Activation of MET promotes resistance to sorafenib in hepatocellular carcinoma cells via the AKT/ERK1/2-EGR1 pathway

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ABSTRACT
Sorafenib is an oral multikinase inhibitor that has become an established therapeutic approach in advanced hepatocellular carcinoma (HCC). However, the benefit of sorafenib in clinical therapy is often affected by drug resistance. Therefore, it is important to explore the mechanisms underlying sorafenib resistance and to develop individualized therapeutic strategies for coping with this problem. In this study, we found that addition of HGF to sorafenib-treated HCC cells activated MET and re-stimulated the downstream AKT and ERK1/2 pathways. Thereby, restored sorafenib-treated HCC cells proliferation, migration and invasion ability, and rescued cells from apoptosis. In addition, we found that HGF treatment of HCC cells induced early growth response protein (EGR1) expression, which is involved in sorafenib resistance. Importantly, the HGF rescued effect in sorafenib-treated HCC cells could be abrogated by inhibiting MET activation with PHA-665752 or by downregulating EGR1 expression with small interfering RNA (siRNA). Therefore, inhibition of the HGF/MET pathway may improve response to sorafenib in HCC, and combination therapy should be further investigated.

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Introduction
Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world and is the second most frequent cause of cancer-related death [1]. Surgical resection, liver transplantation and radiofrequency ablation (RFA) as potentially curative treatment modalities. Unfortunately, more than 80% of patients with HCC are diagnosed at a late stage of the disease, when potentially curative are least effective [2–4]. Sorafenib, a VEGF receptor (VEGFR) inhibitor with activity against platelet-derived growth factor receptor (PDGFR), c-Kit receptor, RAF and p38 signal transduction pathways, has become a standard treatment in patients with advanced HCC [5]. However, even though sorafenib improves the median survival in advanced HCC, the median overall survival remains less than 1 year partly due to that many patients eventually become resistant to this drug [6,7]. Moreover, no effective therapeutic options currently exist in patients who are resistant to sorafenib. Therefore, effective strategies to improve the therapeutic effect of HCC patients with sorafenib resistance are urgently needed.

The MET oncogene, which encodes the tyrosine kinase receptor for hepatocyte growth factor (HGF), has been observed to play an important role in the development of human cancers and drug resistance in cancer cells [8–10]. Binding of HGF to MET results in phosphorylation of the receptor tyrosine kinase domain, then activation of multiple intracellular pathways such as mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and focal adhesion kinase (FAK). MET is found to be overexpressed/activated in approximately 20–30% of HCC and correlated with poor prognosis [11,12]. A recent study revealed that HGF induced resistance to multiple kinase inhibitors by activation of the phosphorylated MET (p-MET) depend on AKT and ERK1/2 pathways [13]. Moreover, our previous report showed that high level of activated MET in HCC is associated with resistance to adjuvant sorafenib treatment [14]. Thus, it is important to clarify that whether HGF-mediated MET activation could rescue HCC cells from sorafenib inhibition and the mechanism involved.

EGR1 is a nuclear protein that is activated by growth factors (e.g. EGF or HGF), cytokines, hormones and environmental stresses [15]. It has been reported that AKT and ERK1/2 are associated with HGF-induced EGR1 expression in HCC cells [16]. According to the report from Chen et al. [17], EGR1 is involved in SLUG expression and the subsequent reduction of sorafenib sensitivity in sorafenib-resistant cells. In this study, we observed that HGF induced resistance of HCC cells...
to sorafenib by restoring signaling through the AKT/ERK1/2-EGR1 signaling pathway. Notably, the anti-proliferative and anti-metastatic effect of sorafenib could be restored by treating cells with MET inhibitor or EGR1 downregulation. These results providing a rationale for combined treatment of sorafenib and MET inhibitor in HCC.

**Material and methods**

**Cell lines and culture conditions**

HepG2 and Huh7 cells were obtained from the American Type Culture Collection (ATCC). The cell lines were maintained in DMEM (GIBCO BRL, Grand Island, NY) supplemented with 10% FBS (fetal bovine serum; GIBCO BRL, Grand Island, NY), 100 U/ml penicillin and 100 U/ml streptomycin. All cell cultures were maintained at 37°C in a 5% CO2 incubator with a controlled humidified atmosphere composed of 95% air and 5% CO2.

**Chemicals and reagents**

Sorafenib and PHA-665752 were obtained from Selleck Chemicals (Houston, TX). Primary antibodies against MET, phospho-MET (Tyr1234/1235), AKT, phospho-Akt (Ser473), ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), EGR1, SLUG and horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Cell Signaling Technology (Beverly, MA). The antibody against GAPDH was a product of Kangchen Biotech (Shanghai, China). The Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies Inc. (Kumamoto, Japan). The recombinant human HGF was obtained from R&D Systems (Minneapolis, MN). The Annexin V-FITC/PI staining Kit was purchased from BD Biosciences (Beckman Coulter, Inc., Miami, FL). The data were analyzed using Kaluza software (Beckman Coulter, Inc., Miami, FL).

**Cell proliferation assay**

Cell proliferation was measured using CCK-8 as previously reported [18]. Briefly, HepG2 and Huh7 cells (5 × 10^4 per well) were plated in 96-well plates in DMEM supplemented with 10% FBS and allowed to adhere for 24 h. Thereafter, the cells were treated for 72 h as indicated in serum-reduced (1% FBS) media. The cells were then incubated for an additional 2 h with CCK-8 reagent (100 μl/medium) and read at 450 nm using a microplate reader (Thermo, Varioskan FlashThermo Fisher Scientific Inc., Waltham, MA). The IC_{50} value, at which 50% of cell growth inhibition compared with control, was calculated by non-linear regression analysis using GraphPad Prism software (San Diego, CA).

**Apoptosis analysis**

Cells were plated at a density of 5 × 10^5 in six-well plates and then treated for 48 h as indicated in serum-reduced (1% FBS) media. Then, the cells were harvested, washed and re-suspended at a density of 5 × 10^5/ml in 100 μl of 1× binding buffer. Annexin V-FITC and PI were then applied to each sample, and the reactions were incubated at room temperature for 15 min. Finally, another 400 μl of 1× binding buffer was added to each sample, and the samples were tested using a flow cytometer (Beckman Coulter, Inc., Miami, FL). The data were analyzed using Kaluza software (Beckman Coulter, Inc., Miami, FL).

**Western blot analysis**

Cell lysates were prepared as described previously [18]. A total of 25–50 μg of protein extracted from cultured cells was separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked and blotted with the relevant antibodies. HRP-conjugated secondary antibodies were detected using an enhanced chemiluminescence reagent (Millipore Corp., Bedford, MA). GAPDH was used as a loading control. All the antibodies were used at a dilution of 1:1000, except the anti-GAPDH antibody, which was used at a dilution of 1:5000.

**Cell migration and invasion assay**

The cell migration and invasion assays were performed using transwell chambers (8 μm pores, Corning Incorporated Costar, Corning, NY) pre-coated without (migration assay) or with (invasion assay) Matrigel (BD Biosciences, San Jose, CA). 5 × 10^4 cells in low serum (1% FBS) DMEM containing 5 μM sorafenib were added to the upper chamber. Then, 0.6 ml of DMEM supplemented with 10% FBS or 1% FBS and 50 ng/ml HGF were added to the lower chamber, and the cells were incubated for 24 h (migration assay) or 48 h (invasion assay). After incubation, the filters were fixed with 4% formaldehyde and stained with 0.5% crystal violet for 30 min. Cells on the upper surface of the filters were removed by wiping with a cotton swab. The stained cells were visualized under an inverted microscope (200×) and counted in four random fields.
The siRNA targeting EGR1 (EGR1 siRNA, sc-29303) and mock siRNA (mock, sc-37007) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Cells were grown to 80% confluence in six-well plates and were transfected on the following day using Lipofectamine 2000 (LF2000; Invitrogen, Carlsbad, CA). After 48 h of transfection, the expression of EGR1 in transfected cells was verified by Western blot.

Statistical analysis
Statistical evaluation of the data was conducted using Student’s t-test. Results were considered as statistically significant at $p < .05$.

Results
HGF reduces sorafenib anti-proliferative effects in HCC cells

The effect of sorafenib on proliferation in each HCC cells is shown in Figure 1. Sorafenib inhibited cell growth in a concentration-dependent manner in HepG2 and Huh7 cells, with $IC_{50}$ values of 6.16 ± 0.06 and 5.75 ± 0.11 μM, respectively. However, the combination treatment with HGF significantly rescued the cells from sorafenib inhibition. The $IC_{50}$ values of the combination treatment in HepG2 and Huh7 cells were 9.02 ± 0.32 and 8.19 ± 0.67, respectively. Our previous research had proven that the selective MET inhibitor PHA-665752 at concentration of 100 nM had no effect on the proliferation of HepG2 and Huh7 cells [19]. When treating HCC cells with indicated combination of sorafenib, HGF and 100 μM PHA-665752, we found that MET inhibition significantly reversed sorafenib resistance mediated by HGF.

HGF protects HCC cells from sorafenib-induced apoptosis

Apoptosis has been proven to be responsive to sorafenib-mediated anti-cancer activities [20]. Accordingly, we investigated the ability of HGF-induced anti-apoptosis of HepG2 and Huh7 cells by using flow cytometric analysis. As illustrated in Figure 2, treatment with 10 μM sorafenib caused

Figure 2. HGF protects HCC cells from sorafenib-induced apoptosis. Apoptosis of HepG2 and Huh7 cells detected by the Annexin V–FITC/PI assay. Cells were treated with or without 50 ng/ml HGF for 24 h, then treated with indicated compounds for 48 h. The rate of apoptosis was determined using a flow cytometer, and data were analyzed using Kaluza software and were reported as the mean ± SD. The results are representative of three independent experiments. $p < .05$. 

EGR1 small interfering RNA (siRNA) and transfection
The siRNA targeting EGR1 (EGR1 siRNA, sc-29303) and mock siRNA (mock, sc-37007) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Cells were grown to 80% confluence in six-well plates and were transfected on the following day using Lipofectamine 2000 (LF2000; Invitrogen, Carlsbad, CA). After 48 h of transfection, the expression of EGR1 in transfected cells was verified by Western blot.
23.15% and 24.31% apoptosis in HepG2 and Huh7 cells, respectively. HepG2 and Huh7 cells showed increased resistance to sorafenib when co-treated in the presence of HGF. The percentage of apoptosis were decreased to 13.14 and 12.86. Importantly, a dose of 100 nM PHA-665752 markedly restored the pro-apoptotic activity of sorafenib in HepG2 and Huh7 cells.

**HGF antagonizes sorafenib anti-metastatic effect in HCC cells**

To evaluate whether HGF could affect sorafenib anti-metastatic effect, transwell assays with or without Matrigel were used. The transwell assay revealed that treated HepG2 and Huh7 cells with 5 μM sorafenib significantly decreased the number of migrated and invaded cells. When treating HepG2 and Huh7 cells with a combination of HGF and sorafenib, we found that HGF could overcome the migration and invasion inhibition induced by sorafenib. MET inhibition with PHA-665752 abrogated the effects of HGF on sorafenib-treated cells and restored the sorafenib-induced inhibition of migration and invasion (Figure 3).

**HGF rescue HCC cells through MET-dependent activation of AKT and ERK1/2 pathways**

We next wished to determine the molecular mechanisms by which HGF rescues HCC cells from sorafenib-induced inhibition of proliferation. On binding of MET, HGF activates several signaling pathways including the AKT and ERK1/2 pathways. As showed in Figure 4, sorafenib inhibits phosphorylation of AKT and ERK1/2 in HepG2 and Huh7 cells. However, treating the cells with a combination of sorafenib and HGF leads to activation of MET and reactivation of AKT and MAPK. When treating the cells with a combination of sorafenib and PHA-665752, inhibition MET activity leads to sustained suppression of the AKT and ERK1/2 phosphorylation despite HGF stimulation. These results suggest that the HGF rescue effect was by p-MET mediated activation of AKT and ERK1/2 pathways.

**Downregulation of EGR1 by siRNA abrogates the HGF-induced sorafenib resistance**

To further elucidate the molecular mechanisms through which HGF stimulates EGR1 expression and SLUG, we performed Western blot analysis using antibodies against EGR1 and SLUG. Our results showed that treatment HepG2 and Huh7 cells with 50 ng/ml HGF for 2 h markedly upregulated the expression of EGR1 and SLUG (Figure 5(A)). To determine whether EGR1 is involved in SLUG expression, HepG2 and Huh7 cells were transfected with si-EGR1. Notably, when the expression of EGR1 was knocked down in the cells, we found that SLUG expression was significantly downregulated (Figure 5(A)). Next, we performed CCK8 and transwell assay to test whether HGF-induced EGR1 expression result in sorafenib resistance. As showed in Figure 5(B), CCK8 assay demonstrated that inhibition of EGR1 expression canceled HGF-induced resistance to sorafenib in HepG2 and Huh7 cells. Transwell assay showed that downregulation of EGR1
Figure 4. HGF rescue HCC cells through MET-dependent activation of AKT and ERK1/2 pathways. HepG2 and Huh7 cells were starved in serum-reduced (1% FBS) media for 12 h before adding either sorafenib (5 μM) or PHA-665752 (100 nM). After incubation for 6 h and stimulation with 50 ng/mL HGF for 10 min, cells were lysed for Western blot analysis. Independent experiments were performed at least three times, and the results from a representative experiment are shown.

Figure 5. Downregulation of EGR1 by siRNA abrogates the HGF-induced sorafenib resistance. (A) HepG2 and Huh7 cells were transfected with EGR1 siRNA for 48 h, followed by the treatment of HGF (50 ng/ml) for 2 h before cell lysis for Western blot analysis. (B) The transfected HepG2 and Huh7 cells were treated with or without HGF (50 ng/ml) for 24 h, then treated with sorafenib (5 μM) for 72 h. The transfected cells were treated with sorafenib (5 μM) alone or HGF (50 ng/ml) and sorafenib. The cells were then incubated for 24 h (migration assay) or 48 h (invasion assay). (C) Migration abilities were measured using a transwell assay without Matrigel. (D) Invasion abilities were measured using a transwell assay with Matrigel. Data shown represent the mean ± SD. *p < .05.
expression restored inhibition effect of sorafenib on migration and invasion in the presence of HGF (Figure 5(C) and (D)).

Discussion

The systemic therapy with sorafenib has become an established therapeutic approach to fighting tumor growth in patients with advanced HCC. Despite sorafenib improves the median survival in advanced HCC, the median overall survival remains less than 1 year partly due to acquire resistance after varying periods of time. To overcome the resistance to sorafenib, it is necessary to clarify its molecular mechanisms.

We focused on the role of HGF/MET pathway in sorafenib resistance, since our previous study revealed that activated MET are associated with poor outcomes of sorafenib therapy in HCC patients [14]. Recent studies reported that long-term sorafenib treatment in HCC cell lines leads to activation of MET pathway with reduced sorafenib sensitivity [17]. In the present study, we found that treatment of HepG2 and Huh7 cells with HGF reduced sorafenib anti-proliferative and anti-apoptotic activity. MET inhibitor PHA-665752 at a concentration of no impact on growth could significantly reverse the HGF-induced sorafenib resistance, which further confirmed the resistance mediated by MET pathway.

MET is capable of triggering metastatic changes, including epithelial mesenchymal transition (EMT), enhancement of motility and invasiveness of various of tumor cells. Several researches reported that establishment of sorafenib resistant HCC cells by long-term sorafenib treatment leads to enhancement of migrated and invaded behavior with activation of EMT process [21,22]. However, the role of HGF/MET pathway in sorafenib anti-metastatic effect is still not clarified. In the transwell assay, we observed that 50 ng/ml HGF dramatically abrogated sorafenib-induced inhibition of migration and invasion. These results indicated that HGF/MET pathway could antagonizes sorafenib anti-metastatic effect in HCC.

Sorafenib executes its anti-HCC activities by targeting the RAF/MEK/ERK1/2 pathway [2]. Activation of PI3K/AKT signaling pathway have been reported to mediate acquired resistance to sorafenib in HCC [23,24]. Our results showed that HGF leads to activation of MET and reactivation of AKT and ERK1/2 in HCC cells despite of sorafenib treatment. These results suggest that HGF-dependent activation of MET would enable HCC cells to escape the inhibitory effects of sorafenib by activating shared downstream pathways.

EGR1 is involved in the development of human tumors and various extracellular signals can induce the transcriptional activation of EGR1 [25]. The ERK1/2, p38 MAPK and PI3K/AKT signaling pathways have been shown to be involved in the regulation of EGR1 [26–28]. A recent study showed that EGR1 expression was knocked down in sorafenib-resistant HCC cells, which resulted in significant repression of SLUG expression and resistance to sorafenib [17]. In support of this, we found that downregulation of EGR1 by siRNA canceled HGF-induced resistance to sorafenib and repression of SLUG expression. Furthermore, downregulation of EGR1 restored inhibition effect of sorafenib on migration and invasion.

In summary, we showed that HGF induced resistance to sorafenib by activating the AKT/ERK1/2-EGFR pathway through activation of MET. The resistance to sorafenib result in rescuing HCC cells from sorafenib-induced inhibition of proliferation, migration and invasion. There are a number of HGF/MET pathway targeting agents that are being tested clinically [29,30]. These results suggest that targeting HGF/ MET pathway may help to improve treatment efficacy in HCC with resistance to sorafenib. Also, the presence of MET activation in HCC is a promising biomarker for predicting the response to sorafenib treatment.

Disclosure statement

No potential conflict of interests was reported by the authors.

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