened a set of five human HCC cell lines with varying degrees of differentiation and found that the HepJ5 was capable of expressing GRP78 ectopically and normoxically, independent of the regulation by hypoxia inducible factor 1-alpha (Fig. 5). With the availability of this unique HCC cells, the problematic interferences caused by inducers of GRP78 expression can be avoided, and thus the effects observed should be a true reflection of the functional attributes of GRP78 itself.

Sorafenib has been demonstrated to inhibit the proliferation of a variety of human cancer cells such as non-small-cell lung cancer (NSCLC), breast, colon, and pancreas carcinoma and has recently gained accelerated approval by the Food and Drug Administration (FDA) for the treatment of advanced HCC.[30–32,38] The proliferative inhibitory abilities of this drug have been shown to be due to the targeting of several kinases, such as Raf kinase. However, the multitude of targets attributed to sorafenib may produce variable effects on different cells. Recently, we discovered that sorafenib targets mitochondria and evokes a ROS-mediated apoptosis. This apoptosis was shown to include intracellular glutathione depletion and mitochondrial calcium overload. It could be inferred that calcium influx into mitochondria may originate from the ER via a stress condition induced by a sudden influx of

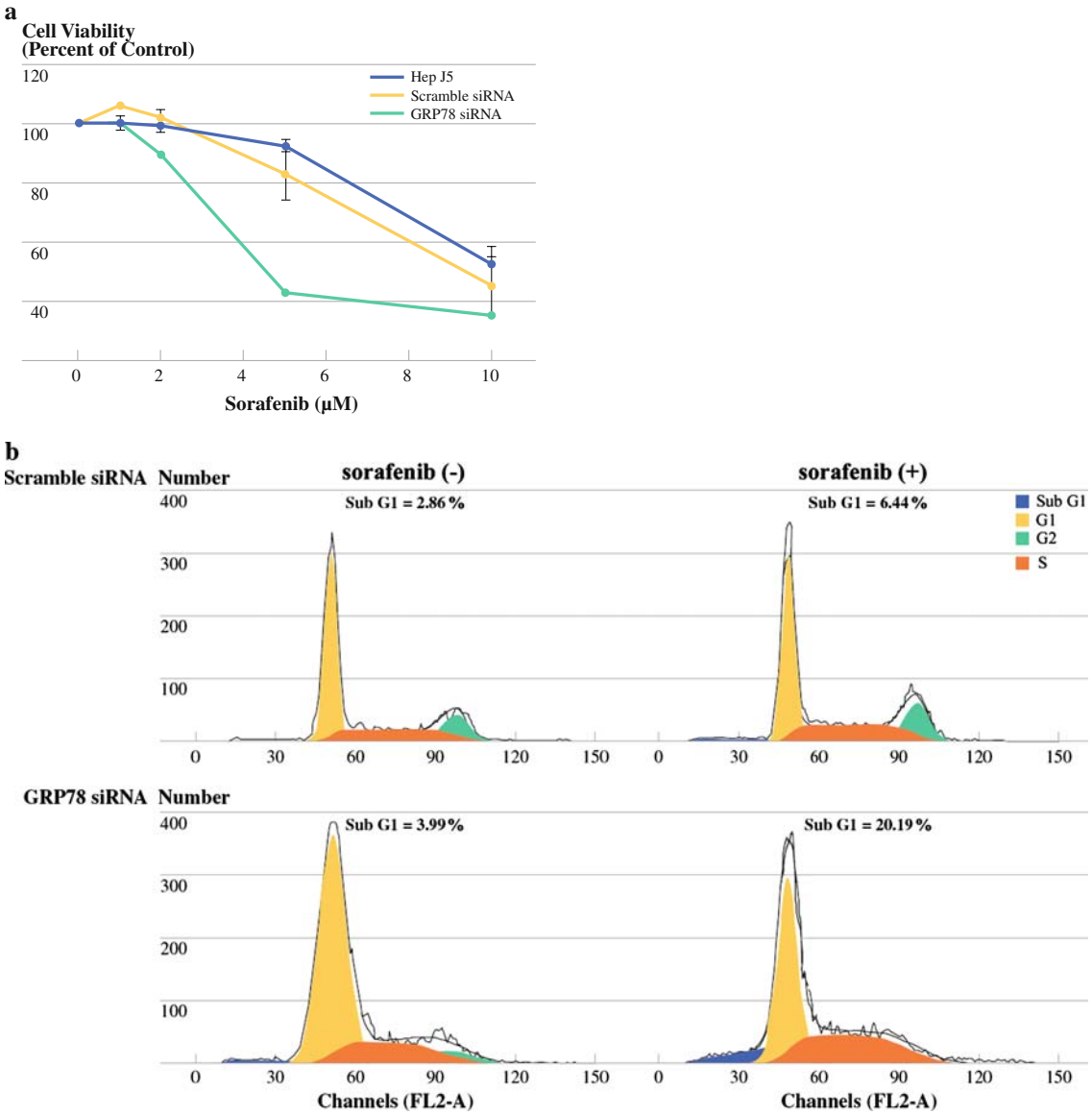

FIG. 6 Knockdown of GRP78 sensitized HepJ5 cells to sorafenib treatment. **a** Cells were treated with different concentrations of sorafenib and cell viability was determined by MTT assay. Silence of GRP78 expression by siRNA increased the sensitivity to sorafenib treatment compared with scramble siRNA and HepJ5 cells. **b** Cell cycle distribution was determined by PI staining and flow cytometry. GRP78 knockdown cells showed an increased sub-G1 cell population after exposure to 20 μM sorafenib compared with scramble siRNA cells

ROS. This possibility was proved to be true by Rahmani et al.,[39] who demonstrated that sorafenib was capable of inducing ER stress in human leukemia and other malignant cells. In addition, Liu et al.[40] reported that mitochondrial calcium overload could cause excess oxidant production and plasma membrane damage which could result in cell death. Based on these studies, we can infer that an agent, either endogenously or exogenously, capable of suppressing ROS accumulation and stabilizing mitochondrial function can offer protection against sorafenib-induced apoptosis. To address this hypothesis, we set out to clarify whether ectopic expression of GRP78 plays a pivotal role in conferring resistance acquisition against sorafenib using GRP78-expressing HepJ5 cells as the cell model. As indicated in Fig. 1, we observed that a distinct difference in resistance ability towards sorafenib existed between GRP78-exppressing HepJ5 cells ($IC_{50} > 20$ μM) and its pair-matched HepG2 control cells ($IC_{50} < 5$ μM). To further prove whether GRP78 itself is responsible for this observed phenomenon, we then proceeded to knock down GRP78 expression in HepJ5 cells with a GRP78-specific siRNA and then determined a dose–response curve with cell viability assays for these transfected cells along with nontransfected parental and scramble siRNA-transfected

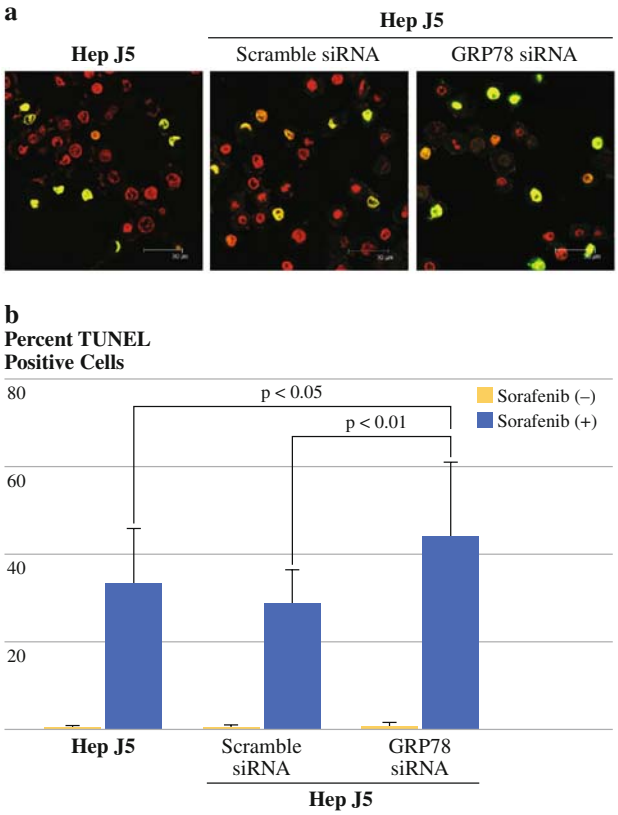

FIG. 7 Increased apoptosis signals to sorafenib treatment after GRP78 knockdown. **a** Apoptosis signals determined by TUNEL assay were increased dramatically after exposure to 20 μM sorafenib in GRP78 siRNA HepJ5 cells. **b** Positive cell numbers in TUNEL assay were measured with confocal microscopy. More positive signals were shown in GRP78 siRNA HepJ5 cells compared with scramble siRNA HepJ5 cells and parental cells after treatment ($P < 0.01$)

control cells. We demonstrated that a marked increase in sorafenib sensitivity could be detected for siRNA-transfected HepJ5 cells as reflected by a drastic reduction of IC_{50} from >20 μM to <5 μM, respectively. In contrast, both parental and vector transfected HepJ5 cells retained high viability and IC_{50} values (Fig. 6a). In addition, substantially higher proportions of apoptotic cells could be observed in sorafenib-treated (20 μM) siRNA-transfected cells compared with both types of control cells. This was reflected by either sub-G1 fractions determined via flow cytometry (Fig. 6b) or TUNEL-positive cells assays and confocal microscopy (Fig. 7).

How did constitutively activating GRP78 contribute to acquisition of resistance to sorafenib-induced apoptosis in HepJ5 cells? We investigated whether GRP78 expression could alleviate sorafenib-induced ROS production by confocal microscopy using DCFDA as a probe. Indeed, the generation of sorafenib-induced ROS production as reflected by the intensities of DCF green fluorescence was found to be universally more abundant in HepG2 cells as compared with that in HepJ5 cells which expressed GRP78 ectopically ($P < 0.05$) (Fig. 2). Can this reduction of oxidative stress by GRP78 help protect mitochondrial function? We then measured mitochondrial membrane potential ($\Delta\psi_m$) using JC-1 as a probe. In comparison, $\Delta\psi_m$ [as reflected by G/R ratio of both low (monomer green fluorescence) and high (J-aggregated red fluorescence)] of JC-1 was severely depolarized in HepG2 cells as compared with that in GRP78-expressing HepJ5 cells (Fig. 3). These data demonstrated that GRP78 is indeed capable of stabilizing mitochondrial membrane potential and thus maintaining stable calcium homeostasis. This finding is consistent with the notion that alleviation of mitochondrial calcium overload can lead to less ROS production, oxidative stress, and plasma membrane damage of renal epithelial cells.[41] Finally, we demonstrated that the protective effect of GRP78 acted through interference of sorafenib-induced apoptosis via suppression of caspase-3/7 activation. As executor caspases, caspase-7 and caspase-3 could act in concert to facilitate apoptosis (Fig. 4). Thus, the ultimate mechanism for sorafenib resistance acquisition by a GRP78-overexpressing HCC cell is mediated through the suppression of mitochondrial caspase-mediated cell death pathway.

In this study, we present the first evidence that GRP78 itself is a positive contributor to acquisition of resistance to sorafenib, a multikinase inhibitor in HCC cells. Targeted suppression of GRP78 expression or function could be a potential strategic approach for increasing the efficacy of sorafenib against GRP78-expressing cancer cells. More importantly, an ever-increasing list of endoplasmic reticulum stress inducers has been compiled that may boast the possibility of increased GRP78 expression in cancer cells and may develop resistance towards sorafenib. Physicians who treat cancer patients must be aware of this potential obstacle. However, we do not know whether GRP78 may communicate with other drug-resistant target genes which bring out resistance to sorafenib in HCC. It may need the performance of more experiments or more clinical information to prove this. In conclusion, we believe that there must be an aggressive search for naturally occurring compounds that inhibit either GRP78 expression or its activity in order to combat sorafenib resistance.

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