Sorafenib Inhibits Migration and Invasion of Hepatocellular Carcinoma Cells Through Suppression of Matrix Metalloproteinase Expression

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Abstract. *Sorafenib increases survival of patients with advanced hepatocellular carcinoma (HCC) by inhibiting RAF kinase and receptor tyrosine kinase activity, but involvement of sorafenib in fibrosis and epithelial–mesenchymal transition (EMT) remains unclear. To elucidate effects of sorafenib on EMT progression and matrix metalloproteinase (MMP) activity, levels of E-cadherin, N-cadherin, and MMPs were evaluated in HepG2 human HCC cells induced by hepatocyte growth factor (HGF). Scratching cell migration assay, matrigel cell invasion assay, and immunohistochemistry were performed to examine effects of sorafenib on tumor metastasis and MMP expression. Sorafenib inhibited HGF-induced EMT and suppressed cell migration and invasion. Treatment with sorafenib significantly reduced HGF-enhanced expression of MMPs, suggesting that inhibition of MMP activity contributes to suppression of cellular motility and invasiveness of HepG2 cells. Neutralization of MMP activity by antibodies to MMP2/9, broad-spectrum MMP inhibitor or selective gelatinase inhibitor resulted in significant suppression of HGF-induced EMT and cell migration/invasion. Sorafenib treatment and MMP inactivation inhibited HGF-induced c-MET and MEK/ERK pathways. Sorafenib reduced MMP activity in this HGF-induced tumorigenic model of HCC. These findings provide in vitro evidence that sorafenib suppresses HGF-induced EMT and cell migration/invasion, as well as HGF-induced c-MET and MEK/ERK pathways.*

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Hepatocellular carcinoma (HCC) is a common human cancer worldwide and is the third leading cause of cancer-related death (1, 2). Most patients with HCC die of metastasis rather than the primary tumor itself and further understanding of the mechanisms underlying HCC metastasis is therefore needed, as well as ways to improve the efficacy of current treatment strategies.

Sorafenib is a multikinase inhibitor that targets several serine/threonine and receptor tyrosine kinases. Sorafenib is reported to inhibit HCC cell growth and metastasis by inhibiting the activity of RAF kinase and receptor tyrosine kinases. However, the possible involvement of sorafenib in fibrosis and epithelial–mesenchymal transition (EMT) remains unclear. EMT is shown to occur during organ fibrosis as well as in the initiation of metastasis during cancer progression (3). EMT-derived hepatic myofibroblasts proliferate and up-regulate their production of fibrillar collagens, which results in an increase in the deposition of fibrotic matrix. EMT and the expression of matrix metalloproteinase (MMP) are two important steps during the metastasis of HCC. MMPs are known to play an important role in cell migration during cancer invasion by degrading extracellular matrix proteins. Regulation of the expression and activation of MMPs is tightly controlled at multiple levels during cellular processes (4-6). Hepatocyte growth factor (HGF)/c-MET binding induces complex biological activities, including cell proliferation and cell invasion (7). Up-regulation of HGF and c-MET have been associated with tumor progression, metastasis and EMT progression in HCC (8).

The multikinase inhibitor, sorafenib, has been used to treat advanced HCC, with established clinical benefit for patients. However, the underlying molecular mechanism of sorafenib in HGF-mediated EMT progression remains elusive. The aim of this study was therefore to investigate the effects of sorafenib on HGF-induced EMT progression and on the
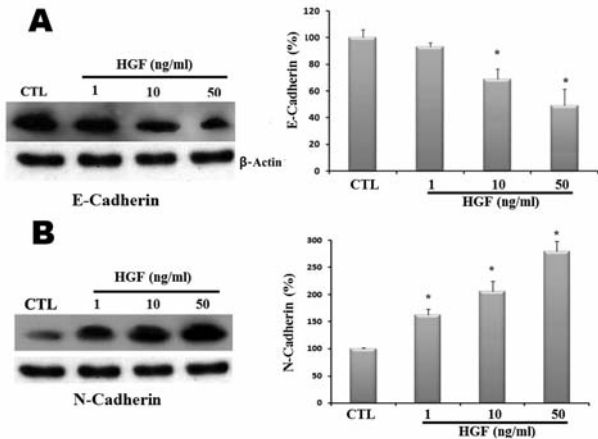
Figure 1. *Hepatocyte growth factor (HGF)-associated expression of E-cadherin (A) and N-cadherin (B). HepG2 cells treated with HGF exhibited decreased E-cadherin and increased N-cadherin expression in a concentration-dependent fashion. CTL, Control; *p<0.001 vs. control.*

activities of MMP family members responsible for metastasis and fibrosis in HCC, and to determine the role of sorafenib in cancer cell adhesion and tumor progression.

Material and Methods

Cell culture. The HCC cell line, HepG2, derived from the liver tissue of a patient with hepatoblastoma, was purchased from the Korean Cell Line Bank. HepG2 cells are adherent, epithelial-like cells that grow as monolayers and in small aggregates, and have a model chromosome number of 55. HepG2 cells were grown in Dulbecco modified Eagle medium (DMEM; GIBCO-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) at 37°C in humidified cell culture conditions with 5% CO_2. Cell culture reagents were purchased from Gibco (Life Technologies, Madrid, Spain). Confluent HepG2 cells growing in 10% FBS complete media were re-plated in 24-well culture dishes, at a density of 5×10^4 per well, in 500 μl of 10% FBS complete medium. The next day, the medium was replaced with serum-free medium and cells were incubated overnight. HepG2 cells were pretreated for 3 hours with HGF (Gibco, Life Technologies, Spain) and then were treated at a final dose of 1 ng/ml, 10 ng/ml and 50 ng/ml in culture media for 48 hours. Sorafenib (Bay-43-9006) was obtained from Cayman Chemical (Ann Arbor, MI, USA) and used at a final concentration of 0.5 μM, 1 μM and 2 μM. In addition, in order to neutralize MMP activation, antibodies to MMP2 and MMP9 purchased from Abcam (Cambridge, MA, USA) were incubated with cells at a final concentration of 5 μg/ml for 48 hours.

Immunocytochemistry. Cells were fixed in 4% paraformaldehyde in phosphate buffer (77.4 ml of 1 M Na_2HPO_4, 22.6 ml of 1 M $Na_2H_2PO_4$ in 900 ml of distilled water) for 30 minutes at room temperature. Fixed cells were permeabilized with 0.5% Triton X-100 (Merck, Darmstadt, Germany) for 5 minutes, and then blocked by incubation with 2% normal goat serum and 2% normal goat serum in phosphate-buffered saline (PBS) for 1 hour at room temperature. Cells were incubated with primary antibodies (E-cadherin, 1:500, Cell Signaling Technology, Danvers, MA, USA and N-cadherin, 1:500, Abcam) overnight at 4°C. Primary antibodies were detected with fluorescein isothiocynate- or CY3 (Invitrogen, Carlsbad, CA, USA)-conjugated anti-rabbit (1:500) or anti-mouse (1:200) secondary antibodies as appropriate for 1 hour at room
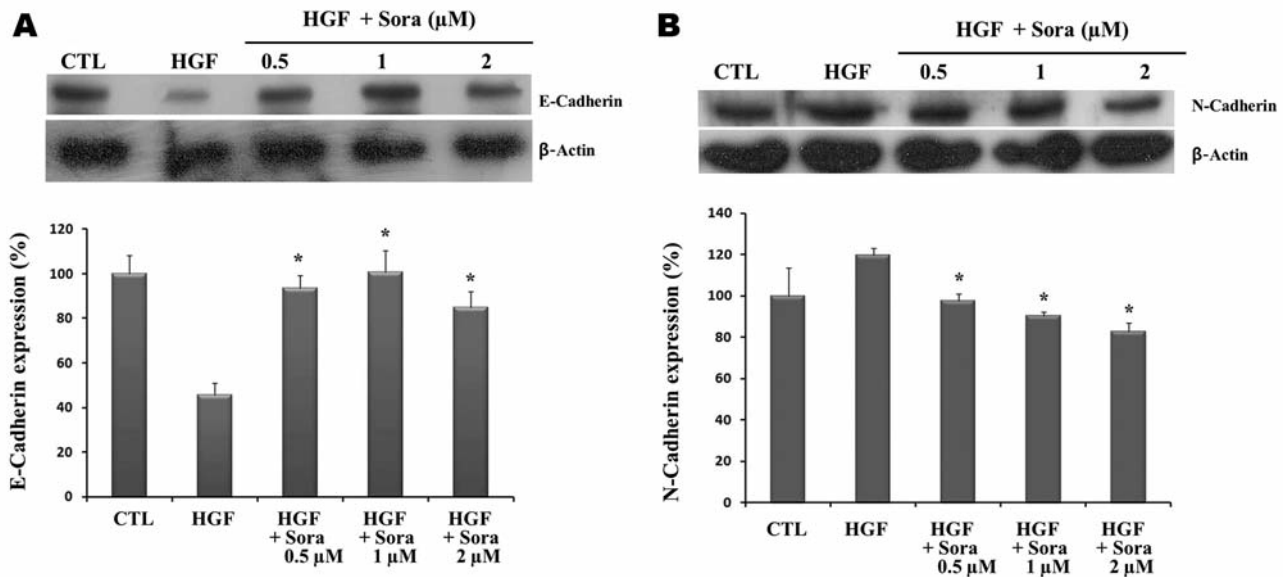

Figure 2. *Sorafenib (Sora) inhibits hepatocyte growth factor (HGF)-induced epithelial-mesenchymal transition phenotype by altering cadherin protein levels. Sorafenib administration clearly increased the expression of E-cadherin (A) and reduced that of N-cadherin (B) in a concentration-dependent fashion, as shown by western blot analysis. Expression of cadherin intensity was measured by an imaging densitometer. CTL, Control; *p<0.001 vs. HGF-treated cells.*
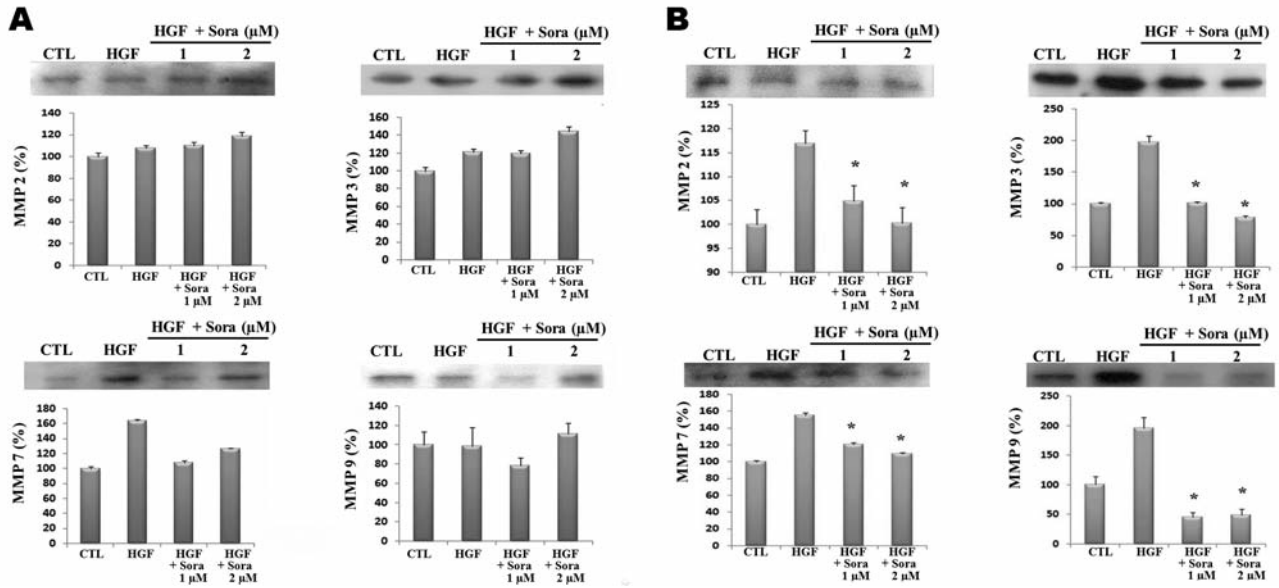
Figure 3. *Sorafenib (Sora) alters matrix metalloproteinase (MMP) protein expression in HepG2 cells. After hepatocyte growth factor (HGF) (10 ng/ml) treatment for 24 hours (A) and 48 hours (B), total cell lysates of each group were examined by western blot analysis, and MMP-2, -3, -7 and -9 proteins were quantified by densitometric analysis. At 24 hours, HGF increased MMP protein levels and sorafenib generally reduced HGF-enhanced MMP protein levels, except some samples. Treatment with sorafenib for 48 hours reduced the expression of HGF-induced MMP proteins, especially of MMP2, -3 and -9. CTL, Control; *p<0.01 vs. HGF-treated cells.*
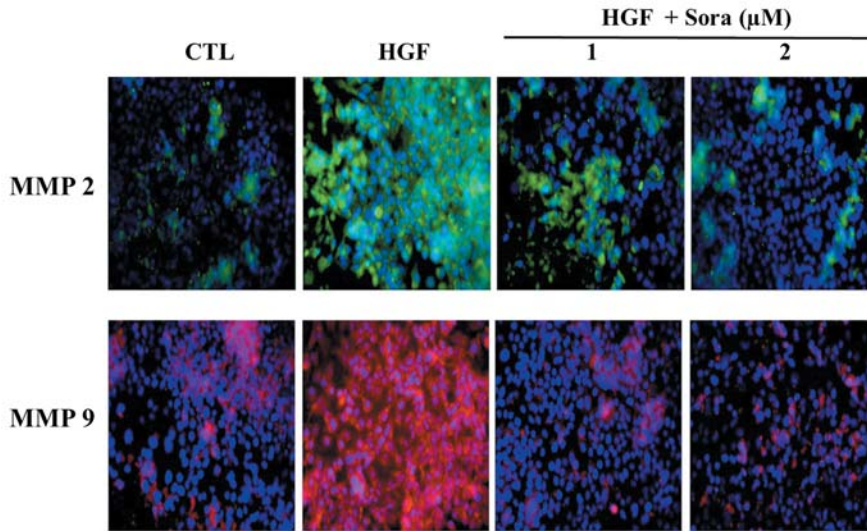

Figure 4. *Sorafenib (Sora) inhibits hepatocyte growth factor (HGF)-promoted matrix metalloproteinase (MMP)-2 and -9 proteins. Hep2 cells were cultured in 24-well plates and grown in medium with 10% fetal bovine serum for 48 hours. The cells were treated with 10 ng/ml HGF, and then 1 and 2 μM of sorafenib for 48 hours. MMP2 (green) and MMP9 (red) were visualized by immunofluorescence staining and nuclei with Hoechst (blue) under a fluorescence microscope.*

temperature. The results were evaluated using a fluorescence microscope (Leica, Heidelberg, Germany).

Western blot analysis. Total protein was extracted from cultures by lysis in Pro-Prep protein extraction solution (iNtRON Biotechnology, Boca Raton, FL, USA) at −20˚C for 30 minutes.

The lysates were centrifuged at 20217×g for 5 minutes at 4˚C. Protein concentration was measured using a standard Bradford assay (Bio-Rad, Hercules, CA, USA). Proteins (20 μg) were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes (Bio-Rad). Membranes were blocked for 1 hour in blocking solution,
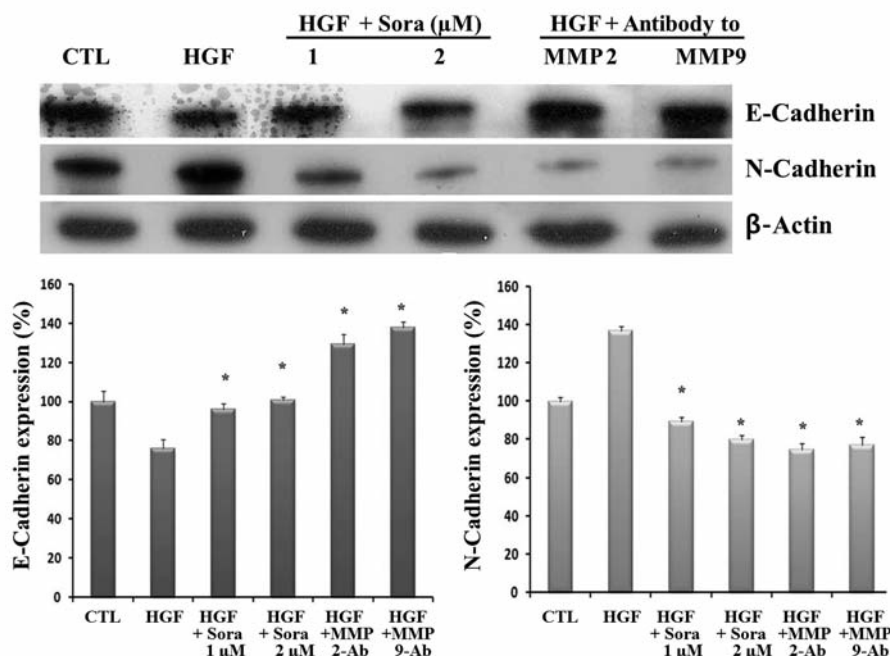
Figure 5 *Sorafenib (Sora) and antibody-mediated neutralization of matrix metalloproteinase (MMP) inhibit hepatocyte growth factor (HGF)-induced epithelial-mesenchymal transition (EMT) phenotype. After HepG2 cells were treated with 10 ng/ml HGF, sorafenib and anti-MMP-2 and 9 monoclonal antibodies (5 μg/ml) were added to the medium for 48 hours. Western blot analysis showed that sorafenib treatment and MMP antibodies effectively blocked HGF-mediated EMT phenotype. CTL, Control; Ab, antibody;* *p<0.001 vs. *HGF-treated cells.*

and then the primary antibody was added and incubated overnight at 4˚C. Primary antibodies used were against E-cadherin (1:1000; Cell Signaling Technology), N-cadherin (1:1000; Abcam, phospho-MET (1:1000; Cell Signaling Technology), MET (1:000; Cell Signaling Technology), phospho-ERK (1:1000; Cell Signaling Technology), MERK (1:000; Cell Signaling Technology), MMP2 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), MMP3 (1:500, Abcam), MMP7 (1:500, Abcam), and MMP9 (1:500, Abcam). Membranes were washed six times in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 5 minutes and then incubated with secondary antibodies, horseradish peroxidase-antimouse (1:2,000; Invitrogen), or anti-rabbit (1:5,000; Invitrogen) for 1 hour at room temperature. After washing six times in TBST for 5 minutes, the protein bands were visualized using electrogenerated chemiluminescent (ECL) reagents (Millipore, Billerica, MA, USA). The optical density of the bands was analyzed by reflectance densitometry on a Bio-Rad GS-670 imaging densitometer.

Scratch migration assay. To evaluate cell motility, HepG2 cells (5×10⁴ per well) were seeded in 24-well plates and grown to 80-90% confluence. After aspiration of the growth medium, the center of each cell monolayer was scraped with a sterile micropipette tip to create a denuded zone (constant width across all wells). Cellular debris was removed by washing with Hank's balanced salt solution, and the HepG2 cells were exposed to HGF (GIBCO-BRL) and either sorafenib or antibody to MMP. After incubation for 0, 24 and 48 hours, the width of the wound was monitored by photographing each well using a camera (Metamorpho) attached to an inverted Leica microscope. To quantify cell migration, two artificial lines fitting the edges of each cell culture were overlaid on the images. The width of the wound in each of 10 randomly selected fields was measured. Analyses were performed in triplicate.

Matrigel invasion assay. A Matrigel Invasion Chamber (8-μm pore size; BD Biosciences, San Jose, CA, USA) was used to measure the effect of sorafenib and antibody to MMP on HGF-induced invasion activity. Matrigel-coated chambers containing an 8-μm pore size filter were fitted into a 24-well tissue culture plate. Cells (2.5×10⁴ cells in 500 μl of medium) were seeded into the upper chambers of the system. The bottom wells were filled with serum-free medium with HGF as a chemoattractant. After 48 hours of incubation with sorafenib and antibody to MMP for each group of upper chambers, non-penetrating cells were removed from the upper surface of the filter with a cotton swab. Cells that invaded the lower surface and penetrated the Matrigel-coated filter were fixed with 100% methanol and stained using 1% toluidine blue. The number of invasive cells on the lower surface of each filter was counted in randomly chosen fields using a Leica microscope at a magnification of ×200. Each experiment was performed in triplicate.

Statistical analysis. Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA, USA) was used to optimize image quality and to prepare figures. All experiments were repeated a minimum of three times. Data were analyzed by one-way ANOVA with a Tukey-Kramer multiple comparison post hoc test using GraphPad Instat 3.05 (GraphPad, San Diego, CA, USA). Each value was expressed as mean±SD, and *p*-values less than 0.01 were considered significant.
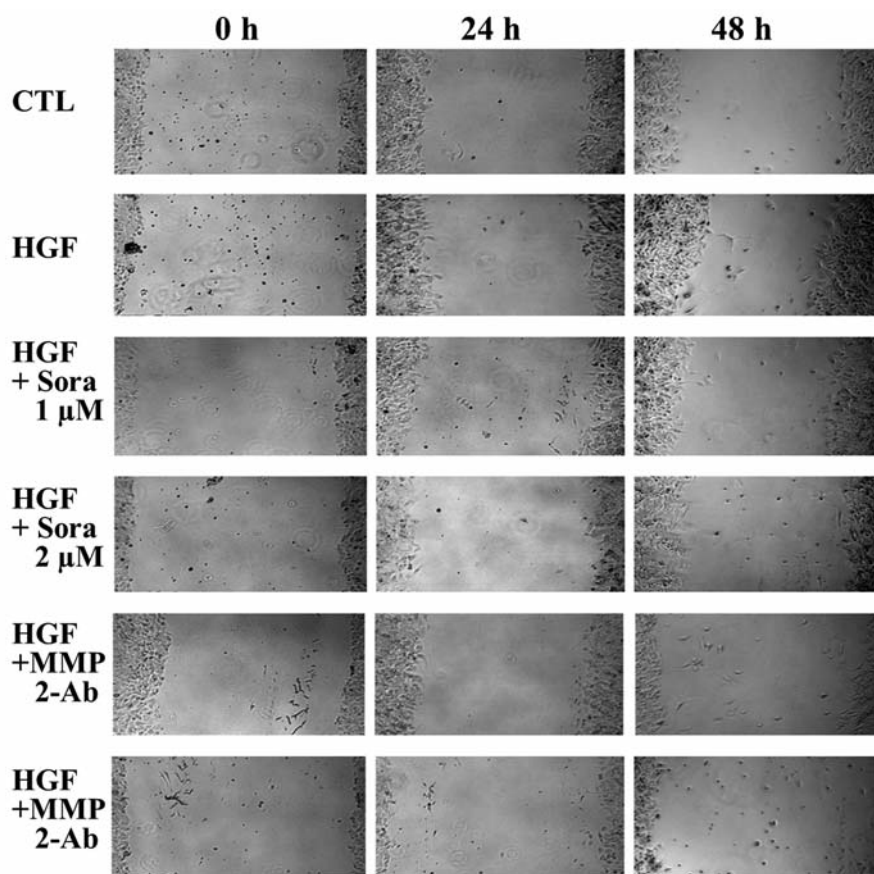
Figure 6. *Sorafenib (Sora) matrix metalloproteinase (MMP) inactivation suppress hepatocyte growth factor (HGF)-promoted cell migration. The migratory ability of HepG2 cells was evaluated using a scratch migration assay. Confluent cells were incubated with 10 ng/ml of HGF, 1 or 2 μM of sorafenib, and 5 μg/ml of MMP2/9 antibody for 48 hours, and a scratch was made with a tip of the pipette at the same time as treatment. Images were taken at 0, 24, and 48 hours after treatment to monitor wound closure. As shown by the scratch assay, sorafenib treatment and MMP inactivation suppressed the HGF-enhanced migratory potential of HepG2 cells. CTL; Ab, antibody.*

Results

HGF/c-MET signaling induces EMT. To determine the effect of HGF on EMT induction in HepG2 cells, we assessed the changes in the expression levels of E-cadherin and N-cadherin. HepG2 cells were incubated with different concentrations of recombinant human HGF (1, 10 and 50 ng/ml) for 48 hours. Western blot analysis and immunocytochemistry demonstrated that HGF strongly enhanced expression of EMT marker and caused cadherin switching (from E-cadherin to N-cadherin) in HepG2 cells. Furthermore, the expression of E-cadherin decreased and the expression of N-cadherin increased in a HGF concentration-dependent manner (Figure 1). These results indicate that HGF/c-MET binding is a strong inducer of EMT in HCC.

Sorafenib inhibits HGF-induced EMT. To assess the effects of sorafenib on HGF-induced EMT, HepG2 cells were treated with sorafenib during HGF treatment. The expression of the EMT marker E-cadherin was significantly higher after sorafenib administration compared to cells treated only with HGF (Figure 2A), with the effects of high-dose sorafenib (5 μM) being minimal, whilst 1 μM and 2 μM sorafenib significantly increased the expression of E-cadherin. We also assessed the effects of sorafenib treatment on N-cadherin expression. Immunofluorescence staining and western blot analysis revealed that sorafenib significantly reduced HGF-induced N-cadherin expression compared to treatment with HGF alone (Figure 2B). These results clearly indicate that sorafenib inhibits HGF-induced expression of EMT marker.

Sorafenib reduces HGF-induced MMP expression. Control of degradation of the extracellular matrix by MMPs is essential during tumor invasion and metastasis. To investigate the importance of sorafenib and HGF/c-MET signaling pathway

in MMP expression, western blot analyses for several MMPs (MMP2, -3, -7, and -9) were performed on isolated samples from 24- and 48-hour treatment cultures. MMP expression was not dramatically affected by sorafenib or HGF at 24 hours (Figure 3A). However, at 48 hours, HGF treatment stimulated MMP protein expression, with sorafenib reducing HGF/c-MET-mediated MMP activity, especially levels of MMP2, 3, and 9 (Figure 3B). Immunohistochemical staining of 48-hour cultures showed that sorafenib significantly suppressed the HGF-promoted expression of MMP2 and MMP9 (Figure 4).

Sorafenib treatment and antibodies to MMPs inhibit HGF-induced EMT phenotype. The aforementioned results demonstrated that sorafenib inhibits HGF/c-MET signaling pathway-mediated EMT and suppresses MMP activity. To better understand MMP activity during HGF-induced EMT, antibodies were used to neutralize MMP2 and MMP9. HGF reduced E-cadherin expression, but sorafenib increased E-cadherin expression and suppressed HGF-promoted expression of N-cadherin. Western blot analysis revealed that neutralization of MMP2 and -9 by antibodies significantly increased the expression of E-cadherin and reduced that of N-cadherin in HepG2 cells (Figure 5). These results show that MMP inactivation using antibodies to MMP2 and -9 is effective in inhibiting HGF-induced EMT phenotype. Hence, the decrease in MMP expression induced by sorafenib may be responsible for suppression of HGF-induced EMT phenotype.

Sorafenib treatment and MMP inactivation attenuate HGF-induced cell migration. Cancer progression is associated with abrogation of the normal controls that limit cell migration and invasion and that eventually lead to metastasis. To examine the effect of sorafenib and MMP inactivation on HGF-induced cell migration, a scratch assay on HepG2 cells was performed. The results show that HGF promoted the migration of HepG2 cells and both sorafenib treatment and treatment with antibodies to MMP2 and -9 inhibited cell migration after 48-hour treatment. Sorafenib treatment and inactivation of MMP significantly blocked the migratory ability of HepG2 cells (Figure 6).

These results suggest that MMP activity may play an important role in HGF-induced cell migration and that sorafenib inhibits HGF-induced cell migration by reducing MMP activation.

Sorafenib treatment and inactivation of MMP attenuate HGF-promoted cell invasion. To assess the effect of sorafenib treatment and MMP inactivation in HGF-promoted cell invasion, Matrigel invasion assays were carried out in the presence of sorafenib or specific anti-MMP-2 and -9 antibodies. Antibodies to MMP2 and -9, as well as sorafenib, reduced HGF-induced HepG2 cell invasion (Figure 7). These results highlight the importance of MMP activity in cell

invasion and suggest that sorafenib inhibits HGF-induced cell invasion *via* inactivation of MMP.

Sorafenib treatment and MMP inhibitors suppress HGF-induced EMT. To study MMP activity during HGF-induced EMT, we used inhibitors for several kinds of MMPs. GM6001 is a broad-spectrum MMP inhibitor including of MMPs 1, 2, 3, 7, 8 and 9, and SB-3CT is a competitive, mechanism-based, and selective inhibitor of MMP2 and 9.

Western blotting for E-cadherin (Figure 8A) showed that GM6001 and SB-3CT increased HGF-reduced E-cadherin expression more than did sorafenib treatment. These data were confirmed by immunohistochemistry for E-cadherin (Figure 8B). Western blotting for N-cadherin (Figure 8C) showed that treatment with sorafenib and MMP inhibitors enhanced the expression of N-cadherin after HGF induction. Immunohistochemistry for N-cadherin (Figure 8D) clearly showed that sorafenib and MMP inhibitors suppressed HGF-induced EMT phenotype. Sorafenib treatment and MMP inhibitors attenuate HGF-induced cell migration.

To examine the effect of sorafenib treatment and MMP inhibition on HGF-induced cell migration, scratch assays were performed. The resulting data clearly confirmed that treatment with sorafenib or inhibition of MMP (GM6001 and SB-3CT) blocked cell migration after 48 hours of treatment (Figure 9A). These results suggest that sorafenib treatment and MMP inactivation have an inhibitory effect on cell migration. MMP inactivation by sorafenib and MMP inhibitors (GM6001 and SB-3CT) after HGF treatment was further confirmed by immunohistochemical staining for MMP2 and MMP9 (Figure 9B-C).

Sorafenib treatment and MMP inactivation have an inhibitory effect on HGF/c-MET signaling. HGF binds to the c-MET receptor, leading to activation of the c-MET signaling pathway. We therefore focused on elucidating the effect of sorafenib on c-MET and extracellular signal-regulated kinase (ERK) pathways. Western blot analysis showed increased phosphorylation of c-MET and ERK in the HGF treatment group, while sorafenib and anti-MMP-2/9 antibody decreased the levels of phosphorylated c-MET and ERK (Figure 10). As expected, sorafenib treatment and MMP inactivation blocked the HGF-induced c-MET and ERK pathway cascade. These results indicate that inhibition of the MEK/ERK pathway contributes to the beneficial effect of sorafenib on tumor progression and that sorafenib seems to inhibit tumor metastasis by reducing MMP activity *via* the MEK/ERK pathway.

Discussion

Sorafenib has been shown to increase the survival rate of patients with advanced HCC (9), and many reports have largely focused on the role of sorafenib in tumor progression
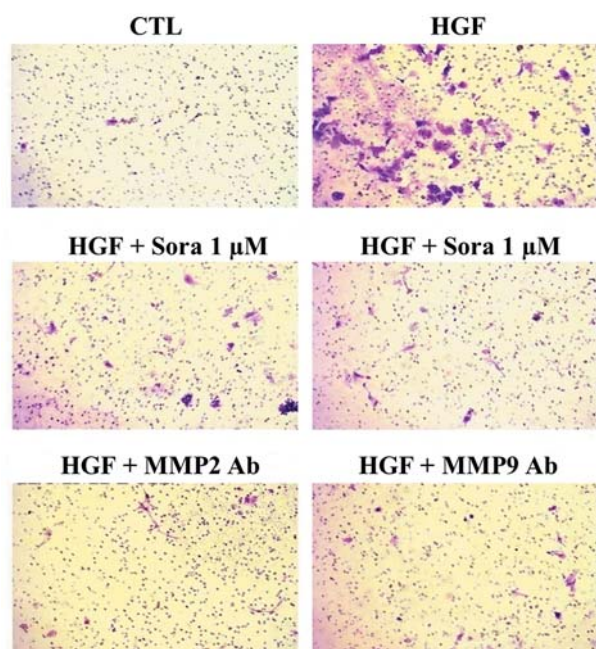
Figure 7. *Sorafenib (Sora) and matrix metalloproteinase (MMP) inactivation inhibit hepatocyte growth factor (HGF)-enhanced cell invasion. A Matrigel invasion assay was used to analyze the inhibitory effects of sorafenib treatment and MMP inactivation on HGF-induced cell invasion. Sorafenib dramatically reduced HGF-promoted cell invasion. The addition of MMP antibodies caused a marked decrease in HGF-induced cell invasion, similarly to sorafenib treatment. CTL, Control; Ab, antibody.*

and apoptotic cell death *via* the blocking of multiple receptor tyrosine kinases (10-12). MMP proteins play a critical role in the invasion and metastasis of cells during tumor progression and are commonly up-regulated in several types of cancer, including HCC (13). Consequently, there is a great need for better understanding of new roles of sorafenib in suppressing MMP activity. Our current study was focused on the effects of sorafenib in inhibiting tumor metastasis and MMP expression in HCC using the HGF-treated HepG2 cell line. Among the growth factors identified to be involved in HCC progression, the HGF/c-MET pathway has been demonstrated to play a key role in the EMT of HCC cells (14). HGF has been shown to be a stromal paracrine mediator that regulates tumor invasiveness and metastasis (15, 16). Our present investigation showed that HGF is also a strong inducer of EMT *via* the down-regulation of E-cadherin and up-regulation of N-cadherin (cadherin switching), which is known to be the most typical cellular event during EMT (17).

High levels of MMPs were detected in HGF-treated HepG2 cells, indicating that activation of the HGF/c-MET pathway enhances MMP expression (MMPs 2, 3, 7, and 9), especially after 48 hours of treatment. In cholangiocarcinoma, the second most common hepatic malignancy after HCC, MMPs 1, 2, 3,

and 9 are highly expressed (18, 19), and higher levels of c-MET and HGF have been detected compared to non-tumor tissues (20, 21). Our present study revealed that HGF is a key molecular regulator of MMP expression in HCC.

Sorafenib inhibited HGF-induced EMT phenotype, which is characterized by morphological changes, cadherin switching, and suppression of cell migration and invasion. These anti-EMT effects of sorafenib occurred through inhibition of phosphorylation of c-MET and ERK in HCC cells. Many reports have shown that tumor formation and metastatic properties are linked to EMT, a process facilitated by several factors. TWIST, a basic helix-loophelix transcription factor, has been shown to play a key role in E-cadherin repression and EMT induction. These EMT-inducing molecules can reduce E-cadherin transcription through their interaction with specific E-boxes in the proximal E-cadherin promoter (22, 23). Our current data showed that a 48-hour treatment with sorafenib significantly reduces the HGF-enhanced activity of MMPs 2, 3, 7, and 9 in HepG2 cells. Sorafenib has been reported to suppress *MMP9* induction concurrently with reduction of JNK phosphorylation, supporting the hypothesis that the RAS/RAC1/CDC42/JNK/ c-JUN/AP1 pathway and *MMP9* induction are important in the pathogenesis of sinusoidal endothelial cell collapse. Blocking of these effects may account for the protective effect of sorafenib in the model of sinusoidal obstruction syndrome (24). Our present data on the suppression of MMP expression by sorafenib further indicates that inhibition of MMPs contributes to suppression of cellular motility and invasiveness of HepG2 cancer cells. These results also confirmed the findings of previous reports that sorafenib attenuates portal hypertension, cirrhosis, and liver fibrosis (25-27).

The addition of MMP2/9-neutralizing antibodies, the broad-spectrum MMP inhibitor (GM6001), or the selective gelatinase inhibitor (SB-3CT) resulted in the clear suppression of HGF-induced EMT and cell migration/ invasion. These results strongly suggest that MMP activity plays an important role in HGF-induced EMT and HGF-enhanced cell migration and invasion. Hence, the reduction in MMP expression levels by sorafenib may be an effective way to suppress HGF-induced metastasis, indicating a therapeutic potential for treatment of liver fibrosis.

There are multiple pathways contributing to the effects of sorafenib on tumor progression. In our current study, c-MET expression levels decreased following sorafenib treatment. In previous *in vitro* analyses, c-MET protein levels were reported to be inhibited in sorafenib-treated HA22T/VGH and HepG2 cells, which may indicate that sorafenib directly or indirectly regulates c-MET expression (25, 28). Since HGF is a potent inducer of EMT and cell migration in HepG2 cells, the anticancer effects of sorafenib may be partly due to its inhibition of HGF/c-MET signaling.
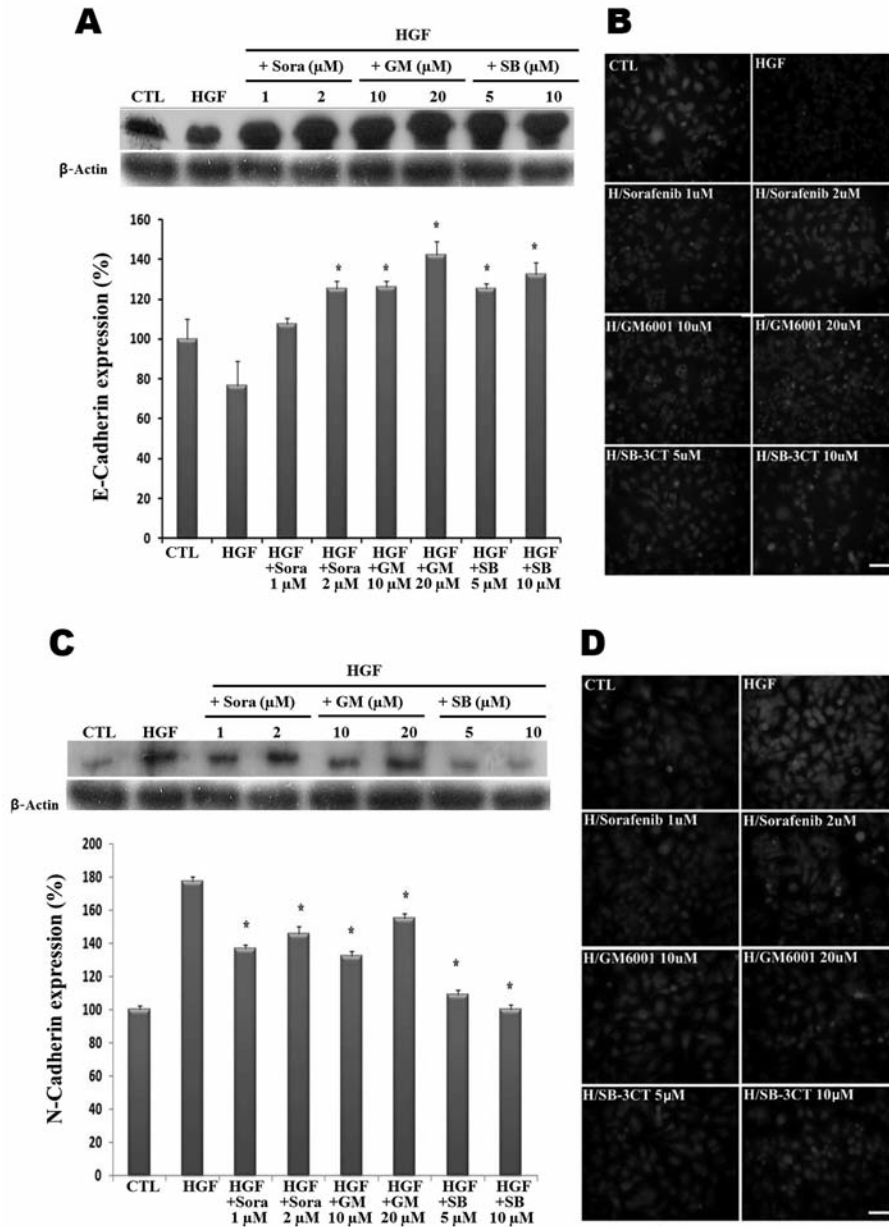
Figure 8. *Sorafenib (Sora) matrix metalloproteinase (MMP) inhibitors suppress hepatocyte growth factor (HGF)-induced epithelial–mesenchymal transition. A: HepG2 cells were treated with 10 ng/ml HGF, followed by the addition of sorafenib (1 and 2 μM) and MMP inhibitors GM6001 (10 and 20 μM) or SB-3CT (5 and 10 μM) for 48 hours. Treatment with sorafenib and MMP inhibitors (GM6001, SB-3CT) enhanced E-cadherin expression reduced by HGF. B: Immunostaining showed that sorafenib and MMP inhibitors effectively abrogated HGF-reduced E-cadherin expression. C: Treatment with sorafenib and MMP inhibitor (GM6001, SB-3CT) reduced HGF-enhanced N-cadherin expression. D: Immunostaining for N-cadherin showed that sorafenib and MMP inhibitors clearly reduced HGF-induced N-cadherin expression. CTL, Control; H, HGF; GM, GM6001; SB, SB-3CT; *p<0.01 vs. HGF-treated cells. The scale bar in the microphotographs corresponds to 50 μm.*

The ERK mitogen-activated protein kinase pathway, also known as MEK-ERK kinase cascade, has been implicated in the regulation of cancer cell proliferation and differentiation. In our current experiments on inhibition of the MEK/ERK pathway, we found that sorafenib reduced HGF-induced EMT and MMP expression in HCC cells through suppression of MEK/ERK activity. In addition, sorafenib exerted anti-EMT effects through inhibition of phosphorylation of c-MET and ERK. Our Western blotting experiments showed that MMP inactivation blocked HGF-
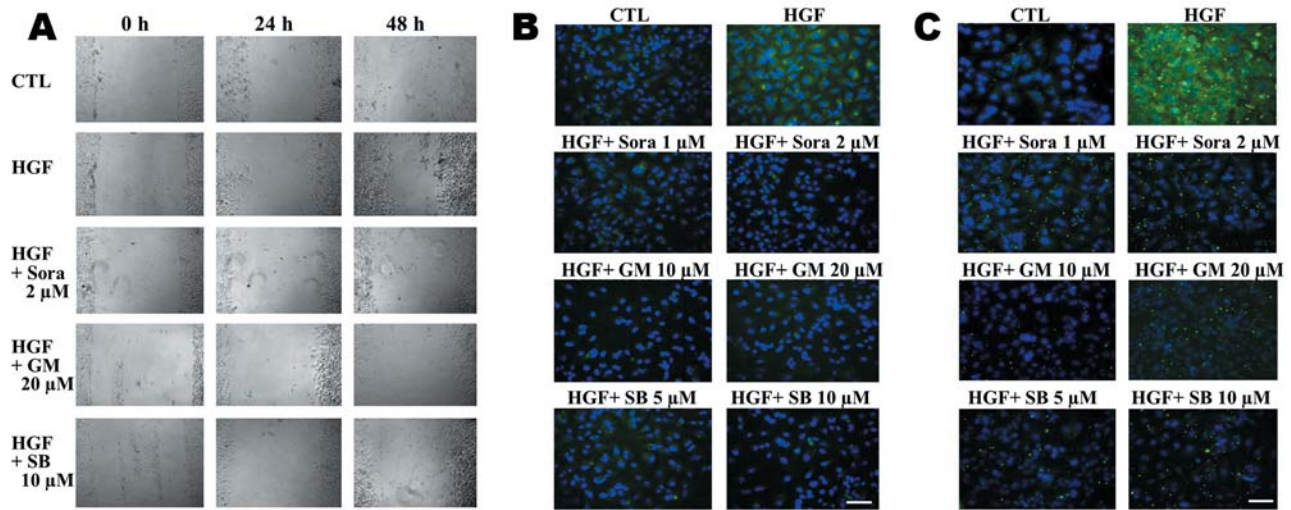
Figure 9. *Sorafenib (Sora) and matrix metalloproteinase (MMP) inhibitors inhibit hepatocyte growth factor (HGF)-induced cell migration. A: A scratch assay showed that sorafenib and MMP inhibitors (GM6001 and SB-3CT) reduced the HGF-enhanced migratory potential of HepG2 cells. B: MMP2 inactivation by sorafenib and MMP inhibitors after HGF treatment. C: MMP9 inactivation by sorafenib and MMP inhibitors after HGF treatment. CTL, Control; GM, GM6001; SB, SB-3CT.*

induced c-MET and MEK/ERK activity. These results suggest that inhibition of the MEK/ERK pathway contributes to the beneficial effects of sorafenib on tumor progression and that sorafenib possibly inhibits tumor metastasis by reducing MMP activity *via* the MEK/ERK pathway.

In conclusion, the results of our present study demonstrate sorafenib exerts beneficial antitumor effects by preventing MMP expression in an *in vitro* HGF-induced tumorigenic model of HCC. Our data provide *in vitro* evidence that sorafenib suppresses HGF-induced EMT and cell migration/invasion by reducing MMP expression. Sorafenib treatment and MMP inactivation suppressed HGF-induced c-MET and MEK/ERK pathways, suggesting that sorafenib inhibits tumor progression through the suppression of MMP activity and the MEK/ERK pathway.

Declaration of Interest

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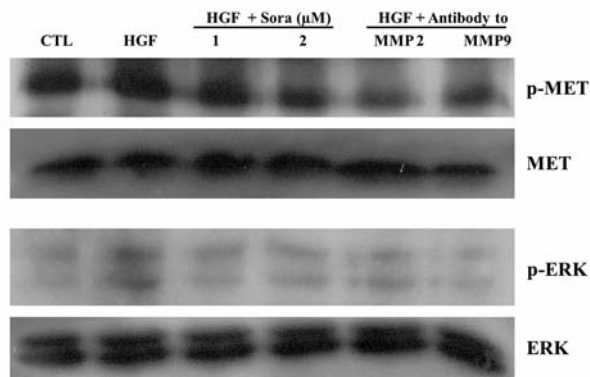

Figure 10. *Sorafenib (Sora) and antibodies to matrix metalloproteinase (MMP) reduce the expression of c-MET and the ERK pathway. After treatment of hepatocyte growth factor (HGF)-treated HepG2 cells with sorafenib and MMP2/9 antibody, cells were collected and phospho-c-MET, c-MET, phospho-ERK, and ERK protein levels were measured by western blotting. Phosphorylation of c-MET and ERK was increased after treatment with HGF and reduced by that with sorafenib and MMP2/9 antibodies. CTL, Control.*

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