CANCER GENOMICS & PROTEOMICS *16*: 569-576 (2019)
doi:10.21873/cgp.20159

Proteomic Profile of Sorafenib Resistance in Hepatocellular Carcinoma; GRP78 Expression Is Associated With Inferior Response to Sorafenib

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Abstract. *Background/Aim: The outcome of patients with advanced hepatocellular carcinoma (HCC) remains poor and therapeutic options, including sorafenib, the first anti-cancer drug proved to prolong survival in patients with advanced HCC, are limited. However, no clinically useful predictive biomarker for sorafenib has been reported. Materials and Methods: We exploited two-dimensional gel electrophoresis coupled with mass spectrometry to find de-regulated proteins by using conditioning of a sorafenib-resistant HCC cell line, Huh7. Tumor samples from 60 patients with HCC treated with sorafenib were analyzed and correlated with survival outcome. Results: Comparative proteomics indicated three proteins including, 78 kDa glucose related protein (GRP78), 14-3-3ε, and heat shock protein 90β (HSP90β). The three proteins were over-expressed in sorafenib-resistant Huh7 cells. In HCC tumor samples from patients treated with sorafenib, 73% of tumor samples had a high expression of GRP78, 18% had high 14-3-3ε expression and 85% had high HSP90β expression. Among these, GRP78 was associated with the shortest progression-free survival of HCC patients treated with sorafenib. Conclusion: GRP78 can be a predictive biomarker in HCC patients treated with sorafenib. Strategies designed to inhibit the GRP78-related pathway may overcome sorafenib resistance.*

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Key Words: Hepatocellular carcinoma, sorafenib, resistance, GRP78.

Hepatocellular carcinoma (HCC) contributed to almost 80% of all liver cancers and was the second leading cause of cancer-related deaths in Taiwan in 2014 (1). More than half of HCC cases are diagnosed late and patients with advanced HCC have a median survival of less than one year (2). Patients with vascular invasion or extrahepatic spread are categorized as having advanced disease and are not suitable candidates for radical therapies. Systemic chemotherapy has not been proven to be consistently efficacious in treating hepatocellular carcinoma (3). Sorefanib, a dual-action Raf kinase and vascular endothelial growth factor (VEGF) inhibitor, prevents tumor growth by two anticancer activities: inhibition of tumor cell proliferation and tumor angiogenesis. This drug has been demonstrated to be well tolerated and clinical benefits have been observed in advanced refractory solid tumors (4). It has been associated with a 37% increase in overall survival (equivalent to a gain of nearly 3 months) as compared with placebo in patients with advanced hepatocellular carcinoma and compensated cirrhosis (5).

The response of sorafenib in HCC treatment is relatively inconsistent and acquired resistance is an emerging issue nowadays. To investigate the acquired resistance to sorafenib, several mechanisms have been proposed such as cross-interaction between PI3K/Akt and JAK-STAT pathways, the

activation of hypoxia-induced factor and epithelial-mesenchymal transition and so on (6). Two-dimensional gel electrophoresis (2DE) is a proteomic approach used to detect differences in protein expression in cells. Previous studies have developed a sorafenib-resistant HCC cell line from Huh7, a human HCC cell line, by long-term exposure of cells to sorafenib treatment (7). Herein, we analyzed the differences in protein expression between the parental cells and sorafenib resistant cells by using 2DE assay. Therefore, potential targets related to sorafenib resistance could be evaluated in human subjects.

Materials and Methods

Cell lines and reagents. The human HCC cell line Huh7 was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. The sorafenib-resistant Huh7 cell line (Huh7-R) which was routinely maintained under constant culture conditions containing sorafenib was established in the laboratory of Dr. Kuen-Feng Chen (7). Sorafenib tosylate was kindly provided by Bayer (Leverkusen, Germany). Anti-GRP78 antibody (C50B12) was purchased from Cell Signaling Technology (Danvers, MA, USA), anti-14-3-3ε antibody (SC-2395) from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and anti-heat shock protein 90β (HSP90β) (ab53497) from Abcam (Cambridge, UK).

Isoelectric focusing and SDS-polyacrylamide gel electrophoresis (SDS-PAGE). We performed 2DE as described before with some modifications (8). The 18 cm immobibline dry strips (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) with a pH range between 4~7 were rehydrated for 16 h at 20˚C with 300 μl rehydration buffer and loaded with 100 μg protein lysates prepared from Huh7 or Huh7-R cells using BioRad Protean IEF Cell. The proteins were isoelectrically focused at 20˚C 50V, 100V, 200 V, 500 V, 1,000 V, 5,000 V, and 8,000 V. The total voltage-hours were 81434. The equilibrated gel was loaded onto the top of a 12.5% w/v polyacrylamide gel. Then, using the Biorad Protein IIxi, proteins were separated at 420 V after equilibrated in equilibration buffer containing 2% w/v DTT and 5% w/v iodoacetamide.

Image analysis and statistical analysis. 2DE gels were stained with LavaPurple™ followed by the image capture of 2DE gel map. Then the image scan was obtained by using Typhoon 9400 fluorescence scanner (GE healthcare) with green laser (green laser PMT: 600 volt and emission filter: 580 BP). A total of 7 pairs of well-focused gel maps collected from control Huh7 cells and Huh7-R cells were compared by PDQuest 8.0.1 (BioRad) software to define the differentially expressed proteins in Huh7-R cells. The results were visually confirmed. The intensity of the spot was measured and normalized as a percentage of the total intensities of all spots in a gel (total normalized volume). These individual protein spots with normalized volumes across replica gels of 0.5 % (v/v) DMSO- or Huh7-R cells were first determined by the normal distribution test and Student's t-Test (STATISTICA, StatSoft, Tulsa, OK, USA) for each differentially expressed protein spot. Log transformation was performed followed by the normal distribution test and Student's *t*-Test if normal distribution was not acquired. To evaluate the statistical variance of sorafenib resistance, the differences were considered statistically significant at $p<0.05$ by Student's *t*-Test. Furthermore, the differentially expressed proteins present at least in 4 out of 7 gel pairs were considered as sorafenib resistance-impacted proteins.

In-gel digestion and protein identification analysis via liquid chromatography-tandem mass spectrometry (LC-MS). The protein spots of interest were selected for in-gel digestion using silver staining. Silver staining, in-gel digestion and mass spectrometric protein identification, were performed as described previously (8, 9). Briefly, we separated the protein digest in LTQ-Orbitrap hybrid tandem mass spectrometer system (ThermoFisher, San Jose, CA, USA). Agilent 1200 nanoflow HPLC system was equipped with LC Packing C18 PepMap 100 (length: 5 mm; internal diameter: 300 μm; bead size: 5 μm) as the trap column. The separating column was Agilent ZORBAX XDB-C18 (length: 50 mm; internal diameter: 75 μm; bead size: 3.5 μm). Extraction of the MS/MS information as well as calculation of the charge and mass for each analyzed peptide were obtained by using File Converter in Xcalibur 2.0SR package (ThermoFisher) and an in-house program. To search the best matched peptides from a non-redundant protein database, TurboSequest program (ver.27, rev.11) was applied using the FASTA sequences downloaded from National Center for Biotechnology Information (http://www.ncku.nim.nih.gov/guide/proteins/#tab-all) on 2010/10/12 with 541927 entries. The mass ranges during the database search were 1 and 3.5 m/z for fragment and precursor ions, respectively, when the tryptic peptides with ≤2 missed cuts were considered. The protein identities were documented only when at least two peptides have matched the conditions listed below: high Xcore (*i.e.*, ≥2.0 for doubly charged peptides and ≥3.0 for triply charged ones) and minimal differences between observed and hypothetical masses (*i.e.*, ΔM <10 ppm). In order to verify the effectiveness of the entire protein identification procedure including in-gel digestion, nanoflow HPLC, MS/MS and informatics analysis, 26 fmol of BSA in gel was obtained for each set of MS/MS analysis. Only when 10 ppm mass accuracy and over 70% coverage was detected for the co-processed BSA sample, the experimental data were confirmed.

Tumor samples. Sixty patients were included retrospectively, who were treated with sorafenib and had a pathological diagnosis of hepatocellular carcinoma between 2006 and 2014 in Chi Mei Medical Center. Pathological, demographic and survival data of these patients were retrieved from medical records. All tumors were harvested from primary liver sites. All tumors were histologically characterized and graded according to Edmondson's scales and grouped as well differentiated, moderate and poorly differentiated. The study was approved by the institutional Review Board of the Chi Mei Medical Center (No. 10212-001).

Immunohistochemical staining. Staining was carried out on formalin-fixed and paraffin-embedded tissue sections using a two-step protocol (Novolink Polymer Detection System, Novocastra) according to the manufacturer's instructions. Briefly, paraffin sections were deparaffinized first and then hydrated. After microwave antigen retrieval, as required, slides were washed for 5 min in phosphate buffered saline (PBS). We used peroxidase block for 5 min to neutralize endogenous peroxidase. Following incubation, the sections were washed in PBS (2×5 min washes). Sections were incubated with protein block for 5 min and washed again in PBS (2×5 min washes). Primary rabbit polyclonal

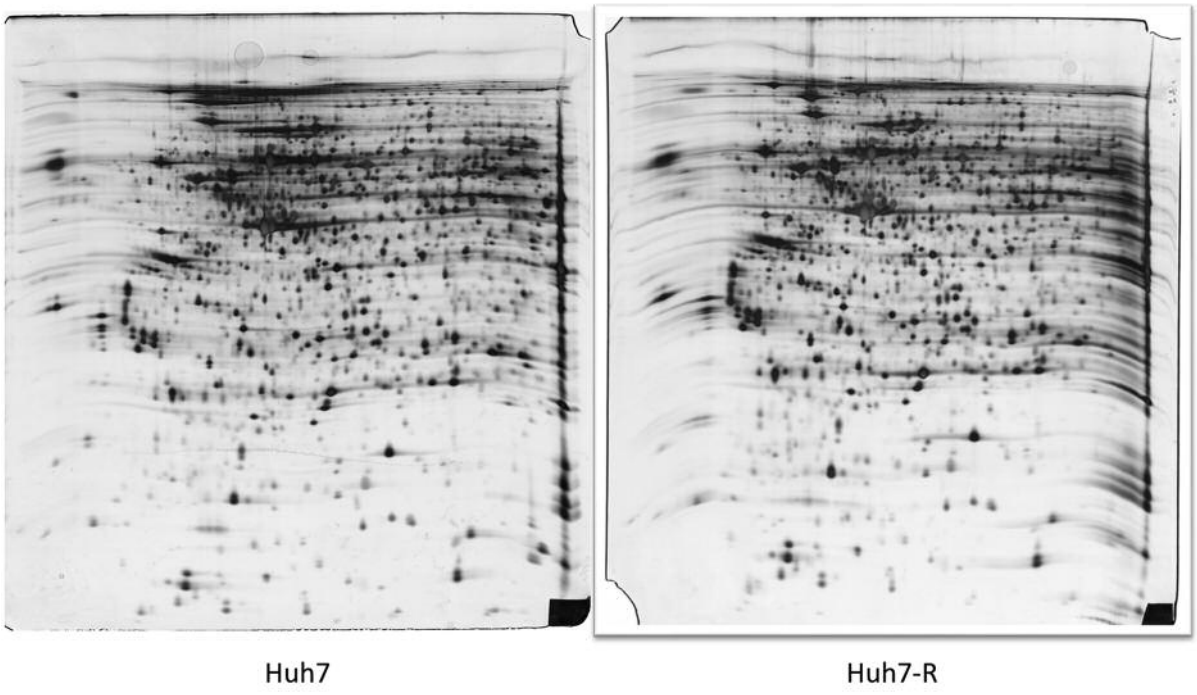

Figure 1. *Silver stained 2D gel electrophoresis of parental Huh7 cells and Huh7-R cell lysates. Protein lysates were prepared from Huh7 cells (left) and Huh7-R cells (right), as described in Materials and Methods.*

antibodies used were anti-human-14-3-3ε (3570, Cell Signaling Technology), HSP90βab (MA5-14057, Thermo Fisher Scientific, Cheshire, UK) and GRP78 (ab12158, Abcam, Cambridge, UK). After serial incubation with primary antibodies overnight at 4˚C, the sections were washed in PBS (2×5 min washes) and incubated with post-primary for 10 min. In the next steps, slides were washed in PBS (2×5 min washes), incubated with Novolink polymer for 10 min and then washed in PBS (2×5 min with gentle rocking on an orbital shaker). 3-3-diaminobenzidine working solution was applied to the slides for 3 min. Slides were washed in PBS, counterstained with hematoxylin for 1 min and washed in di-H_2O for 5 min before dehydration, clearing and mounting. To evaluate the expression of indicated proteins, slide scorings were based on intensity of stain (0-3). The intensities of expression 0 and 1 were categorized as low expression. On the other hand, 2 and 3 were defined as high expression.

Immunoblotting. Immunoblotting analysis was performed according to a standard method. The primary antibodies used for immunoblotting analysis included anti-human-14-3-3ε (3570, Cell Signaling Technology), HSP90βab (MA5-14057, Thermo Fisher Scientific, Cheshire, UK), GRP78 (ab12158, Abcam) and β-actin (Sigma-Aldrich). Following incubation with the secondary antibodies for 2 h, protein-antibody complexes were detected using the ECL system (Millipore) and visualized with a Biospectrum AC imaging system (UVP, Cambridge, UK).

Statistical analysis. Survival was statistically analyzed by the Kaplan–Meier survival curve and a log-rank test performed with SigmaPlot 12.0 software (Systat Software Inc., San Jose, CA, USA).

Results

Two-dimensional gel electrophoresis of Huh7-R cells and parental Huh7 cells. Previously, we have established Huh7-R HCC cells by long-term exposure to sorafenib at low doses escalating to higher doses for a long period of time. Furthermore, parental Huh7 cells showed apoptosis in the presence of sorafenib in a dose-dependent manner. In contrast, Huh7-R cells revealed resistance to sorafenib-induced apoptosis, even at 10 μM (the highest clinical achievable concentration) (7). To analyze the mechanism of acquired resistance to sorafenib, the protein expression profile of parental Huh7 and Huh7-R cell lines was analysed by 2DE coupled LC-MS/MS. To prevent the gel-gel variation, seven replicate gel pairs were collected from different protein harvests. The representative 2D maps of parental Huh7 cells and Huh7-R cells are depicted in Figure 1.

Identification of differential expressed proteins between parental Huh7 cells and Huh7-R cells. After the proteomic comparison, over-expressed proteins and under-expressed proteins were identified. Among these, 13 proteins were differentially expressed with a magnitude near or higher than two-fold between Huh7 and Huh7-R cells, in at least 4/7 gel pairs. The protein spots of interest were picked up for in-gel digestion using silver staining. The protein spots

Table I. *Differentially expressed proteins identified by tandem mass spectrometry.*

Protein identity	Incidence	Experiment PI/MW (kDa)	Theoretical PI/MW (kDa)	Matched peptide number[a]	Coverage (%)[b]	Accession number (NCBI)	Fold
78 kDa glucose-regulated protein	4/7	5.0/88.1	5.01/82.6	29	62.1	14916999	6.8
ATP synthase subunit beta, mitochondrial	6/7	5.7/57	5.26/56.6	8	28.7	114549	–2.9
Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial precursor	5/7	6.6/34.3	8.16/35.8	4	27.1	70995211	4.3
14-3-3 protein epsilon	5/7	4.5/32.7	4.63/29.2	11	62.7	51702210	2.4
Annexin A3	7/7	6.1/35.3	5.58/36.4	10	44.6	113954	4.1
Triosephosphate isomerase 1, isoform CRA-b	4/7	6.6/29.4	5.65/30.8	9	41.3	119609128	–2.5
NADH-ubiquinone oxidoreductase 23 kDa subunit	5/7	5.4/27.4	6/23.71	4	28.6	2499325	4.8
COMM domain-containing protein 10	4/7	6.6/26.8	6.09/22.97	6	43.1	51316122	–2.2
Heat shock protein HSP 90-beta	4/7	4.8/95.4	5.07/98.2	26	54.6	17865718	4.2
Actin-like 6A, isoform CRA-c	4/7	5.9/52.3	5.39/47.5	12	36.8	119598807	2.6
Capping protein (actin filament), gelsolin-like, isoform CRA-a	4/7	6.4/41.2	5.83/41.8	6	36.5	119619923	2.5
Chain A, Crystal Structure Of A Complex Between Protein Phosphatase 1 Alpha (pp1), The Pp1 Binding And Pdz Domains Of Spinophilin And The Small Natural Molecular Toxin Nodularin-r	4/7	6.3/39.2	6.21/37.3	6	24.6	291463445	–2.8
Chromosome 12 open reading frame 10, isoform CRA-b	4/7	6.2/41.1	6.35/42.5	6	19.7	119617094	–2.4

[a]Matched peptide number: Number of peptides matched with protein in mass spectrometry/mass spectrometry query; [b]Coverage: Total percentage of amino acid sequence covered by peptides identified by mass spectrometry/mass spectrometry query.

of interest were identified by mass spectrometric protein identification and the results are listed in Table I. Among these deregulated proteins, three proteins were targeted based on their high-fold alterations, including GRP78, 14-3-3ε protein, and HSP90β.

Immunohistochemical staining of indicated proteins in HCC tissues. Tumor samples from sixty advanced HCC patients treated with sorafenib were collected for immunohisto-chemical analysis of GRP78, 14-3-3ε, and HSP90β. The clinical characteristics of patients are listed in Table II. First, expression of HSP90β, GRP78 and 14-3-3ε in parental Huh7 and Huh7-R cells was examined by immunoblotting. Stronger expression of HSP90β, GRP78 and 14-3-3ε were detected in Huh7-R cells compared with parental Huh7 cells (Figure 2A). Second, expression of HSP90β, GRP78 and 14-3-3ε in patient samples was examined by immunocyto-chemistry. The expression of indicated proteins was categorized as high expression or low expression based on intensity (Figure 2B). 73% of tumor samples had high expression of GRP78, 18% had high 14-3-3ε expression and 85% high HSP90β expression.

High expression of GRP78 correlated with shorter progression free survival in HCC patients treated with sorafenib. Patients with high expression of GRP78 HCC had shorter progression free survival (PFS) following sorafenib treatment. The median PFS in the high GRP78 expression group was 2.20 months (95% confidence interval=2.42-5.10) significantly shorter compared with 4.73 months (95%CI=4.45-14.27) in the low GRP78 expression group (p=0.008). Furthermore, low expression of 14-3-3ε tended to associate with poor progression-free survival, but it did not reach statistical significance. Regarding HSP90β, the expression intensity did not correlate with survival (Figure 3).

Table II. *Demographics of HCC patients treated with sorafenib.*

	n (%)	n (%)	n (%)
Age (<60:≥60)	26 (43)	34 (57)	
Gender (M:F)	49 (82)	11 (18)	
BCLC[a] (A:B:C)	3 (5)	15 (25)	42 (70)
Diabetes (Y:N)	17 (28)	43 (72)	
Hepatitis B (Y:N)	35 (58)	25 (42)	
Hepatitis C (Y:N)	20 (33)	40 (67)	
GRP78 expression (L:H)	16 (27)	44 (53)	
14-3-3ε expression (L:H)	49 (82)	11 (18)	
HSP90β expression (L:H)	9 (15)	51 (85)	

[a]BCLC: Barcelona Clinic Liver Cancer staging system.

Discussion

Sorafenib had been demonstrated to involve several targets in inhibiting cancer cell growth: Serine-threonine kinase Raf-1 and B-Raf in RAF/MEK/ERK pathway, RET, FLT-3, the receptor tyrosine kinase activity of vascular endothelial

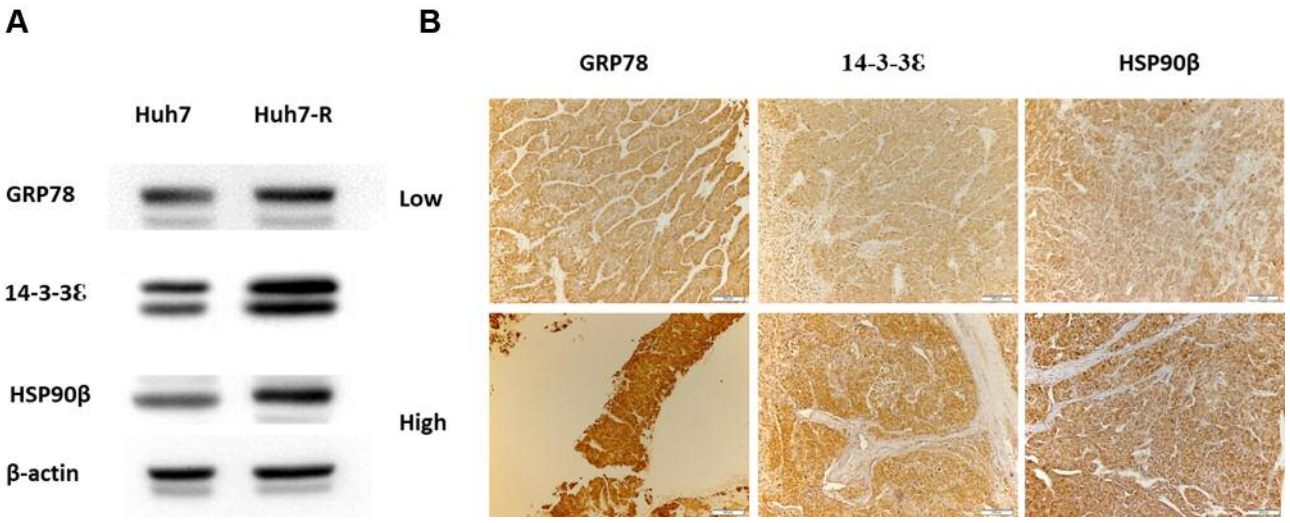

Figure 2. *A. Immunoblotting of the expression of GRP78, 14-3-3ε and HSP90β in parental Huh7 and Huh7-R cells. B. Immunohistochemistry stainins of GRP78, 14-3-3ε and HSP90β in human HCC tumors. The indicated protein expression was categorized as low or high.*

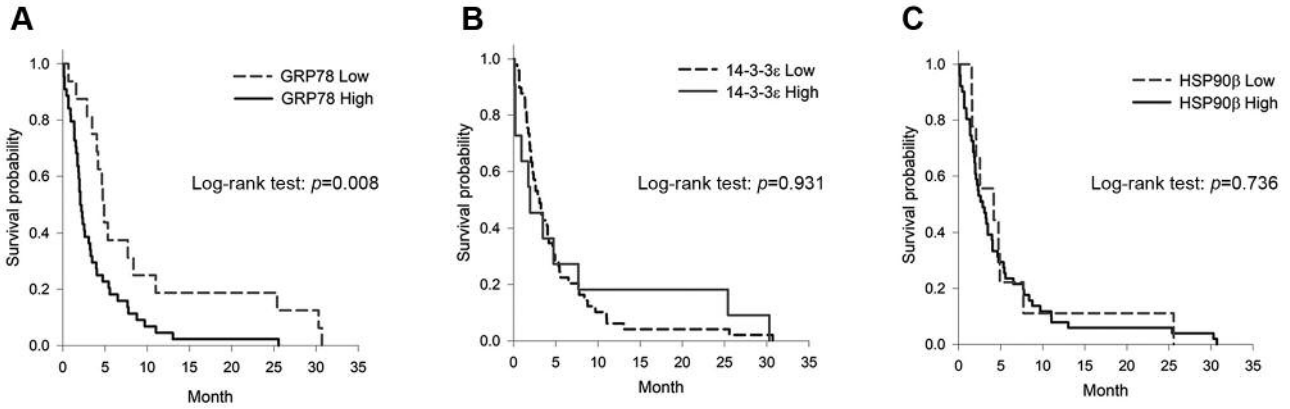

Figure 3. *Progression-free survival categorized by the expressions of indicated proteins. A: GRP78, B: 14-3-3ε, C: HSP90β.*

growth factor receptors (VEGFRs) 1, 2, 3 and platelet-derived growth factor receptor-β (PDGFR-β)(10). The clinical efficacy of sorafenb in treating patients with Child-Pugh A stage C HCC has been documented by two phase III randomized, placebo-controlled trials: the SHARP study and the Asia-Pacific study (ORIENTAL) in terms of benefit on overall survival, time to radiologic progression and disease control rate (5, 11). Sorafenib seems to be effective to prolong the median survival time with limited side effects in patients with advanced HCC, but the primary resistance and acquired resistance to sorafenib has become an obstacle to extend the clinical efficacy of sorafenib.

More than half of HCC patients have epidermal growth factor receptor (EGFR) over-expression and abnormal activation. Abnormal activation of EGFR/human epidermal receptor type 3 (HER3) as well as overexpression of both EGFR and its ligand can inhibit the effect of sorafenib on suppressing tumor growth. Overexpression of EGFR or its ligand in HCC cells may relate to primary resistance of sorafenib (12). In addition to EGFR and its ligand, downstream signaling molecules also contributed to sorafenib resistance. Down-regulation of pERK and expression of c-Jun N-terminal kinase can predict the sensitivity to sorafenib (13, 14).

In regards to the acquire resistance to sorafenib, many mechanisms have been extensively studied, including PI3K/Akt pathway, autophagy, epithelial-mesenchymal transition, tumor microenvironment, and epigenetic regulation. An emerging theory focuses on the angiogenic strategy to acquire resistance to cancer treatment, including sorafenib. It proposes vessel co-option, where the tumor hijacks the existing vasculature in organs such as the lung and liver, limiting the tumor's need for angiogenesis. An orthotopic human HCC model revealed that up to 75% of total vessels were provided by vessel co-option in resistant tumors relative to 23% in untreated control (15).

GRP78 is a molecular chaperone involved in endoplasmic reticulum stress response and can be regulated by the transcription factors ATF6 and XBP1 which play important role in regulation of tumor proliferation, invasion and metastasis (16, 17). GRP78 was up-regulated in HCC tumors and correlated with Granulin-epithelin precursor expression (18). The development of acquired resistance to systemic treatment limits the extension of survival of patients with advanced HCC. Treatment human leukemia cells (U937) with sorafenib can induce endoplasmic reticulum stress by immediate cytosolic calcium mobilization, GADD34 protein induction, PKR-like ER kinase and eukaryotic initiation factor 2α phosphorylation, XBP1 splicing, and a general reduction in protein synthesis as assessed by [^{35}S]methionine incorporation. It was accompanied with pronounced generation of reactive oxygen species through a mechanism dependent on cytosolic calcium mobilization and a significant decline in GRP78/Bip protein levels (19). In agreement, the expression of GRP78 has been associated with a wide range of therapies, including chemotoxic, anti-hormonal, DNA damaging and anti-angiogenesis agents in several cancers (20). The contribution of GRP78 in acquired resistance to sorafenib has been studied in HCC cells. Another study used HepJ5 (a GRP78-overexpressing HCC cells) and HepG2 cells as a paired matched control to determine the acquired resistance to sorafenib in HCC cells (21). Further studies suggested that GRP78 can be detected in serum samples of HCC patients. Also, secreted GRP78 facilitated proliferation and inhibited apoptosis induced by sorafenib both in HCC cell lines and xenografts. GRP78 can interact with EGFR, activate EGFR-SRC-STAT3 pathway, conferring the resistance of sorafenib (22). Questiomycin, a suppressor of GRP78 by promoting its degradation, enhanced the anti-tumor effect of sorafenib in HCC xenograft models (23). Importantly, high expression of GRP78 was associated with inferior survival of HCC patients in our study. It substantially supported the role of GRP78 in acquired resistance to sorafenib therapy of HCC.

14-3-3 proteins comprise seven isoforms and share highly conserved homology among all eukaryotic cells. The biological functions of 14-3-3 proteins influence cell development, cell cycle regulation, DNA repair, cell proliferation, apoptosis, adhesion, motility, and tissue response to injury (24). Several studies have demonstrated that 14-3-3β, 14-3-3ε, 14-3-3γ, 14-3-3σ and 14-3-3ζ isoforms are overexpressed in HCC and expression of 14-3-3ε is associated with inferior overall and progression free survival (25). Among these 14-3-3 proteins, 14-3-3ζ is conjugated with αB-Crystallin to promote HCC progression and has been demonstrated to be involved in sorafenib resistance in vitro and patients with HCC (26). In our study, 14-3-3ε was overexpressed in Huh7-R cells but it failed to contribute to sorafenib resistance in patients with HCC. The effect of 14-3-3ε on sorafenib resistance remains uncertain based on our analysis and previous studies (25). HSP is normally maintained at low levels but it is induced under protein-damage conditions. HSP70, not HSP90, is downstream of heat shock fctor-1α which is activated by 14-3-3σ and β-catenin. The biological functions of HSP70 include enhancement of cell migration and endothelial-mesenchymal transition (27, 28). Up to date, only clusterin, an ATP-independent molecular chaperone with HSPs-like properties, has been reported to confer resistance to sorafenib from an unpublished data (28).

The overall treatment for advanced HCC remains unsatisfied and novel agents targeting sorafenib resistance or other pathways are strongly desired. Recently clinical trials have indicated that selective c-Met inhibitors have anti-tumor activity in HCC with acceptable safety and tolerability in patients with Pugh A liver reserve. It is required to continue the exploration of the clinical benefit of c-Met inhibition in HCC treatment. Before this achievement, it is mandatory to conquer sorafenib resistance by targeting GRP78 and further research focused on this strategy should be encouraged.

Conclusion

In conclusion, we enforced the evidence that GRP78 contributes to the resistance to sorafenib in HCC by *in vivo* and clinical analysis. The results are consistent with other preliminary studies and present a primary target for overcoming sorafenib resistance.

Conflicts of Interest

All Authors have no potential conflicts of interest to declare regarding this study.

Authors' Contributions

YHF, CJT and TFW wrote the manuscript. YHF and CLT conducted the process of experiments. YHF, YCS, CJT and TFW designed the study and data interpretation. YHF, YCS and CLT prepared the organization of figures and statistical analysis. All Authors verified the content of the manuscript.

Acknowledgements

The Authors appreciate the generous donation of the cell lines, Huh7-R from Dr. Kuen-Feng Chen (National Taiwan University).

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Received June 18, 2019
Revised July 24, 2019
Accepted July 25, 2019