Activation of phosphatidylinositol 3-kinase/Akt signaling mediates sorafenib-induced invasion and metastasis in hepatocellular carcinoma

HAIYONG WANG[1,2], LITAO XU[1,2], XIAOYAN ZHU[1,2], PENG WANG[1,2], HUIYING CHI[3] and ZHIQIANG MENG[1,2]

[1]Department of Integrative Oncology, Fudan University Shanghai Cancer Center, Shanghai 200032;
[2]Department of Oncology, Shanghai Medical College, Fudan University, Shanghai 200032;
[3]Shanghai Geriatric Institute of Chinese Medicine, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 200031, P.R. China

Received March 27, 2014; Accepted June 24, 2014

DOI: 10.3892/or.2014.3352

Abstract. Sorafenib, an antiangiogenic agent, can promote tumor invasion and metastasis. The phosphatidylinositol 3-kinase (PI3K)/Akt/Snail-dependent pathway plays an important role in tumor invasion and metastasis. Yet, little is known concerning the role of the PI3K/Akt/Snail-dependent pathway in sorafenib-induced invasion and metastasis of hepatic carcinoma (HCC). A human HCC orthotopic xenograft model was established, and sorafenib (30 mg/kg/day) was administered orally. Tumor growth and intrahepatic metastasis were assessed, and immunohistochemistry was applied to analyze the activation of the PI3K/Akt/Snail-dependent pathway. HCC cell lines were treated with sorafenib (1, 5 and 10 μM), and proliferation, migration and invasion were assessed. Western blotting and real-time polymerase chain reaction (RT-PCR) were used to examine the related gene expression of epithelial-mesenchymal transition (EMT) markers and the PI3K/Akt/Snail-dependent pathway. Sorafenib inhibited tumor growth and promoted intrahepatic invasion and metastasis of the orthotopic tumors grown from SMMC7721-GFP cells *in vivo*. Additionally, sorafenib promoted EMT and invasion and metastasis of HCC cells *in vitro*. Importantly, sorafenib enhanced PI3K and Akt activation and upregulation of the expression of transcription factor Snail, a critical EMT mediator. The upregulation of transcription factor Snail expression by sorafenib may be related to activation of the PI3K/AKT signaling pathway. The PI3K/Akt/Snail-dependent pathway may mediate the pro-invasive and pro-metastatic effects of sorafenib on HCC by inducing EMT.

Correspondence to: Professor Zhiqiang Meng, Department of Integrative Oncology, Fudan University Shanghai Cancer Center, 270 Dong An Road, Shanghai 200032, P.R. China
E-mail: mengzhq@gmail.com

Key words: sorafenib, invasion and metastasis, EMT, PI3K/Akt, Snail

Introduction

Hepatic carcinoma (HCC) is the fifth most common malignancy worldwide and the second leading cause of cancer-related death in Asia generally and in China in particular (1). Currently, surgical resection and liver transplantation offer the best potential for treating HCC (2-4), but most HCC patients are diagnosed in advanced stages. At present, sorafenib, a multikinase inhibitor with antiangiogenic and antiproliferative effects, currently sets the new standard for advanced HCC (5,6). However, the survival benefit is only 2.8 months.

Antiangiogenic therapy has been thought to hold significant potential for the treatment of cancer (7). However, clinical and preclinical observations indicate that these therapies may have limited efficacy. Although these agents typically produce inhibition of primary tumor growth, lasting responses are rare, with only a moderate increase in progression-free survival and little benefit in overall survival (8). In addition, recent reports describe that treatment of tumor-bearing mice with antiangiogenic drugs leads to increased local tumor cell invasion and enhanced distant metastasis after prolonged treatment or after only short-term treatment (9,10). Notably, sorafenib, the only approved molecular-targeted drug for HCC, was found to promote invasion and metastasis of HCC by increased intrahepatic metastasis, lung metastasis, and circulating tumor cells in tumors with higher expression of HTATIP2 in xenograft models (11). Therefore, it is important to clarify the molecular mechanisms of invasion and metastasis caused by sorafenib from all aspects in HCC.

Epithelial-mesenchymal transition (EMT) plays a key role in tumor invasion and metastasis. During this process, epithelial cells lose their epithelial signatures while acquiring the characteristics of mesenchymal cells including morphology, cellular structure and biological function (12). Transcription factor Snail has also been shown to confer survival properties either concomitantly with induction of EMT or independent of EMT (13-15). Snail, Slug and Twist transcription factors can act as E-box repressors and block E-cadherin transcription (16). In addition, Snail transcription factor can mediate an increase in expression of mesenchymal markers such as

vimentin, fibronectin, matrix metalloproteinases (MMPs) and RhoA (17-20). The overall effect of Snail is increased migration and invasion (18,19).

Numerous signaling pathways are involved in the regulation of EMT. PI3K/Akt signaling is an important survival/proliferative pathway involving various growth factors, cytokines and activation of receptors (21). In addition, the PI3K/Akt signaling pathway plays a key role in the control of cell invasion and metastasis and the activation of PI3K/AKT is a central feature of EMT in the development of cancer (22-27). On the one hand, the PI3K/AKT signaling pathway can increase the expression of matrix metalloproteinases to induce EMT (28,29). On the other hand, the PI3K/AKT signaling pathway can upregulate the expression of transcription factor Snail to induce EMT (30-32). Notably, activation of the PI3K/Akt signaling pathway plays a key role in mediating resistance to sorafenib. The combination of MK-2206, an Akt inhibitor, and sorafenib overcomes such resistance (33). Yet, little is known concerning the role of the PI3K/Akt signaling pathway on the invasion and metastasis induced by sorafenib in HCC.

In the present study, we tested and verified that sorafenib promotes invasion and metastasis of HCC by inducing EMT. More importantly, we showed that activation of the PI3K/Akt/Snail-dependent pathway may play a key role in this process.

Materials and methods

Reagents and antibodies. Sorafenib was purchased from Bayer Corporation (West Haven, CT, USA). Antibodies against E-cadherin, N-cadherin, vimentin, Snail and GAPDH were purchased from Epitomics (Burlingame, CA, USA); antibodies against p-PI3K and p-AKT were purchased from Bioworld Technology (Minneapolis, MN, USA).

Cell culture. The human HCC cell lines SMMC7721 and HCCLM3 originated from the American Type Culture Collection (ATCC) and were cultured in RPMI-1640 containing 10% fetal bovine serum (FBS; Biochrom, Berlin, Germany) in 5% CO_2 at 37˚C. SMMC7721-GFP cells were SMMC7721 cells transfected with green fluorescence protein (GFP) and were labeled as SMMC7721-GFP cells.

Cell proliferation, migration and invasion assays. Cell proliferation analysis was performed as previously described by us (34). Briefly, cells were plated at 5,000/well in 96-well microtiter plates and incubated overnight at 37˚C in a humidified incubator containing 5% CO_2. On the following day, various concentrations of sorafenib were added to the wells, and cultures were incubated for an additional 24, 48 and 72 h. Cell viability was determined using a Cell Counting Kit-8 (Dojindo, Gaithersburg, MD, USA) according to the manufacturer's instructions. For cell migration assay, cell migration was assessed using the Transwell assay (Boyden chambers; Corning, Cambridge, MA, USA). Cells ($5x10^4$) were seeded in serum-free medium in the upper chamber and allowed to migrate toward the lower chamber that contained 10% FBS. After 48 h, cells that had traveled through and adhered to the underside of the membrane were counted at x200 magnification. The cell invasion assay was carried out similarly, except that 50 μl Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) diluted 1:6 with serum-free medium was added to each well overnight before cells ($2x10^5$) were seeded onto the membrane.

Animal models and treatments. Six-week-old BALBc nu/nu female mice were obtained from the Shanghai Institute of Material Medica, Chinese Academy of Science. All mice were bred in laminar flow cabinets under specific pathogen-free conditions. We followed internationally recognized guidelines on animal welfare. The study design was approved by the Animal Ethics Committee, and the experiments were undertaken in accordance with the Ethical Principles of Animal Experimentation of Fudan University. SMMC7721-GFP cells [$5x10^6$/0.2 ml phosphate-buffered saline (PBS)] were subcutaneously inoculated into the right flanks of 6-week-old BALBc nu/nu female mice. After 4 weeks, non-necrotic tumor tissue was cut into 1 mm^3 pieces and orthotopically implanted into the liver. Treatment was started 2 weeks after orthotopic implantation of the tumors. Mice were randomly separated into two groups with 6 mice in each group. Mice in the experimental group received 30 mg/kg/day sorafenib, whereas the control mice received vehicle alone. Animal weight was measured twice a week for 4 weeks. At the end of the experiment, mice were sacrificed, tumors were excised from each mouse, weighed and snap-frozen for further analysis.

Detection of metastasis. Tumors were excised and their largest (a) and smallest (b) diameters were measured to calculate tumor volume ($V = ab^2/2$). The livers were also excised, and green fluorescent protein-positive metastatic foci were imaged by Lumazone imaging system (Mag Biosystems, Tucson, AZ, USA). Hematoxylin and eosin staining (H&E) was further applied to detect liver metastasis.

Western blot analysis. Cells were washed with cold PBS and lysed in culture dishes using PhosphoSafe™ Extraction Reagent (Merck, Darmstadt, Germany) containing 1% protease inhibitor cocktail (EDTA-Free; Thermo, San Jose, CA, USA). Protein concentrations were then determined using Bio-Rad detergent compatible protein assays (Bio-Rad, Hercules, CA, USA). A total of 30 μg protein from each of the control and treated cell lysates was loaded on 8-12% gradient NuPAGE gels (Novex, San Diego, CA, USA), electrophoresed under reducing conditions, and transferred onto polyvinylidene difluoride membranes (0.22 Å; Millipore). Western blot analysis was carried out as previously described (34).

Immunohistochemistry. Procedures for the immunohistochemistry were previously described (35). Briefly, the tumor sections were stained with rabbit anti-p-Akt, and rabbit anti-p-PI3K at 4˚C overnight. Goat anti-rabbit IgG/horseradish peroxidase was applied as the secondary antibody according to the standard protocols provided by the manufacturer. For negative controls, primary antibodies were replaced with PBS. The procedures were performed by two independent investigators, both of whom were blinded to the model/treatment type for the series of experiments.

Real-time polymerase chain reaction. RT-PCR analysis was performed as previously described by us (36). The following

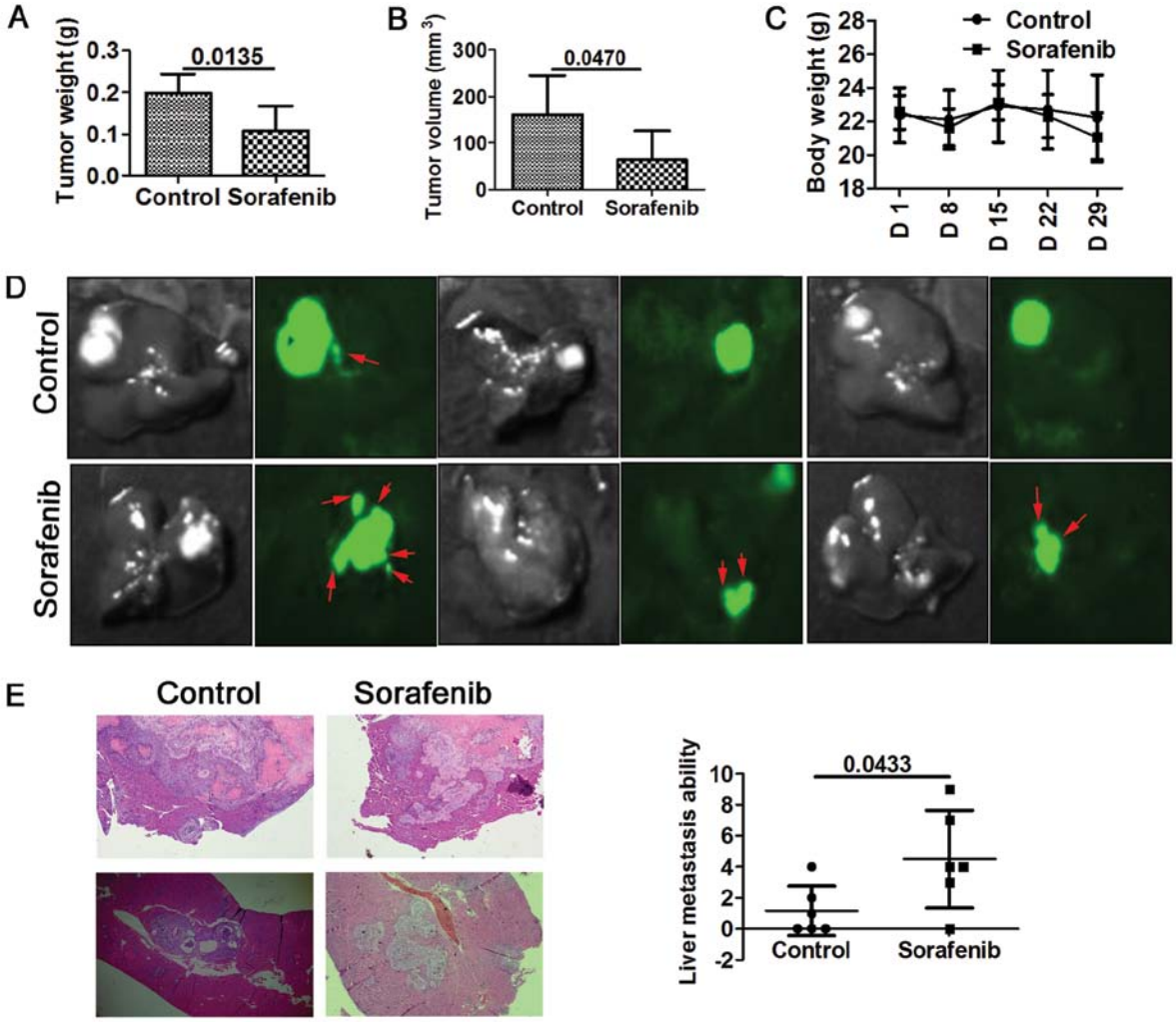

Figure 1. Sorafenib suppresses primary tumor growth and promotes intrahepatic metastasis of hepatic tumors *in vivo*. Mice were orthotopically implanted with SMMC7721-GFP cells and treated with sorafenib (30 mg/kg/day, i.g.) for four weeks. (A and B) Weight and volume of the hepatic tumors. At the end of the experiment, primary tumors from the control and sorafenib-treated mice were carefully excised. Weight and volume were measured and are presented as bar graphs. P<0.05. (C) Body weights of the control and sorafenib-treated mice are presented as a line graph. Values are means ± SD. (D) At the end of the experiment, animals from the control and sorafenib-treated groups were imaged to visualize intrahepatic metastasis by Lumazone imaging system, and representative images are presented (red arrows represent micrometastases). (E) Liver tumors and liver tissues were analyzed using H&E staining (magnification, x40). Liver metastases were quantified by counting the number of metastatic colonies in one histological section of the mid-portion of each liver sample from each mouse. Representative images and dot plots are shown. Data are expressed as the means ± SD (P<0.05). GFP, green fluorescence protein; H&E, hematoxylin and eosin staining.

primers for amplification of human genes were used: E-cadherin forward, 5'-AGCCCCGCCTTATGATTCTCTG-3' and reverse, 5'-TGCCCCATTCGTTCAAGTAGTCAT-3'; N-cadherin forward, 5'-CCACGCCGAGCCCCAGTAT-3' and reverse, 5'-GGCCCCCAGTCGTTCAGGTAAT-3'; vimentin forward, 5'-CCTTGACATTGAGATTGCCACCTA-3' and reverse, 5'-TCATCGTGATGCTGAGAAGTTTCG-3'; Snail forward, 5'-CAGCCTGGGTGCCCTCAAGAT-3' and reverse, 5'-GCACACGCCTGGCACTGGTA-3'.

Statistical analysis. All analyses of the results were performed using the GraphPad Prism software version 5.0 (GraphPad Software, San Diego, CA, USA) and the SPSS 19.0 software package (SPSS, Inc., Chicago, IL, USA). Statistical analyses were performed using the Student's t-test and analysis of variance (ANOVA) models. Differences were considered statistically significant at P<0.05.

Results

Sorafenib promotes invasion and migration in vivo. To elucidate the effects of sorafenib on HCC invasion and migration, mice were orthopically implanted with SMMC7721-GFP cells and treated with 30 mg/kg/day sorafenib. Tumor growth and metastasis were monitored. Our results showed that sorafenib substantially reduced the primary tumor growth compared with the control tumors. Tumor weight and volume were reduced in the sorafenib-treated mice (Fig. 1A and B). Additionally, sorafenib was well tolerated by the mice as no apparent weight loss was noted (Fig. 1C). Unfortunately, sorafenib-treated mice developed more intrahepatic metastatic lesions and exhibited irregular tumor margins as detected by green fluorescence imaging (Fig. 1D). To further explore the effect of sorafenib on the invasion and metastasis of HCC, liver metastatic nodules were evaluated by H&E staining as observed under a micro-

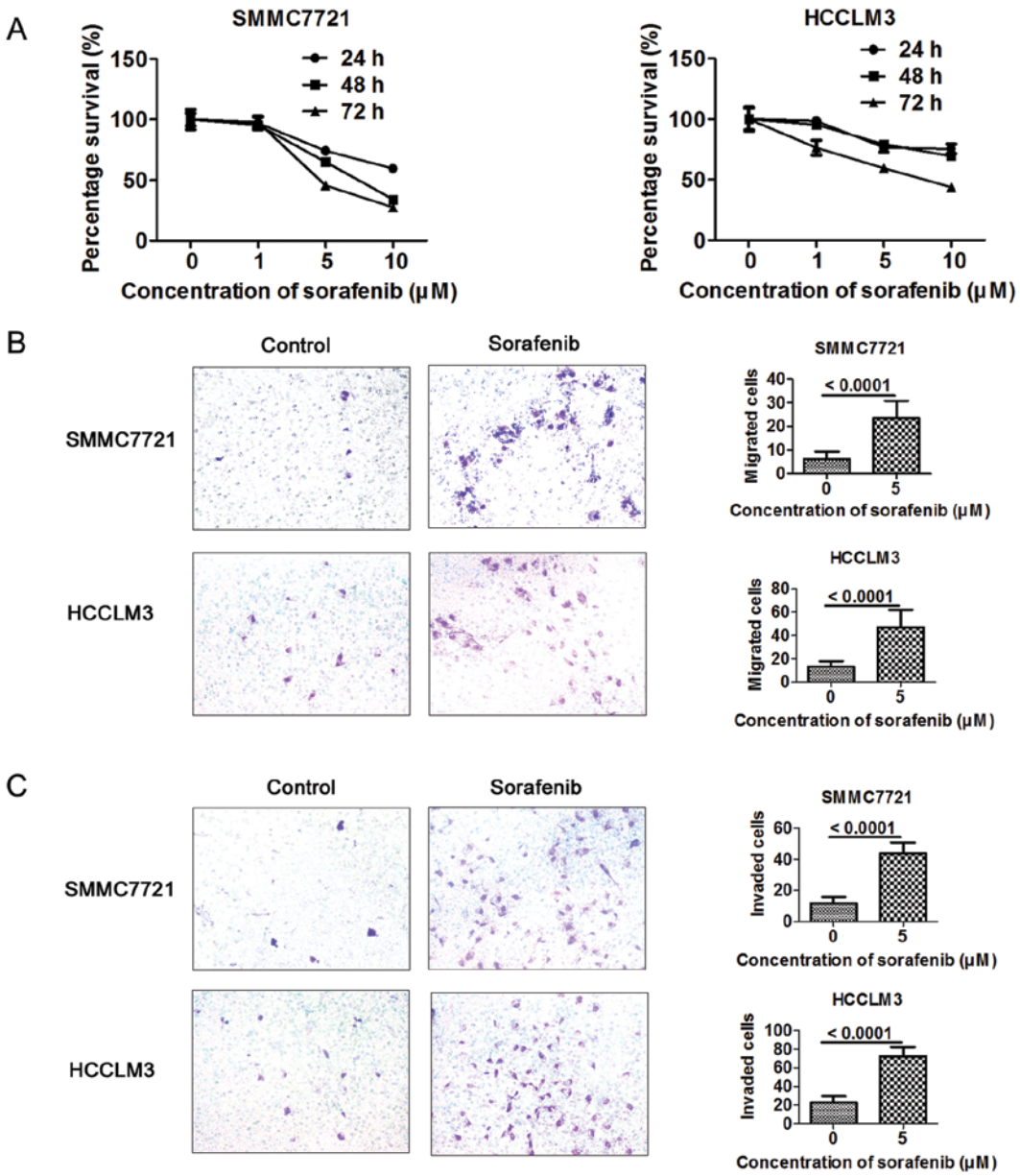

Figure 2. Sorafenib inhibits proliferation and promotes migration and invasion of HCC cells. (A) SMMC7721 and HCCLM3 cells were plated in a 96-well plate, treated with various concentrations of sorafenib and cell viability was measured by Cell Counting Kit-8. (B) Migration of HCC cells was measured by Transwell assay according to the manufacturer's instructions. Values are means ± SD ($P<0.05$). (C) Invasion of HCC cells was also measured by Transwell assay according to the manufacturer's instructions. Values are means ± SD ($P<0.05$).

scope. The number of metastatic nodules was then statistically analyzed. A higher number of intrahepatic metastatic nodules was detected in the sorafenib-treated mice (Fig. 1E).

Sorafenib promotes invasion and migration of HCC cells. As sorafenib promoted invasion and migration *in vivo*, we wanted to further validate whether sorafenib could promote the invasion and migration *in vitro*. Cell proliferation assay was applied to assess the proliferative effect on hepatoma cells after sorafenib treatment. The antiproliferative effect of sorafenib on HCC cells was dose- and time-dependent at a concentration of 1-10 μM in the SMMC7721 and HCCLM3 cells (Fig. 2A). Sorafenib at a concentration of 5 μM, with little effect on cell proliferation, was applied to assess the effect of sorafenib on the migration and invasion of HCC cells. Cells ($5x10^4$ or $2x10^5$) were seeded in the upper chamber. A higher number of metastatic and invasive cells were detected in the sorafenib-treated HCC cells as assessed by Transwell assay (Fig. 2B and C).

Sorafenib promotes EMT in HCC cells. EMT is well known to closely correlate with cancer metastasis. To test and verify whether 5 μM sorafenib promotes the EMT process, we evaluated the expression of EMT markers in the sorafenib-treated and the control cells. As expected, SMMC7721 and HCCLM3 cells treated with 5 μM sorafenib underwent significant morphological changes and displayed the mesenchymal phenotype (Fig. 3A). Importantly, epithelial marker E-cadherin was downregulated and mesenchymal markers N-cadherin and vimentin were upregulated in the sorafenib-

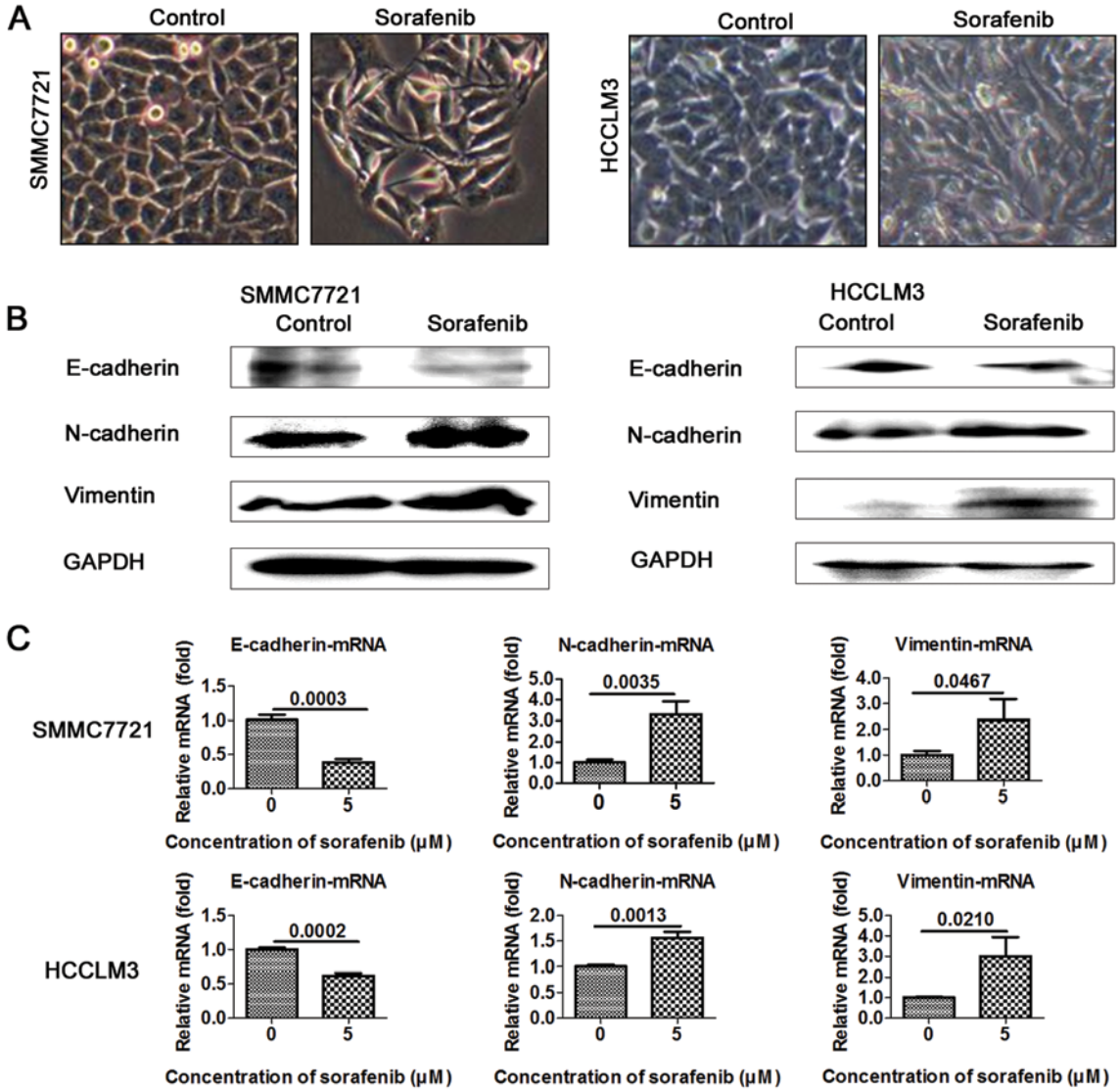

Figure 3. Sorafenib promotes EMT *in vitro*. Cells were treated with 5 μM sorafenib, and EMT markers were evaluated by western blotting and RT-PCR. (A) After 72 h of treatment, morphological changes were evaluated in the SMMC7721 and HCCLM3 cells. (B and C) EMT markers, including E-cadherin, N-cadherin and vimentin, were assessed by western blotting and RT-PCR in the SMMC7721 and HCCLM3 cells. GAPDH was used as a loading control in the western blot analysis. EMT, epithelial-mesenchymal transition.

treated cells (Fig. 3B). RT-PCR assay further confirmed the decreased levels of epithelial marker E-cadherin and the increased levels of mesenchymal markers N-cadherin and vimentin in the SMMC7721 and HCCLM3 cells (Fig. 3C).

Sorafenib upregulates the expression of Snail in vitro. As zinc-finger transcriptional repressor Snail plays a key role in EMT-mediated tumor invasion and metastasis, we ascertained whether Snail is involved in sorafenib-mediated EMT. HCC cells were treated with 5 μM sorafenib and western blot analysis and RT-PCR were carried out to measure Snail expression. As anticipated, transcription factor Snail was upregulated in the SMMC7721 and HCCLM3 cells, when compared to the controls (Fig. 4A and B).

Sorafenib activates the PI3K/AKT signaling pathway in vivo and vitro. As a highly conserved cellular program, EMT has been documented to involve several important pathways.

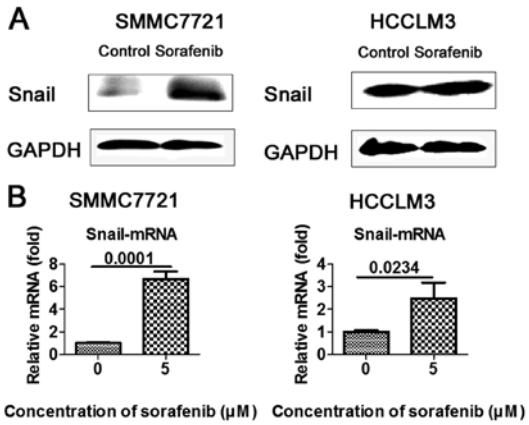

Figure 4. Sorafenib promotes the expression of transcription factor Snail *in vitro*. (A and B) After 72 h of treatment with 5 μM sorafenib, the expression of transcription factor Snail was assessed by western blotting and RT-PCR in SMMC7721 and HCCLM3 cells. GAPDH was used as a loading control in the western blot analysis.

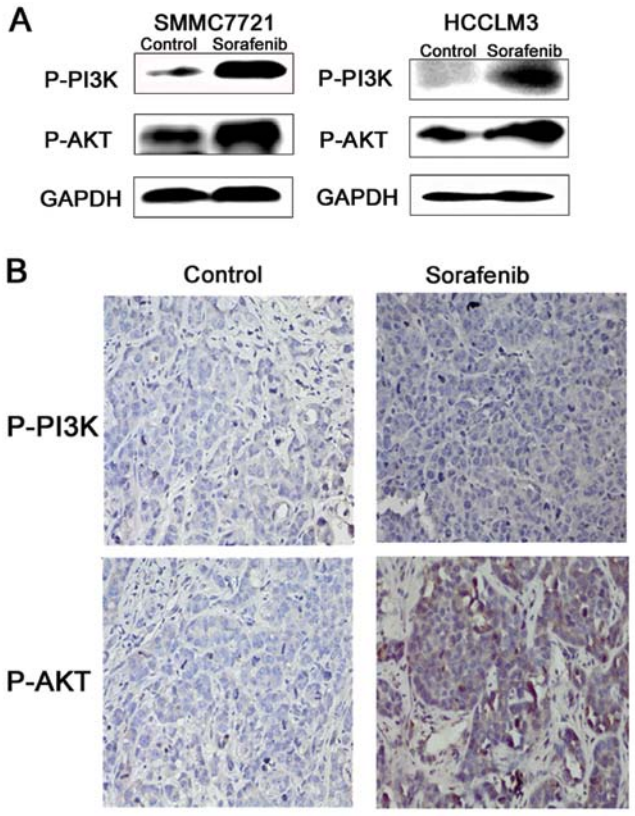

Figure 5. Sorafenib activates the PI3K/AKT signaling pathway *in vitro* and *in vivo*. (A) After 72 h of treatment with 5 μM sorafenib, the phosphorylation of PI3K/AKT was analyzed by western blotting in SMMC7721 and HCCLM3 cells. GAPDH was used as a loading control in the western blot analysis. (B) At the end of the experiment, the control and sorafenib-treated tumors were excised for immunohistochemistry for P-PI3K and P-AKT.

Accumulating research suggests that PI3K/Akt activation plays a pivotal role in tumor progression via induction of EMT. The PI3K/Akt/GSK-3β/Snail-dependent signaling pathway is involved in HCC. Thus, we detected the activity of PI3K/AKT in the sorafenib-induced invasion and metastasis of HCC. The results showed that the PI3K/AKT signaling pathway was activated in the HCC cells treated with 5 μM sorafenib (Fig. 5A). In addition, the marginal tissues of the xenografts were analyzed by immunohistochemical staining as described earlier. PI3K/AKT phosphorylation levels were also upregulated (Fig. 5B).

Discussion

As a result of the SHARP and ORIENTAL trials, sorafenib has become the new standard therapy for patients with advanced hepatic carcinoma (HCC) (5,6). However, the survival benefit is only a few months. Furthermore, tumors may progress during sorafenib treatment (9-11). In the present study, we demonstrated that sorafenib exerted an antitumor effect and inhibited tumor growth in mouse models of cancer. However, sorafenib also promoted invasion and metastasis of HCC in this tumor model by inducing EMT. Similar observations were reported by other authors. Importantly, we found that sorafenib upregulated the expression of transcription factor Snail and activated the PI3K/Akt signaling pathway.

In a previous study, increased local invasion and distant metastasis during or after treatment with sorafenib were observed (11). EMT plays a key role in tumor invasion and metastasis. EMT is also reported to be involved in the progression of HCC and is correlated with the prognosis of patients (37). In the present study, more metastatic lesions in the livers of nude mice were detected in the sorafenib treatment group. In addition, HCC cell lines, including SMMC7721 and HCCLM3, were treated with 5 μM sorafenib, with little effect on cell proliferation as confirmed by Cell Counting Kit-8. Surprisingly, morphology of the cells underwent significant changes and presented a mesenchymal phenotype after treatment for 72 h. Then EMT-related markers were analyzed. As anticipated, mesenchymal markers were significantly upregulated and epithelial markers were markedly decreased in the sorafenib-treated cells. Transwell assay was also used to analyze the ability of hepatoma cell invasion and migration. Invasion and migration capacity of the HCC cell lines was enhanced. Therefore, these data indicate that sorafenib may promote HCC invasion and metastasis by the induction of EMT, consistent with other reports.

The Snail transcription factor, a member of the Snail superfamily, is a zinc finger protein that can mediate EMT through downregulation of cell adhesion molecules such as E-cadherin by binding several E-boxes located in the promotor region (16). Snail has also been shown to confer survival properties either concomitantly with induction of EMT or independent of EMT. Snail plays an important role in inducing EMT in HCC cells (38). In cancer patients, an EMT-phenotype transcriptome profile, with increased Snail expression correlates with invasive tumors. Phosphorylation and subsequent degradation of Snail is controlled by GSK-3β, which is predominantly regulated by PI3K/Akt (39). The PI3K/Akt/GSK-3β/Snail-dependent signaling pathway can mediate invasion and metastasis of HCC (40,41). Increasing evidence also demonstrates that activation of the PI3K/Akt pathway plays a central role in the EMT process and correlates with an aggressive phenotype in several types of malignancies (22-27). Several signaling pathways that induce EMT and metastasis often converge at or activate PI3K/Akt, which itself is frequently activated during tumor progression. Hyperactivation of Akt is closely associated with elevated invasion and metastasis, resulting in a poor prognosis and a greater probability of relapse in many different cancer types (42-46). The PI3K/Akt signaling pathway plays a key role in invasion and metastasis of HCC. It was therefore of significance to investigate whether the PI3K/Akt/Snail-dependent signaling pathway participates in sorafenib-induced EMT. The PI3K/Akt signaling pathway was analyzed in the human HCC SMMC7721 and HCCLM3 cells. Notably, we found that 5 μM sorafenib activated the PI3K/Akt signaling pathway and upregulated the expression of transcription factor Snail. Immunohistochemical staining was then applied to the xenograft marginal tissues. As anticipated, these results were further confirmed *in vivo*.

In conclusion, the present study showed that sorafenib upregulated the expression of transcription factor Snail and activated the PI3K/AKT signaling pathway. Importantly, these may be associated with sorafenib-induced invasion and metastasis of HCC. Therefore, inhibition of the expres-

sion of transcription factor Snail or combined with PI3K/AKT signaling pathway inhibitors may enhance the effectiveness of sorafenib treatment of HCC. Currently, relevant studies are being carried out. The present study may provide the theoretical basis for the combined treatment of sorafenib and PI3K/AKT signaling pathway inhibitors to treat HCC.

Acknowledgements

We thank Te Liu (Shanghai Geriatric Institute of Chinese Medicine, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai) and Ning Zhang (Liver Cancer Institute and Zhongshan Hospital, Fudan University, Shanghai) for the experimental technical assistance.

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. CA Cancer J Clin 61: 69-90, 2011.
2. Gao JJ, Song PP, Tamura S, *et al*: Standardization of perioperative management on hepato-biliary-pancreatic surgery. Drug Discov Ther 6: 108-111, 2012.
3. Belghiti J and Fuks D: Liver resection and transplantation in hepatocellular carcinoma. Liver Cancer 1: 71-82, 2012.
4. Lee Cheah Y and KH Chow P: Liver transplantation for hepatocellular carcinoma: an appraisal of current controversies. Liver Cancer 1: 183-189, 2012.
5. Cheng AL, Kang YK, Chen Z, *et al*: Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. Lancet Oncol 10: 25-34, 2009.
6. Llovet JM, Ricci S, Mazzaferro V, *et al*: Sorafenib in advanced hepatocellular carcinoma. N Engl J Med 359: 378-390, 2008.
7. Folkman J: Tumor angiogenesis: therapeutic implications. N Engl J Med 285: 1182-1186, 1971.
8. Bergers G and Hanahan D: Modes of resistance to anti-angiogenic therapy. Nat Rev Cancer 8: 592-603, 2008.
9. Ebos JM, Lee CR, Cruz-Munoz W, Bjarnason GA, Christensen JG and Kerbel RS: Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. Cancer Cell 15: 232-239, 2009.
10. Pàez-Ribes M, Allen E, Hudock J, *et al*: Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. Cancer Cell 15: 220-231, 2009.
11. Zhang W, Sun HC, Wang WQ, *et al*: Sorafenib down-regulates expression of HTATIP2 to promote invasiveness and metastasis of orthotopic hepatocellular carcinoma tumors in mice. Gastroenterology 143: 1641-1649, 2012.
12. Lee JM, Dedhar S, Kalluri R and Thompson EW: The epithelial-mesenchymal transition: new insights in signaling, development, and disease. J Cell Biol 172: 973-981, 2006.
13. Barrallo-Gimeno A and Nieto MA: The Snail genes as inducers of cell movement and survival: implications in development and cancer. Development 132: 3151-3161, 2005.
14. Martínez-Alvarez C, Blanco MJ, Pérez R, *et al*: Snail family members and cell survival in physiological and pathological cleft palates. Dev Biol 265: 207-218, 2004.
15. Emadi Baygi M, Soheili ZS, Schmitz I, Sameie S and Schulz WA: Snail regulates cell survival and inhibits cellular senescence in human metastatic prostate cancer cell lines. Cell Biol Toxicol 26: 553-567, 2010.
16. Thiery JP, Acloque H, Huang RY and Nieto MA: Epithelial-mesenchymal transitions in development and disease. Cell 139: 871-890, 2009.
17. Cano A, Pérez-Moreno MA, Rodrigo I, *et al*: The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat Cell Biol 2: 76-83, 2000.
18. Zhang AL, Wang QS, Zhong YH, *et al*: Effect of transcriptional factor snail on epithelial-mesenchymal transition and tumor metastasis. Ai Zheng 24: 1301-1305, 2005 (In Chinese).
19. Jordà M, Olmeda D, Vinyals A, *et al*: Upregulation of MMP-9 in MDCK epithelial cell line in response to expression of the Snail transcription factor. J Cell Sci 118: 3371-3385, 2005.
20. Yokoyama K, Kamata N, Fujimoto R, *et al*: Increased invasion and matrix metalloproteinase-2 expression by Snail-induced mesenchymal transition in squamous cell carcinomas. Int J Oncol 22: 891-898, 2003.
21. Liu P, Cheng H, Roberts TM and Zhao JJ: Targeting the phosphoinositide 3-kinase pathway in cancer. Nat Rev Drug Discov 8: 627-644, 2009.
22. Bakin AV, Tomlinson AK, Bhowmick NA, Moses HL and Arteaga CL: Phosphatidylinositol 3-kinase function is required for transforming growth factor β-mediated epithelial to mesenchymal transition and cell migration. J Biol Chem 275: 36803-36810, 2000.
23. Grille SJ, Bellacosa A, Upson J, *et al*: The protein kinase Akt induces epithelial mesenchymal transition and promotes enhanced motility and invasiveness of squamous cell carcinoma lines. Cancer Res 63: 2172-2178, 2003.
24. Altomare DA and Testa JR: Perturbations of the AKT signaling pathway in human cancer. Oncogene 24: 7455-7464, 2005.
25. Larue L and Bellacosa A: Epithelial-mesenchymal transition in development and cancer: role of phosphatidylinositol 3' kinase/AKT pathways. Oncogene 24: 7443-7454, 2005.
26. Wang H, Quah SY, Dong JM, Manser E, Tang JP and Zeng Q: PRL-3 down-regulates PTEN expression and signals through PI3K to promote epithelial-mesenchymal transition. Cancer Res 67: 2922-2926, 2007.
27. Song LB, Li J, Liao WT, *et al*: The polycomb group protein Bmi-1 represses the tumor suppressor PTEN and induces epithelial-mesenchymal transition in human nasopharyngeal epithelial cells. J Clin Invest 119: 3626-3636, 2009.
28. Yoo YA, Kang MH, Lee HJ, *et al*: Sonic hedgehog pathway promotes metastasis via activation of AKT, EMT, and MMP-9 in gastric cancer. Cancer Res 71: 7061-7070, 2011.
29. Zuo JH, Zhu W, Li MY, *et al*: Activation of EGFR promotes squamous carcinoma SCC10A cell migration and invasion via inducing EMT-like phenotype change and MMP-9-mediated degradation of E-cadherin. J Cell Biochem 112: 2508-2517, 2011.
30. Qiao M, Sheng S and Pardee AB: Metastasis and AKT activation. Cell Cycle 7: 2991-2996, 2008.
31. Emadi Baygi M, Soheili ZS, Essmann F, *et al*: Slug/SNAI2 regulates cell proliferation and invasiveness of metastatic prostate cancer cell lines. Tumour Biol 31: 297-307, 2010.
32. Bolós V, Peinado H, Pérez-Moreno MA, Fraga MF, Esteller M and Cano A: The transcription factor Slug represses *E-cadherin* expression and induces epithelial to mesenchymal transitions: a comparison with Snail and E47 repressors. J Cell Sci 116: 499-511, 2003.
33. Chen KF, Chen HL, Tai WT, *et al*: Activation of phosphatidylinositol 3-kinase/Akt signaling pathway mediates acquired resistance to sorafenib in hepatocellular carcinoma cells. J Pharmacol Exp Ther 337: 155-161, 2011.
34. Gao Y, Li HX, Xu LT, *et al*: Bufalin enhances the anti-proliferative effect of sorafenib on human hepatocellular carcinoma cells through downregulation of ERK. Mol Biol Rep 39: 1683-1689, 2012.
35. Wang P, Chen Z, Meng ZQ, *et al*: Dual role of *Ski* in pancreatic cancer cells: tumor-promoting versus metastasis-suppressive function. Carcinogenesis 30: 1497-1506, 2009.
36. Li H, Wang P, Gao Y, *et al*: Na^+/K^+-ATPase α3 mediates sensitivity of hepatocellular carcinoma cells to bufalin. Oncol Rep 25: 825-830, 2011.
37. Yang MH, Chen CL, Chau GY, *et al*: Comprehensive analysis of the independent effect of twist and snail in promoting metastasis of hepatocellular carcinoma. Hepatology 50: 1464-1474, 2009.
38. Zucchini-Pascal N, Peyre L and Rahmani R: Crosstalk between beta-catenin and snail in the induction of epithelial to mesenchymal transition in hepatocarcinoma: role of the ERK1/2 pathway. Int J Mol Sci 14: 20768-20792, 2013.
39. Zhou BP, Deng J, Xia W, *et al*: Dual regulation of Snail by GSK-3β-mediated phosphorylation in control of epithelial-mesenchymal transition. Nat Cell Biol 6: 931-940, 2004.
40. Wen W, Ding J, Sun W, *et al*: Cyclin G1-mediated epithelial-mesenchymal transition via phosphoinositide 3-kinase/Akt signaling facilitates liver cancer progression. Hepatology 55: 1787-1798, 2012.

41. Shi GM, Ke AW, Zhou J, *et al*: CD151 modulates expression of matrix metalloproteinase 9 and promotes neoangiogenesis and progression of hepatocellular carcinoma. Hepatology 52: 183-196, 2010.
42. Thiery JP and Sleeman JP: Complex networks orchestrate epithelial-mesenchymal transitions. Nat Rev Mol Cell Biol 7: 13-42, 2006.
43. Pérez-Tenorio G and Stål O; Southeast Sweden Breast Cancer Group: Activation of AKT/PKB in breast cancer predicts a worse outcome among endocrine treated patients. Br J Cancer 86: 540-545, 2002.
44. Scheid MP and Woodgett JR: PKB/AKT: functional insights from genetic models. Nat Rev Mol Cell Biol 2: 760-768, 2001.
45. Vivanco I and Sawyers CL: The phosphatidylinositol 3-kinase AKT pathway in human cancer. Nat Rev Cancer 2: 489-501, 2002.
46. Xue G, Restuccia DF, Lan Q, *et al*: Akt/PKB-mediated phosphorylation of Twist1 promotes tumor metastasis via mediating cross-talk between PI3K/Akt and TGF-β signaling axes. Cancer Discov 2: 248-259, 2012.