Synergistic Effect of Sorafenib and Vitamin K on Suppression of Hepatocellular Carcinoma Cell Migration and Metastasis

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Abstract. *Vitamin K plays a role in controlling cell growth. Anti-angiogenic effects of sorafenib lead to impairment of vitamin K uptake and induction of des-γ-carboxyprothrombin release by hepatocellular carcinoma (HCC) cells. We examined sorafenib and vitamin K individually and in combination regarding their ability to suppress migration and metastatic potential of HCC cells. HepG2 cells (HCC cell line) were treated with hepatocyte growth factor (HGF). E-Cadherin expression, phospho-MET (p-MET), and phospho-extracellular signal-regulated kinase (p-ERK) levels and cell migration were evaluated. HGF-stimulated HepG2 cells, which were treated with a combination of sorafenib and vitamin K, showed significantly increased expression of E-cadherin and impairment of migration ability compared to when treated with either agent alone. This combination therapy also induced marked inhibition of epithelial– mesenchymal transition phenotype; inhibition of HGF-stimulated cell proliferation, invasion and migration; and inhibition of HGF/c-MET signaling pathway. Levels of p-MET and p-ERK were also significantly reduced by this combination. Our experimental study demonstrated that sorafenib and vitamin K can function synergistically to inhibit the migration and proliferation of HCC cells. Combination therapy with sorafenib and vitamin K appears to be worthy of clinical trial with expectation of synergistic therapeutic effects.*

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Liver cancer ranks sixth in incidence and third in mortality among all types of cancer worldwide (1). The most common type of primary liver cancer, hepatocellular carcinoma (HCC), accounts for 70-85% of all liver cancer. Despite recent improvements in HCC treatment, survival outcomes for HCC patients are still poor due to high rates of local recurrence and metastasis.

Cancer progression is associated with an abrogation of the normal controls that limit cell migration and invasion, eventually leading to metastasis. The epithelial–mesenchymal transition (EMT) is proposed as a crucial step in promoting cell migration, tumor invasiveness and metastasis. EMT is a transient and reversible switch from a polarized, epithelial phenotype to a fibroblastic or mesenchymal cellular phenotype, the latter exhibiting highly motile and invasive properties (2, 3).

Vitamin K has the ability to prevent the development of HCC in women with viral cirrhosis (4). A number of findings *in vitro* have indicated that vitamin K may play a role in controlling cell growth, including the growth of HCC cells (5-7). In the absence of vitamin K or in the presence of vitamin K antagonists, abnormal prothrombin [*des*-γ-carboxyprothrombin (DCP); or protein induced by vitamin K absence antagonist II (PIVKA-II)] is released into the blood. DCP is a well-established tumor marker for HCC. The precise mechanism by which HCC produces DCP remains unclear. After chemical induction of EMT, impairment of vitamin K uptake *via* cytoskeletal rearrangement causes HepG2 cells to produce DCP (8), suggesting that HCC cells may shift to a DCP-producing phenotype when they gain migratory or invasive properties.

Sorafenib is a multikinase inhibitor that targets several serine/threonine and tyrosine receptor kinases (9). Despite its therapeutic efficacy, sorafenib treatment in patients with HCC causes DCP levels to increase, and paradoxically, the elevation of DCP may also indicate a highly therapeutic

effect of sorafenib (10, 11). This induction may represent tumor suppression due to the anti-angiogenic effects of sorafenib because hypoxia stimulation is known to impair vitamin K uptake and to induce DCP in HCC (12). These findings led us to hypothesize that a synergistic therapeutic effect against HCC could be achieved by combining sorafenib with vitamin K treatment.

Clinically, we had administered oral vitamin K2 analog for HCC patients with an expectation of some antitumor effects. We experienced favorable outcomes in a few patients with metastatic HCC, but meta-analyses including a randomized controlled study failed to prove preventive and therapeutic effects (13, 14). Currently, sorafenib is the only effect-proven therapeutic agent for patients with HCC lesions which are not manageable with locoregional treatments (15, 16), but its therapeutic response is often disappointing.

In this experimental study, we examined the effects of vitamin K and sorafenib, individually and in combination, in suppressing the migration and metastatic potential of HCC cells. The end-point was to quantify the antitumor effects of sorafenib and vitamin K in terms of inhibition of EMT and metastasis in HCC, and to determine whether the combination of these agents might block the hepatocyte growth factor (HGF)/c-MET pathway in HCC cells.

Materials and Methods

Cell culture. The HCC line, HepG2, derived from the liver tissues 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's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum in a humidified incubator with 5% CO_2 at 37°C.

Immunocytochemistry. Untreated HepG2 cells were fixed for 30 minutes at room temperature with 4% paraformaldehyde in phosphate buffer (77.4 ml of 1 M Na_2HPO_4, 22.6 ml 1 M $Na_2H_2PO_4$ in 900 ml of distilled water). Fixed cells were permeabilized with 0.5% Triton X-100 (Merck, Darmstadt, Germany) for 5 minutes, 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, Cambridge, MA, USA) overnight at 4°C. The primary antibody was detected with fluorescein isothiocynate- or CY3 (Invitrogen Life Technologies, Carlsbad, CA, USA)-conjugated antirabbit (1:500) or anti-mouse (1:200) secondary antibodies as appropriate for 1 hour at room temperature. The results were evaluated using a fluorescence microscope (Leica Microsystems Inc., Heidelberg, Germany).

Western blot analysis. For western blotting, total protein was extracted from untreated 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. The 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 and incubated with primary antibody overnight at 4°C. The 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), and snail homologue 1 (*SNAI1*) (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were washed six times in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 5 minutes and incubated with secondary antibodies, horseradish peroxidase anti-mouse (1:2000; Invitrogen Life Technologies) or anti-rabbit (1:5000; Invitrogen Life Technologies) for 1 hour at room temperature. After washing six times in TBST for 5 minutes, the protein bands were visualized using electro-generated chemiluminescent (ECL) reagents (EMD Millipore, Billerica, MA, USA). The optical density of bands was analyzed by reflectance densitometry on a Bio-Rad GS-670 imaging densitometer.

Cell migration assay. The Oris Cell Migration Assay (Platypus Technologies, Madison, WI, USA) was used to investigate the effects of vitamin K1/K2 and sorafenib, individually and in combination, on cell migration after treatment with HGF. Oris Cell Seeding Stoppers were inserted in each well to prevent attachment of the cells in the center region of the well. Cells were seeded at a density of 10×10^5 cells/well and allowed to attach overnight onto plates coated with collagen solution. Then they were incubated for 24 hours to permit cell adhesion. Once the cells had formed a confluent monolayer, the silicone stoppers were carefully removed and the cells were treated with HGF, vitamin K1/K2, and sorafenib in serum-free medium. Following 48-hour incubation, images of the migrated cells in the detection zones were acquired using a Leica fluorescence microscope. The fractional area (% area) of cell migration was analyzed using the Image J system (1.43u; NIH, Bethesda, MD). Each experiment was repeated at least three times, and data are presented as the mean±standard deviation (SD).

Scratch assay. To evaluate cell motility, HepG2 cells (2.5×10^5 per ml) were seeded in 6-well plates and grown to 80-90% confluence. After aspirating the growth medium, the center of each cell monolayer was scraped with a sterile micropipette tip to create a denuded zone (with a constant width across all wells). Cellular debris was removed by washing with PBS, and the HepG2 cells were exposed to HGF (Gibco Life Technologies, Grand Island, NY, USA) and vitamin K1/K2 or sorafenib individually or in combination. After 24-hour incubation, the width of the wound was monitored by photographing each well using a digital camera (Nikon, Tokyo, Japan) attached to an inverted Leica microscope. To quantify the cell migration, two artificial lines fitting the edges of each cell culture were overlaid on the images. The width of the wound (in μm) in each of 10 randomly selected fields was counted. Analyses were performed in triplicate.

Cell proliferation assay. To determine the activity of sorafenib and vitamin K on cell proliferation, we used the cell proliferation reagent WST-1 (Roche Diagnostics GmbH, Mannheim, Germany). HepG2 cells were seeded at a density of 1×10^4 cells/well on a 96-well plate with 100 μl of DMEM containing sorafenib with/without
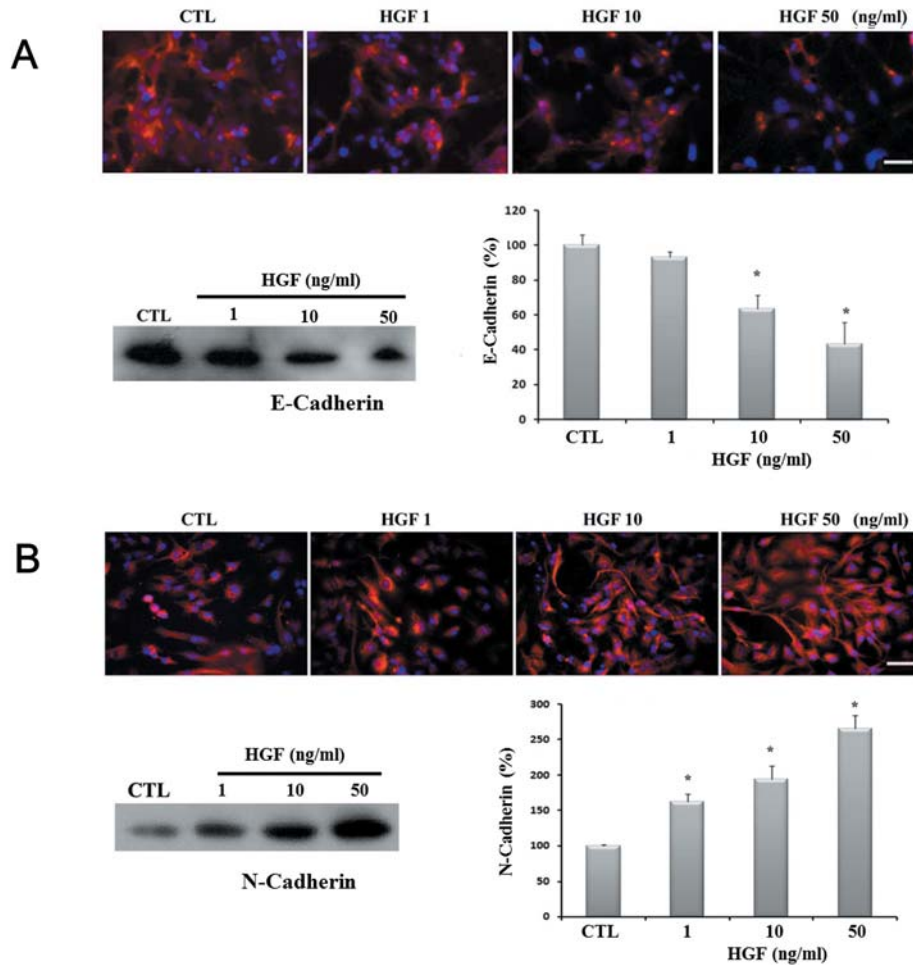
Figure 1. *Hepatocyte growth factor (HGF)-associated expression of E-cadherin (A) and N-cadherin (B). HepG2 cells treated with HGF exhibited decreased E-cadherin and increased N-cadherin expression in a concentration-dependent fashion. CTL, Control; *p<0.001 vs. control.*

vitamin K. After 48-hour incubation, 10 μl of WST-1 proliferation reagent was added to each well, and cells were incubated for 4 hours at 37°C under 5% CO_2. The absorbance of the samples against a background control was measured by spectrophotometry at 450-690 nm.

F-Actin staining. To determine the activity of sorafenib and vitamin K on the cytoskeletal morphological change of HGF-stimulated HepG2 cells, we used an F-actin visualization kit (Cytoskeleton Inc., Denver, CO, USA). HGF-treated HepG2 cells grown on fibronectin-coated cover slips or plates containing sorafenib with/without vitamin K were fixed with paraformaldehyde (4%), permeabilised with Triton X-100 (0.1%), and stained with tetramethylrhodamine-conjugated phalloidin overnight at 4°C. After washing with PBS, the cells were mounted and documented using a Nikon digital camera.

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 vitamin K 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 (5×10^4 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/vitamin K 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. The data were analyzed by one-way ANOVA with Tukey-Kramer multiple comparison *post-hoc* test in GraphPad Instat 3.05 (GraphPad, La Jolla, CA, USA). Each value was expressed as the mean±standard deviation (SD), and *p*-values less then 0.01 were considered statistically significant.
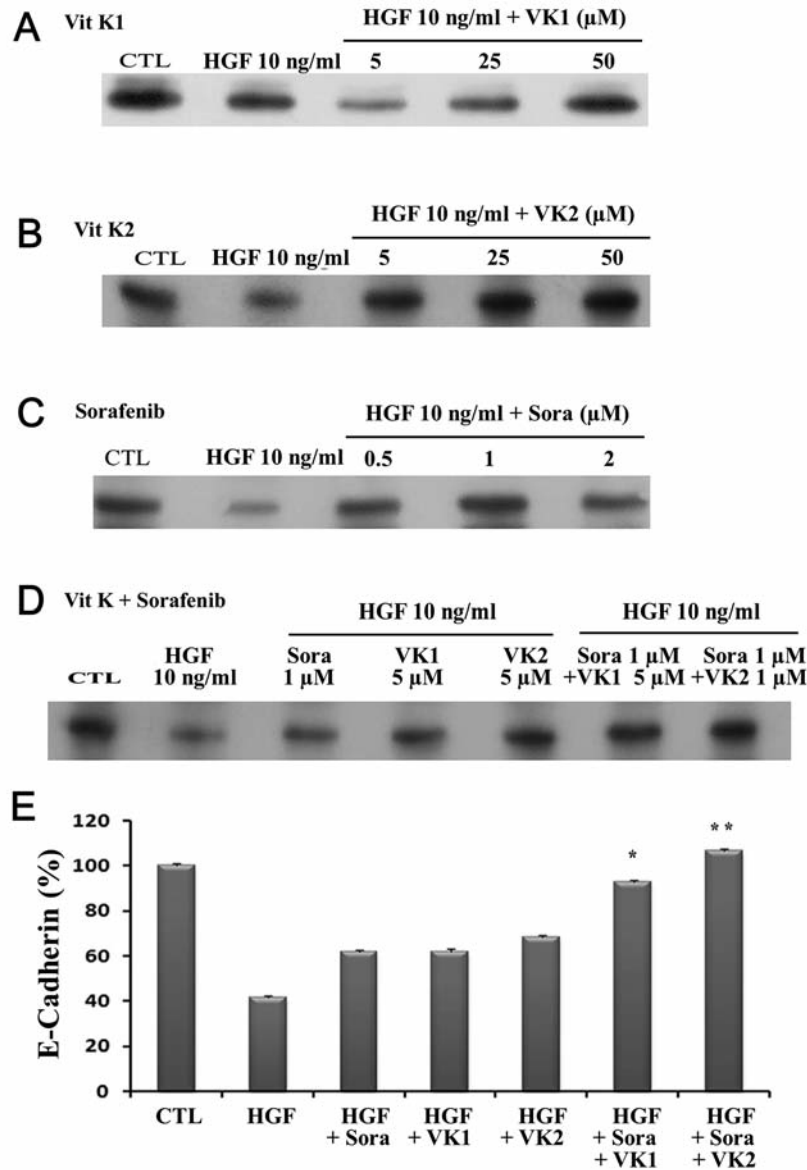
Figure 2. *Inhibition of hepatocyte growth factor (HGF)-induced cell scattering through increased expression of E-cadherin following sorafenib (Sora) and vitamin K treatment. HepG2 cells treated with HGF and vitamin K1/K2 (A, B) or sorafenib (C) showed increased expression of E-cadherin with increasing concentrations of the agents. The combination of sorafenib (1 μM) and low-dose vitamin K (5 μM) (D, E) significantly increased the expression of E-cadherin compared to treatment with either agent alone. CTL, Control; VK1, vitamin K1; VK2, vitamin K2; *p<0.01 vs. HGF+Sora or HGF+VK1; **p<0.01 vs. HGF+Sora or HGF+VK2.*

Results

Inhibition of EMT phenotype by combining sorafenib with vitamin K. We used HGF to induce human HepG2 cell invasion to examine the effect of HGF on expression of mesenchymal characteristics in HepG2 cells. Western blot analysis and immunohistochemical staining revealed that increasing concentrations of HGF (1, 10 and 50 ng/ml) were associated with decreased expression of E-cadherin and increased expression of N-cadherin in a concentration-dependent manner (Figure 1). To assess the effect of combining sorafenib with vitamin K, HepG2 cells that had been previously treated with HGF (10 ng/ml) were additionally treated with the study agents, either vitamin K1/K2 or sorafenib, individually and in combination. The expression of E-cadherin was increased in the presence of both vitamin K and sorafenib in a concentration-dependent manner. The effects of low-dose vitamin K1/K2 (5 μM) were
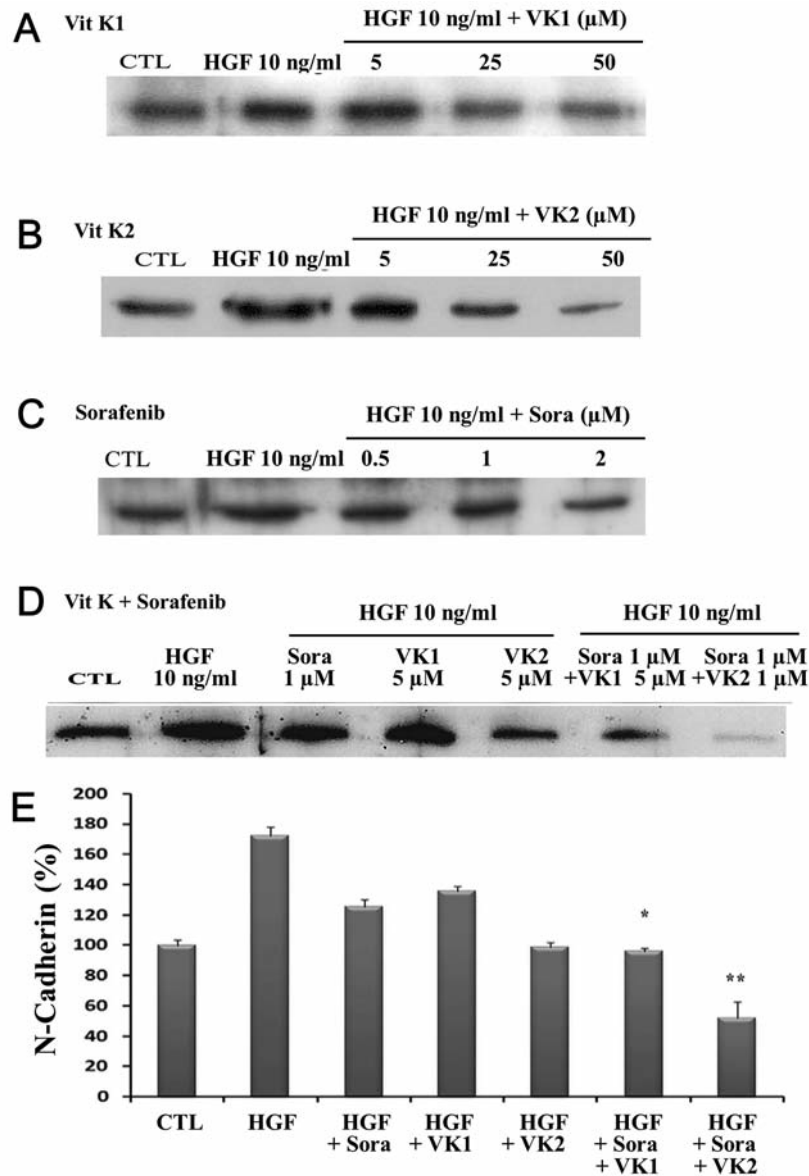
Figure 3. *Inhibition of hepatocyte growth factor (HGF)-induced cell scattering through decreased expression of N-cadherin following sorafenib (Sora) and vitamin K treatment. HepG2 cells treated with HGF and vitamin K1/K2 (A, B) or sorafenib (C) displayed decreased expression of the N-cadherin upon exposure to increasing concentrations of these agents. Combination of sorafenib (1 μM) and low-dose vitamin K (5 μM) (D, E) significantly reduced the expression of N-cadherin compared to treatment with either agent alone. CTL, Control; VK1, vitamin K1; VK2, vitamin K2; *p<0.01 vs. HGF+Sora or HGF+VK1; **p<0.01 vs. HGF+Sora or HGF+VK2.*

minimal compared to the controls, whereas the combination of sorafenib (1 μM) and low-dose vitamin K (5 μM) significantly increased expression of E-cadherin compared to the single-agent treatments (Figure 2).

We also tested whether vitamin K1/K2 and sorafenib affected the expression of N-cadherin. The results were comparable to those with E-cadherin in the degree of synergy but in the opposite direction. Immunohistochemical staining revealed that combining sorafenib with vitamin K significantly reduced HGF-stimulated N-cadherin expression compared to both single-agent treatments (Figure 3).

Inhibition of HGF-stimulated cell migration by combining sorafenib with vitamin K. We investigated the effect of vitamin K1/K2 and sorafenib on the invasiveness of HGF-treated HepG2 cells using the Oris invasion assay. In control
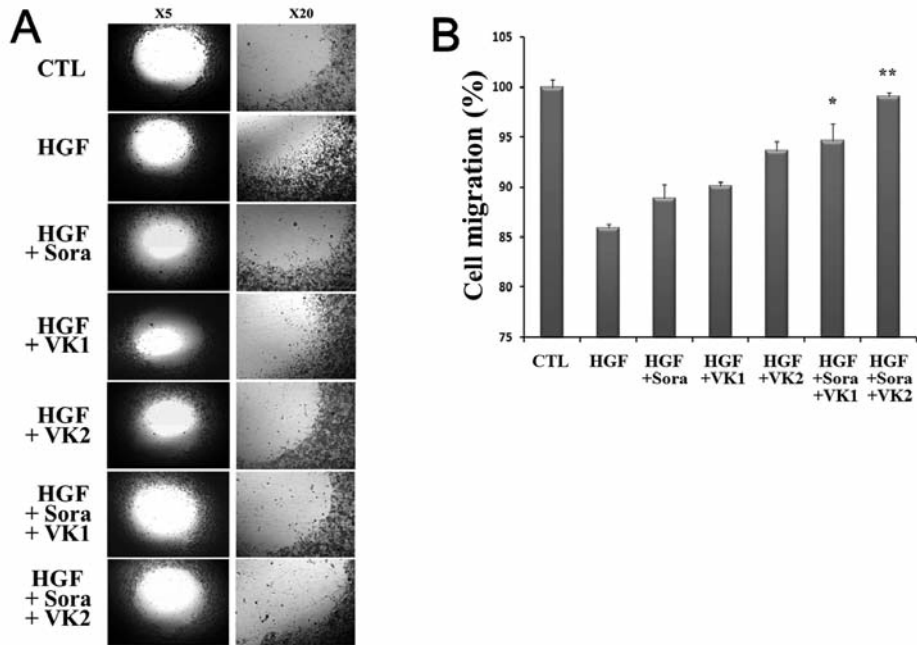
Figure 4. *Oris invasion assay showing suppression of hepatocyte growth factor (HGF)-stimulated cell migration. A: Cells treated with vitamin K, sorafenib (Sora), and a combination of these two agents showed compromised migration ability compared with control cells. B: Combining sorafenib with vitamin K inhibited cell migration to a greater degree than either agent alone. CTL, Control; VK1, vitamin K1; VK2, vitamin K2;* $^{*}p<0.01$ *vs. HGF+Sora or HGF+VK1;* $^{**}p<0.01$ *vs. HGF+Sora or HGF+VK2.*

cells, HGF promoted migration of HepG2 cells. Twenty-four hours after treatment, obvious cell migration was seen in control cells, but cells treated with vitamin K or sorafenib showed compromised migration ability. Combining sorafenib with vitamin K caused a more potent inhibition of cell

migration than either agent alone (Figure 4). We also performed a scratch assay as an independent test of cell migration. Similar to the Oris invasion assay, HGF-stimulated HepG2 cells treated with a combination of sorafenib and vitamin K showed greatly compromised migra-
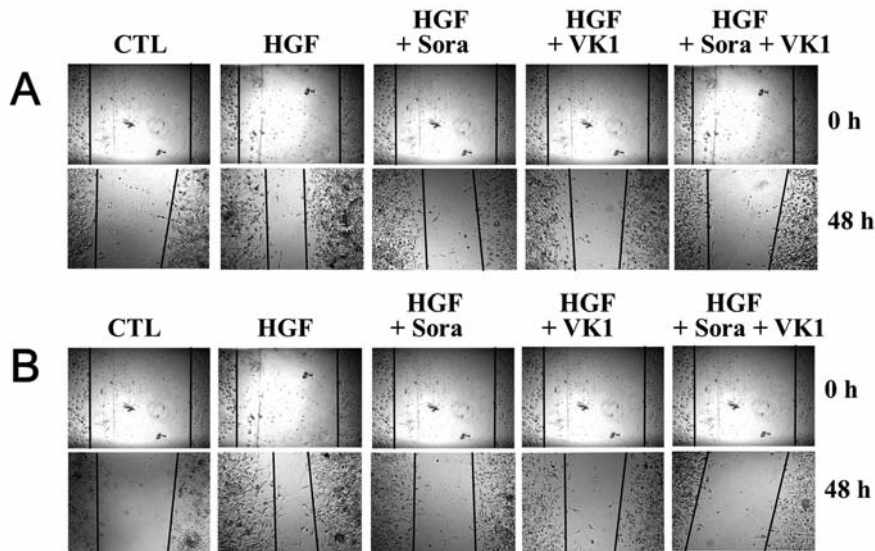

Figure 5. *Scratch assay showing suppression of hepatocyte growth factor (HGF)-stimulated cell migration. CTL, Control; VK1, vitamin K1; VK2, vitamin K2; Sora, sorafenib.*
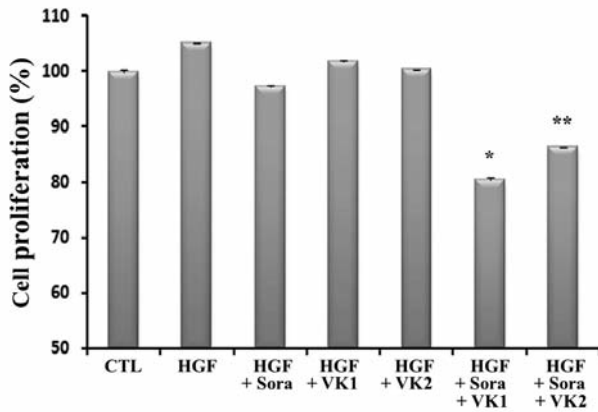
Figure 6. *Suppression of hepatocyte growth factor (HGF)-stimulated cell proliferation through combination therapy. Combination of sorafenib (Sora) and vitamin K suppressed HGF-stimulated cell proliferation more potently than either agent alone. CTL, Control; H, HGF; VK1, vitamin K1; VK2, vitamin K2; *p<0.01 vs. HGF+Sora or HGF+VK1; **p<0.01 vs. HGF+S or HGF+VK2.*

tion ability. Simultaneously, a significant reduction in cell density, and morphological changes were observed in the cells treated with this combination (Figure 5).

Inhibition of HGF-promoted cell proliferation by combining sorafenib with vitamin K. To determine the effects of vitamin K1/K2 and sorafenib on cell proliferation, a WST-1 assay was performed. Combination of sorafenib with vitamin K showed greater inhibition of cell proliferation than treatment with either agent alone (Figure 6).

Cytoskeletal morphological changes in HCC cells following combined treatment with sorafenib and vitamin K. Based on F-actin staining, the structure of the cytoskeleton in HGF-stimulated HepG2 cells was altered by treatment with vitamin K1/K2 and sorafenib, both individually and in combination. HGF-treated HepG2 cells became scattered and adopted a fibroblast-like appearance, but the cells treated with vitamin K1/K2 or sorafenib were rounded and clustered. In contrast, cells treated with the combination of sorafenib and vitamin K displayed greater clustering compared with single agent-treated cells (Figure 7).
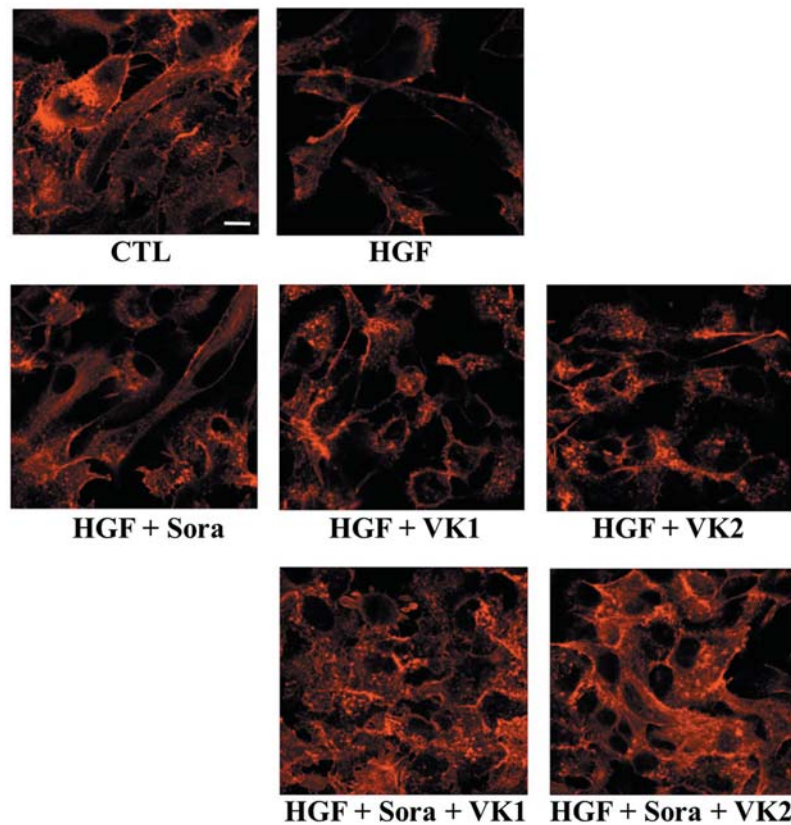

Figure 7. *Suppression of hepatocyte growth factor (HGF)-stimulated cell scattering. HGF-stimulated HepG2 cells treated with combination of sorafenib (Sora) and vitamin K showed greater clustering and agglomerated morphological characteristics compared with the cells treated with either agent alone. CTL, Control; VK1, vitamin K1; VK2, vitamin K2.*

Inhibition of HGF-stimulated cell invasion by combining sorafenib with vitamin K. We evaluated the effects of vitamin K and sorafenib on HGF-treated HepG2 cell invasion *in vitro*. Invasion of HepG2 cells was inhibited by sorafenib and vitamin K when used alone, and a combination of sorafenib with vitamin K further suppressed the invasiveness of HepG2 cells after HGF-stimulation (Figure 8).

Increased inhibition of the HGF/c-MET signaling pathway by combining sorafenib with vitamin K. We assessed the expression of p-MET and p-ERK by Western blot analysis of HGF-stimulated HepG2 cells treated with vitamin K1/K2 or sorafenib for 24 hours. Vitamin K1/K2 and sorafenib treatments alone resulted in decreased p-MET and p-ERK levels, but in combination resulted in dramatically reduced phosphorylation of both MET and ERK, although the total MET and ERK levels were not altered (Figure 9A and B) The transcription of *SNAI1* was somewhat suppressed by vitamin K1/ K2 or sorafenib alone, but was dramatically suppressed by combining these two compounds (Figure 9C).

Discussion

The escape of carcinoma cells from a solid tumor can be due to de-differentiation of epithelial cells *via* the loss of cell-to-cell contacts and the concomitant gain of migratory and invasive abilities (17). This phenotypic conversion of cells, designated as EMT, has been described in different types of carcinoma cells including HCC (18, 19). HCC cells change their cytoskeletal morphologies during EMT, in which the dynamic polymerization of endogenous F-actin plays a crucial role (8). E-Cadherin is an important molecule required to suppress invasion during cancer progression and to prevent EMT induction (20). In a variety of human cancer types, E-cadherin loss is linked to poor prognosis, tumor progression, and metastasis. In parallel, N-cadherin expression, which replaces E-cadherin during EMT, enhances metastasis (21, 22). The expression of the transcription factor *SNAI1*, which is a key inducer of the EMT, is positively correlated with poor clinical outcomes in various types of cancer, including HCC.

Accumulating evidence suggests that the EMT plays a pivotal role in the dissemination of malignant hepatocytes during HCC progression. A recent study by Dooley *et al*. aimed to clarify the role of hepatocytes in hepatic fibrosis and demonstrated that primary mouse hepatocytes lose their epithelial phenotype in response to transforming growth factor (TGF)-β through the loss of E-cadherin, a major component of epithelial adherens junctions, and up-regulation of *SNAI1,* which is a potent transcriptional repressor of E-cadherin (19). During EMT, the activity of the adherens junctions is highly modified mainly due to
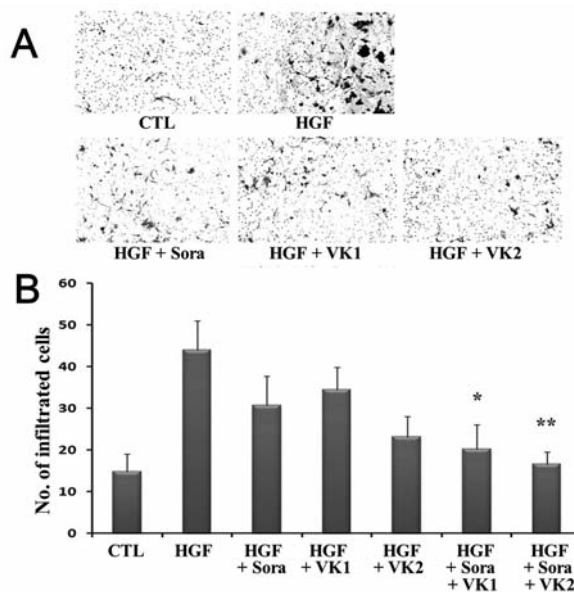

Figure 8. *Suppression of hepatocyte growth factor (HGF)-stimulated cell invasion. A: Invasion of HepG2 cells into the upper compartment of the trans-well culture chambers in the presence of sorafenib (Sora) with/without vitamin K was assessed after 24 hours of treatment. B: Combining sorafenib with vitamin K significantly suppressed the HGF-stimulated invasion of HepG2 cells compared to either agent alone. CTL, Control; H, HGF; VK1, vitamin K1; VK2, vitamin K2; *p<0.01 vs. HGF+Sora or HGF+VK1; **p<0.01 vs. HGF+Sora or HGF+VK2.*

replacement of E-cadherin by N-cadherin, a process referred to as cadherin switching (23). This loss of E-cadherin expression is a critical step in EMT, and E-cadherin is therefore emerging as one of the caretakers of the epithelial phenotype. E-Cadherin is required for maintenance of the stable junctions; antibodies to E-cadherin can disrupt these contacts and induce a mesenchymal phenotype (24).

HGF is a major driver of EMT and cancer progression in HCC (25). HGF binding to the c-MET receptor activates c-MET tyrosine kinase activity and induces phosphorylation of multiple sites on the receptor. The HGF/c-MET signaling pathway induces EMT in HCC cell lines (26, 27). Although HGF/c-MET signaling does not play a role in liver homeostasis under normal physiological conditions, HGF has important roles in liver regeneration, hepatocyte survival, and tissue remodeling after acute injury. ERK and phosphoinositide 3-kinase (PI3K) signal transduction pathways, which regulate cell proliferation, survival, and invasion, are activated downstream of the c-MET signaling pathway (28).

Our current study findings demonstrate that E-cadherin expression increases in HCC cells in response to HGF in a concentration-dependent manner. Concurrent treatment of these cells with HGF and vitamins K1/K2 inhibits cadherin
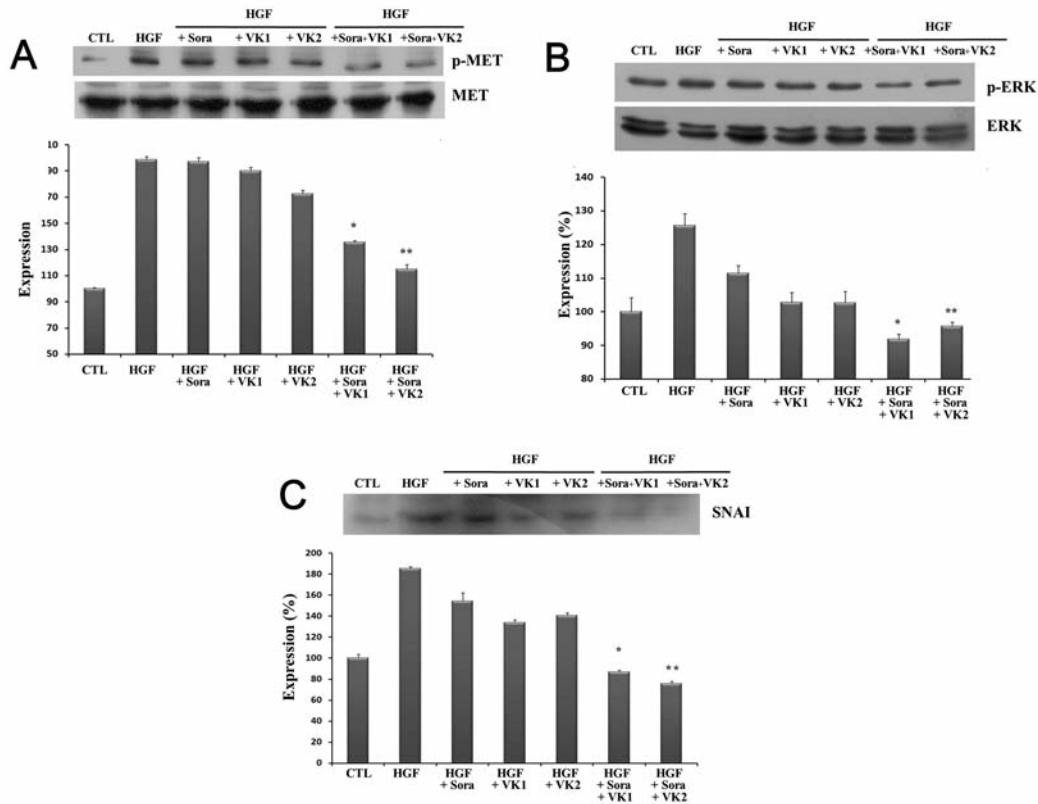
Figure 9. *Inhibition of the hepatocyte growth factor (HGF)/c-MET signaling pathway. A, B: Sorafenib in combination with vitamin K dramatically reduced phosphorylation of MET and ERK, although the total MET levels were unchanged. C: Transcription of SNAI1 was dramatically suppressed by combining sorafenib (Sora) with vitamin K. CTL, Control; VK1, vitamin K1; VK2, vitamin K2; *p<0.01 vs. HGF+Sora or HGF+VK1; **p<0.01 vs. HGF+Sora or HGF+VK2.*

switching. Our findings indicate that there is a synergism between vitamin K and sorafenib in HGF-induced cell scattering by enhancing the expression of E-cadherin and reducing the expression of N-cadherin, both of which play crucial roles in EMT. Combination of sorafenib and vitamin K also enhances inhibition of HGF-induced cell proliferation and migration/invasion *in vitro* to a greater extent than either agent alone. We speculate that combining sorafenib with vitamin K inhibits HGF/c-MET signaling pathway effectively, compared with treatments using either agent alone.

A number of studies to date have implicated HGF and its receptor, the c-MET proto-oncogene, in tumor progression and metastasis in HCC (28, 29). The RAS-ERK pathway plays a critical role in numerous cellular processes, including proliferation, differentiation, survival, and motility (30). Deregulation of the RAS-ERK pathway has been implicated in multiple types of human cancer, including HCC (31). Previous studies have also established a critical role for receptor tyrosine kinase signaling and the RAS pathway in EMT (32, 33). We demonstrated here that vitamin K1/K2 or

sorafenib alone reduces p-MET and p-ERK levels, but vitamin K at low concentrations (5 μM) in combination with sorafenib (1 μM) produces a greater reduction of p-MET and p-ERK levels. Both vitamin K and sorafenib alone can mediate a modest inhibition of phosphorylation of MET and ERK, but a clear and significant synergistic effect is evident from combination treatment with these agents.

The results of our present study strengthen our previous hypothesis that a synergistic therapy for HCC might be achieved by adding vitamin K to sorafenib, which impairs vitamin K uptake due to its anti-angiogenic effects. The mechanism by which combination of sorafenib and vitamin K1/K2 synergistically inhibits HGF-stimulated HepG2 cells appears to be complex. Down-regulation of the c-MET and RAF/MEK/ERK pathways has been suggested to hold great promise for preventing tumor cell growth (28). Our current results suggest that the synergistic effect of EMT inhibition by combining sorafenib with vitamin K is, at least in part, attributable to inhibition of the c-MET signaling pathway.

Positive clinical trials have now established that sorafenib improves survival in patients with advanced HCC

compared with a placebo. However, sorafenib monotherapy offers only limited survival benefits in a small proportion of patients with HCC (23, 24) Especially after liver transplantation, an aggressive pattern of multiple HCC recurrence is very common, thus systemic chemotherapy is often indicated (34). However, sorafenib causes multiple human toxicities, including anorexia, gastrointestinal bleeding and hand-foot syndrome (15). Modulation of its actions to reduce its toxicity levels is a desirable goal. Therefore, development of more efficacious combination therapies involving sorafenib and vitamin K are a potentially attractive approach for HCC treatment.

In parallel with our current experimental study, we have performed a clinical study involving combination therapy with sorafenib and vitamin K2 in patients with HCC expecting beneficial effects similar to those demonstrated in this study. At this time, it is not possible for us to estimate the survival benefit objectively, but an interim analysis of 72 patients revealed that there are nearly no adverse side-effects after intake of vitamin K2 analog (data not presented here). Such safety of vitamin K2 analog intake in patients with HCC is already well known because this agent has been very widely used for treatment of osteoporosis (13, 14). We also found that vitamin K added to sorafenib did not increase the adverse side-effects of sorafenib. Interestingly, we observed exceptionally favorable outcomes in a small proportion of patients with HCC metastasis after vitamin K therapy alone or with sorafenib. Solitary administration of oral vitamin K to patients with metastatic HCC has sporadically led to a favorable outcome, but meta-analyses including a randomized controlled study, failed to prove its antitumor effects (13, 14).

In conclusion, the results of our present study support our assumption that supplementing sorafenib with vitamin K may produce synergistic therapeutic effects against HCC. We believe the main mechanism by which a combination of sorafenib and vitamin K causes HCC cell suppression *in vitro* is the inhibition of the HGF/c-MET pathway. Thus, sorafenib and vitamin K can work synergistically to inhibit the migration and proliferation of HCC cells. Because oral administration of vitamin K2 analog is quite safe, a combination therapy with these two agents appears to be worthy of further clinical trials with an expectation of synergistic therapeutic effects.

Declaration of Interest

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