Knockout of ADAM10 enhances sorafenib antitumor activity of hepatocellular carcinoma *in vitro* and *in vivo*

WEI ZHANG, SONGYANG LIU, KAI LIU, BAI JI, YINGCHAO WANG and YAHUI LIU

Department of Hepatobiliary and Pancreatic Surgery, The First Hospital of Jilin University,
Changchun, Jilin 130021, P.R. China

Abstract. Sorafenib (SOR), a vascular endothelial growth factor receptor (VEGFR) inhibitor, is in wide clinical use for the treatment and prevention of liver cancer. However, extended SOR administration for hepatocellular carcinoma (HCC) induces drug resistance thereby limiting its efficacy and highlighting the need for improved therapeutic strategies. It has previously been demonstrated that knockout of a disintegrin and metalloproteinase 10 (ADAM10) via siRNA induced cancer apoptosis and decreased chemotherapy drug resistance. However, whether knockout of ADAM10 is able to decrease SOR resistance remains to be determined. Therefore, in the present study, the effect of siRNA-ADAM10 in combination with SOR was analyzed in HCC cell lines (HepG2) by inhibiting tumor growth and simultaneously reducing doses of SOR. Cell proliferation, apoptosis, migration, invasion and involvement in receptor signaling were determined after siRNA-ADAM10 was applied in combination with SOR treatment. Tumor growth ability in nude mice was also detected. The results showed that siRNA-ADAM10 in combination with SOR treatment in HCC cancer cells significantly suppressed proliferation, migration and invasion, and induced tumor apoptosis *in vitro*, and suppressed tumor growth *in vivo*. In addition, the results showed that knockout of ADAM10 by siRNA inhibited the constitutive phosphorylation of PI3K and AKT, which may contribute to the reduction of SOR resistance. Collectively, our experimental results indicate that knockout of ADAM10 by siRNA increased the SOR antitumor activity of liver cancer *in vitro* and *in vivo*, and that this additive combination is a promising drug candidate for treatment of HCC.

Correspondence to: Professor Yahui Liu, Department of Hepatobiliary and Pancreatic Surgery, The First Hospital of Jilin University, 71 Xin Min Street, Changchun, Jilin 130021, P.R. China
E-mail: liuyahui588@sina.com

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third most frequent cause of cancer-related mortality globally, affecting >600,000 individuals annually (1,2). Although there have been developments in surgical strategies and percutaneous techniques such as ethanol injection and radiofrequency ablation and transcatheter arterial chemo-embolization (TACE), the overall outcome for HCC patients remains poor as HCC is commonly detected at a late stage when therapeutic options are limited (3). Conventional cytotoxic therapies may result in significant morbidity in patients with solid tumors including HCC since these drugs affect rapidly dividing normal and malignant cells, thereby limiting the survival of normal cells (4). Therefore, it is necessary to improve anticancer therapies that effectively and specifically target liver tumor cells while minimizing the toxic side-effects commonly associated with conventional cytotoxic therapies.

Sorafenib (SOR) (Nexavar, BAY 43-9006), an oral multi-kinase small molecule inhibitor, has been shown to have significant antitumor activity against various types of cancer including HCC (5-7). SOR blocks tumor cell proliferation and angiogenesis by targeting the Raf/mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling pathway and receptor tyrosine kinases (RTKs), such as vascular endothelial cell growth factor receptor (VEGFR)-2, VEGFR-3, platelet-derived growth factor receptor-β, fms-like tyrosine kinase receptor-3 (FLT3), RET and c-KIT (5,6). The results of phase III trials in Europe and Asia showed that SOR increased the survival rate in patients with advanced HCC (8,9). Although results of SOR for patients with advanced HCC are encouraging, treatment outcomes are poor due to unfavorable pharmacokinetics, low tumor accumulation and other adverse effects (10). Several studies have demonstrated that combination therapies of SOR and TRAIL or other chemotherapeutic agent are effective for HCC (11-18). However, chemotherapeutic drug resistance often occurs and the management of multi-drug resistant and recurrent or refractory tumors pose a challenge for clinical oncologists.

A class of disintegrins and metalloproteinases, known as ADAMs, has been shown to be involved in a variety of signaling events that are aberrant in cancers as well as during tumor progression (19). A disintegrin and metalloproteinase 10 (ADAM10), a member of the ADAM family,

has been found to be upregulated in various types of cancer and contributes to cancer progression and metastasis (20,21). Consistent with these findings, our recent results showed that ADAM10 is overexpressed in HCC tissues and there were significant associations between the protein levels of ADAM10 and tumor grade, amount of tumor differentiation, tumor size and the presence of metastasis (22). In addition, the RNA interference (RNAi)-mediated downregulation of endogenous ADAM10 was found to decrease the cell migration and invasion of HCC (23). Yang *et al* found that ADAM10 plays an important role in modulating the chemosensitivity of HCC cells to doxorubicin (24). Findings of that study suggested that ADAM10 is involved in HCC progression and metastasis and is important in modulating the chemosensitivity of HCC. However, little is known regarding the role of ADAM10 in the modulation of chemosensitivity of HCC cells to SOR. Thus, in the present study, we investigated the effects of modulating ADAM10 expression on the sensitivity of HCC cells to SOR treatment and examined the molecular pathways involved. In addition, tumor growth ability in nude mice was detected to define SOR in combination with the siRNA-ADAM10 treatment effect on tumorigenesis *in vivo*.

Materials and methods

Reagents. SOR (BAY 43-9006; Nexavar, LC Laboratories, Woburn, MA, USA) was dissolved in sterile dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO, USA) for the *in vitro* experiments. DMSO was added to cultures at 0.1% (v/v) final concentration as a vehicle control. PI3K and phosphorylated PI3K (p-PI3K; Tyr458); Akt and phosphorylated Akt (p-Akt; S473) primary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ADAM10, MMP-2 and MMP-9 were purchased from Sigma-Aldrich (St. Louis, MO, USA). The HRP-conjugated goat anti-mouse IgG secondary antibody was obtained from Amersham Biosciences (Uppsala, Sweden).

Cell culture. The human HCC cell lines, HepG2, was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) supplemented with 1% penicillin/streptomycin (Gibco-BRL, Grand Island, NY, USA) and 10% heat-inactivated fetal calf serum (FCS) (Invitrogen, Carlsbad, CA, USA).

Preparation of plasmid-based ADAM10 shRNA vector and transfect in HepG2. The ADAM10 small-interfering RNA (siRNA) (CAGTGTGCATTCAAGTCAA) and scrambled control (AATTCTCCGAACGTGTCACGT) sequences, which do not target any gene product or have any significant sequence similar to the human gene sequences, were designed using siRNA Target Designer software (Promega, Madison, WI, USA). The human ADAM10 and scrambled control short hairpin RNA (shRNA) were synthesized (Shanghai GeneChem Co., Ltd., China) and cloned into the pSUPER siRNA expression plasmid with the U6 promoter (Oligoengine, Seattle, WA, USA) as previously described (25) and designated as psi-ADAM10 and psi-Scramble, respectively.

HepG2 cells were transduced with the plasmid psi-ADAM10 and psi-Scramble using Lipofectamine™ 2000 transfection reagent according to the manufacturer's instructions. G418 (300 μg/ml; Sigma) was used to screen stably transfected clones. The expression of ADAM10 was examined by quantitative RT-PCR (RT-qPCR) and western blotting with an antibody against ADAM10 to validate the silencing efficiency of the target gene after RNAi.

Quantitative RT-PCR. RT-qPCR for ADAM10 transcripts in HepG2 cells was performed. First, total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA was subsequently reverse-transcribed into cDNA by a PrimeScript™ RT reagent kit according to the manufacturer's instructions (Takara, Dalian, China). RT-qPCR was conducted using the SYBR-Green fluorescent dye method, and a Rotor Gene 3000 real-time PCR apparatus. ADAM10 gene-specific amplification was confirmed by PCR with specific primers (sense, 5'-CTGCCCAGCATCTGACCCTAA-3' and antisense, 5'-TTGCCATCAGAACTGGCACAC-3') and subjected to melting curve analysis. GAPDH was used as an internal control for standardization. The primer sequences used were: β-actin, forward: 5'-GATCATTGCTCCTCCTGAGC-3' and reverse: 5'-ACTCCTGCTTGCTGATCCAC-3'. The PCR conditions were as follows: pre-denaturation at 95˚C for 2 min, followed by 40 cycles of denaturation at 95˚C for 10 sec, and annealing/extension at 55˚C for 20 sec. All the RT-qPCR tests were performed in triplicate and after the third day of plasmid transfections. The data were analyzed using the comparative Ct method.

Western blotting. The cells were collected and homogenized in a lysis buffer (Tris-HCl 50 mmol/l, EDTA 5 mmol/l, NaCl 150 mmol/l, sodium deoxycholate 1%, Na$_3$VO$_4$ 500 μmol/l, Triton X-100 0.5%, AEBSF 10 μmol/l, NaF 10 mmol/l) on ice for 30 min. Cell lysates were clarified by centrifugation (10,000 x g, 15 min), and protein concentrations were determined using the Bradford reagent (Sigma-Aldrich, Taufkirchen, Germany). Equal amounts of protein (15 μg/lane) from the cell lysates were separated on an 8-15% SDS-polyacrylamide gel (SDS-PAGE) and transferred onto nitrocellulose membranes (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The membrane was incubated for 2 h in phosphate-buffered saline (PBS) plus 0.1% Tween-20 and 5% non-fat skim milk to block non-specific binding. The membranes were then incubated overnight at 4˚C with primary antibodies. After washing, the proteins were visualized using an ECL detection kit with the appropriate HRP-conjugated secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for 2 h. All the assays were performed after the third day of drug treatment.

Cell proliferation assay. The MTT assay was used to determine for cell proliferation. Briefly, the cells were seeded in 8- of 96-well plates at a density of 2x10^3 cells/well. After being cultured for 24 h, the cells were treated with psi-ADAM10, psi-Scramble, SOR or psi-ADAM10 in combination with SOR, respectively. At 48 h after treatment, 20 μl of MTT (5 mg/ml; Sigma) was added to each well followed by incubation at 37˚C

for 48 h. Centrifugation was then performed at 2,000 x g for 10 min. The supernatant was removed, and 200 μl of DMSO was added to each well followed by agitation for 10 min. The absorbance was measured using a microplate reader at a wavelength of 490 nm. The experiment was repeated three times. The inhibition rate was calculated according to the formula: Inhibition rate (%) = [1-(average absorbance of experimental group/average absorbance of blank control group)] x 100%. The mean proliferation of cells without any treatment was expressed as 100%.

Colony formation assay. HepG2 cells were seeded in 6-well culture plates at $1x10^4$ cells/well, and were treated with specific drugs when cells reached the logarithmic growth phase. After the cells were incubated at 37°C for 10 days, and the medium was replaced every 3 days. After washing three times with PBS, the colonies were fixed with ice methanol for 30 min and stained with Giemsa for 10 min. The visible colonies were then counted.

TUNEL assay. To measure the effect of psi-AMAD10 in combination with SOR on cell apoptosis, a TUNEL assay was carried out. Briefly, after HepG2 cells were treated with psi-ADAM10, psi-Scramble, SOR or psi-ADAM10 in combination with SOR for 24 h, cellular DNA fragmentation was measured with the ApoTag Red In Situ Apoptosis Detection kit (Chemicon International, Temecula, CA, USA) according to the manufacturer's instructions. To quantify the apoptotic cells, the terminal deoxynucleotidyl transferase-mediated nick end-labeling (TUNEL)-positive cells were counted using confocal microscopy.

In addition, at the molecular level, we detected apoptosis related to protein, survivin and Bcl-2 protein expression by western blotting as an additional indicator of apoptosis.

Caspase-8 activity assay. HepG2 cells were collected and lysed in lysis buffer (10 mM Tris-HCl, pH 7.5, 130 mM NaCl, 1% Triton X-100 and 10 mM sodium pyrophosphate) following specific treatment. Cell lysates were clarified by centrifugation at 14,000 x g for 5 min at 4°C and clear lysates containing 50 μg of protein were incubated with 100 μM of enzyme-specific substrate (Ac-IEVD-pNA for caspase-8) in assay buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA and 10 mM EGTA) at 37°C for 1 h. Caspase-8 activity was determined by the cleavage of colorimetric substrate monitored at 405 nm.

Wound-healing assay. To assess the effect of psi-ADAM10 in combination with SOR psi-ADAM10 on cell migration, a wound-healing assay was performed. Briefly, $1x10^5$ HepG2 cells were plated in 12-well plates in DMEM containing 10% fetal bovine serum (FBS). After 24 h, a scratch was made through the confluent cell monolayer, and the cells were treated with psi-ADAM10, psi-Scramble, SOR or psi-ADAM10 in combination with SOR, respectively, in 3 ml of complete medium. After 48 h treatment, the cells were stained with hematoxylin and eosin (H&E). Cells invading the wound line were observed under an inverted phase-contrast microscope using x20, Leica DMR, Germany. Experiments were performed in triplicate.

Transwell invasion assay. Cell invasion was determined using Transwell chambers produced from polycarbonate membrane filters with a pore size of 8-μm according to the manufacturer's instructions (Costar, USA) and the upper chambers were coated with Matrigel (BD Biosciences, USA). The cells were transferred to the upper chamber of each prepared Transwell chamber at a density of $4x10^5$ cells/ml (100 μl) and treated with psi-ADAM10, psi-Scramble, SOR or psi-ADAM10 in combination with SOR, respectively. The lower chamber contained DMEM supplemented with 10% FBS. The cells were allowed to migrate for 24 h at 37°C. Non-invading cells were removed from the top surfaces with a cotton swab. The membranes were fixed in 95% ethanol and stained with 0.1% crystal violet. The cells that had penetrated to the bottom surface of each membrane were counted with 10 random fields on each microscope slide. In addition, cells were quantified by measuring the absorbance of dye extracts at 570 nm in 100 ml of Sorenson's solution (9 mg trisodium citrate, 305 ml distilled water, 195 ml 0.1 N HCl, and 500 ml 90% ethanol). Experiments were performed in triplicate.

Tumor xenograft assay. Fifty female BALB nude mice aged 4-6 weeks and weighing 18-20 g, were purchased from the Institute of Laboratory Animal Science, Jilin University (Changchun, Jilin, China), and were maintained under specific pathogen-free (SPF) conditions and provided with food and water *ad libitum*. The animal experiments were carried out according to the standards of animal care as outlined in the Guide for the Care and Use of Experimental Animals of Jilin University. The study protocol was approved by the Ethics Committee, The First Hospital of Jilin University.

Exponentially growing HepG2 cells were harvested and a tumorigenic dose of $2x10^6$ cells was injected intraperitoneally into the BALB mice. Approximately 20 days after the inoculation of HepG2 cells, the average tumor volume was at 120.28±8.23 mm³, and the mice were divided randomly into 5 groups (10 mice/group). The control group received 1% polysorbate resuspended in deionized water. The remaining four groups were treated with psi-Scramble (30 μg/50 μl/mouse), SOR (80 mg/kg body weight), psi-ADAM10 (30 μg/50 μl/mouse) or SOR plus psi-ADAM10 (SOR, 40 mg/kg body weight; psi-ADAM10; 15 μg/50 μl/mouse) intraperitoneally on alternative days for 3 weeks. The tumor size was measured using calipers prior to administration of the treatment injections and on the 7th, 14th and 21st days of treatment. On the 21st day, the animals were sacrificed using chloroform and their spleen tissue was collected and cultured for a splenocyte surveillance study as previously described (26). Sections of each tumor tissue were wax-embedded for H&E staining to study cell apoptosis *in vivo* by TUNEL.

Statistical analysis. Data are presented as means ± SD. A statistical comparison of more than two groups was performed using one-way ANOVA followed by a Tukey's post-hoc test. Statistical analyses were undertaken using the GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA, USA) for Windows®. $P<0.05$ was considered to indicate a statistically significant result. The images shown in the present study were obtained from at least three independent experiments with similar results.
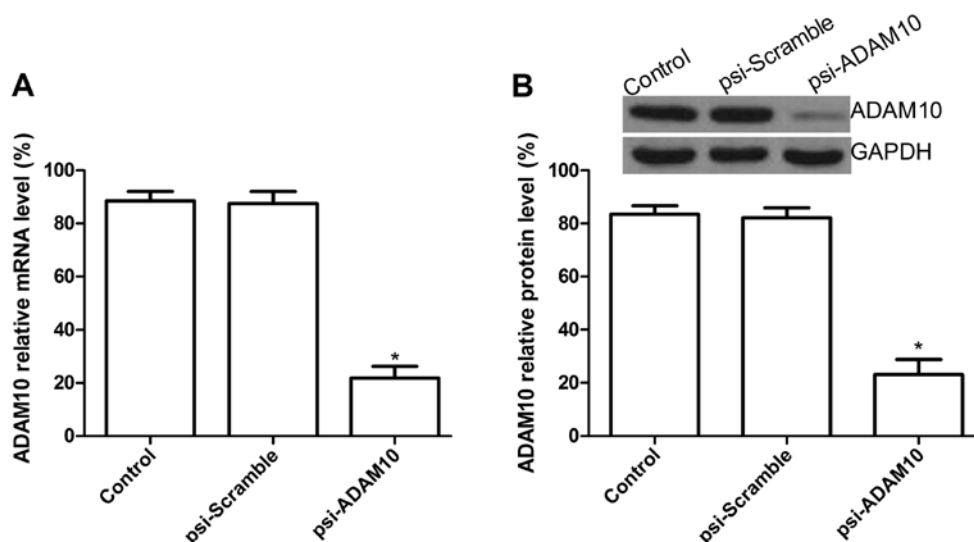
Figure 1. Silencing ADAM10 inhibits ADAM10 expression in HepG2 tumor cells. (A) ADAM10 mRNA expression was determined by quantitative PCR (RT-qPCR) 72 h after transfection of plasmid psi-ADAM10. (B) Western blot analysis and RT-qPCR of ADAM10 protein expression 72 h after the transfection of plasmid psi-ADAM10. *P<0.05 vs. control. ADAM10, a disintegrin and metalloproteinases 10.
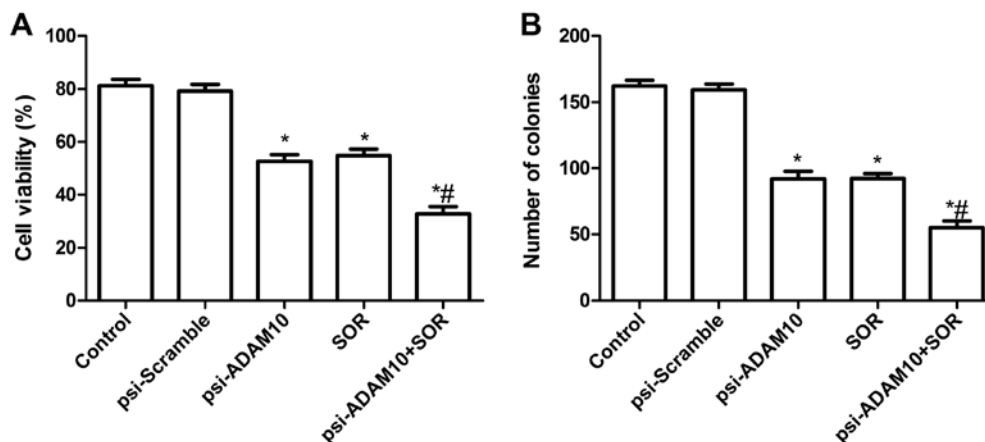

Figure 2. Effect of SOR and psi-ADAM10 alone and in combination on cell proliferation and clone formation. (A) Cell proliferation and (B) cell clone formation of HepG2 cells were determined following treatment with SOR and psi-ADAM10 alone or in combination. *P<0.05 vs. control, #P<0.05 vs. SOR alone. SOR, sorafenib; ADAM10, a disintegrin and metalloproteinases 10.

Results

Downregulation of ADAM10 expression by plasmid psi-ADAM10. To silence ADAM10 expression, we constructed a recombinant plasmid-based shRNA against ADAM10, psi-Scramble. Subsequently, we assessed the silencing ability of psi-ADAM10 in the HCC cell line by RT-qPCR and western blotting following the treatment of HepG2 cells with plasmid psi-ADAM10 for three days. RT-qPCR results showed no significant inhibition in ADAM10 mRNA expression in the control and psi-Scramble groups. Compared to the psi-Scramble and control groups, mRNA expression in the psi-ADAM10 group was significantly decreased (Fig. 1A, P<0.01). On the protein level, no significant inhibition in ADAM10 protein expression was found in the psi-Scramble and control groups (P>0.05), while the band density markedly decreased in the psi-ADAM10 group as compared with the psi-Scramble and control groups (P<0.01) (Fig. 1B). These results demonstrated

that silencing ADAM10 significantly decreased ADAM10 expression in the HCC cell line.

Effects of SOR and psi-ADAM10 alone or in combination on HepG2 cell proliferation and cell colony formation. To evaluate the effect of psi-ADAM10 and SOR alone or their combination on the viability of HCC cells *in vitro*, an MTT assay was performed for 48 h when HepG2 cells were treated with psi-ADAM10 and SOR alone or both. It was found that the inhibitory rates of psi-ADAM10 and SOR alone or the combination treatment were higher than those of the control and psi-Scramble groups (P<0.01, Fig. 2A). No significant difference between the psi-Scramble and control groups was observed (P>0.05, Fig. 2A). In addition, the inhibitory rates of psi-ADAM10 in combination with the SOR group were higher than the single treatment group (P<0.05, Fig. 2A).

The effects of psi-ADAM10 and SOR alone or in combination on the HepG2 cell colony formation were then analyzed.
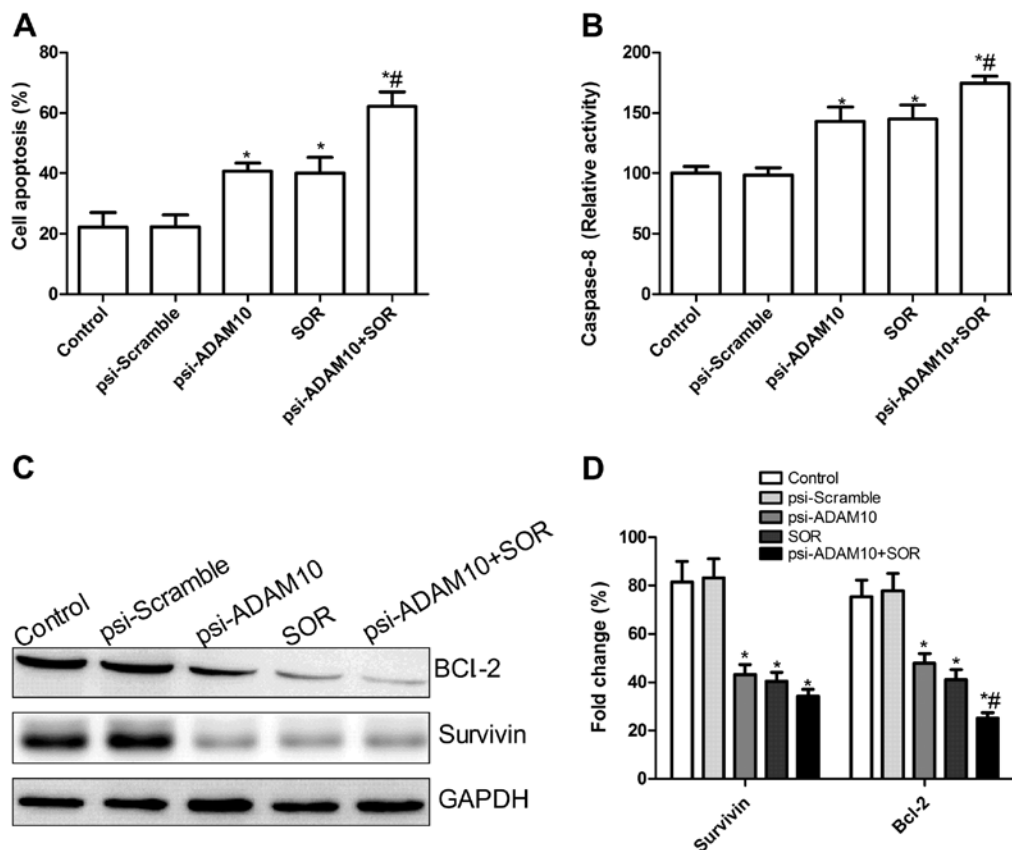
Figure 3. Effect of SOR and psi-ADAM10 alone and in combination on cell apoptosis in HepG2 cells. (A) Apoptosis of HepG2 cells was determined following treatment with SOR and psi-ADAM10 alone or in combination. (B) Caspase-8 activity was determined following treatment with SOR and psi-ADAM10 alone or in combination. (C and D) Expression of survivin and BCl-2 in HepG2 cells was determined by western blotting. Data are presented as the means ± SD. *P<0.05 vs. control, #P<0.05 vs. SOR alone. SOR, sorafenib; ADAM10, a disintegrin and metalloproteinases 10.

Compared with the control and psi-Scramble groups, the number of tumor cells per colony were significantly reduced in psi-ADAM10 and SOR alone or the combination groups (P<0.01, Fig. 2B). The psi-Scramble in combination with SOR resulted in an even greater percentage of reduction than the higher doses of either drug alone (P<0.01, Fig. 2B).

Effects of SOR and psi-ADAM10 alone or in combination on HepG2 cell apoptosis. To investigate whether the psi-ADAM10 and SOR alone or in combination induced apoptosis, the apoptosis was analyzed after treatment with the specific drug. It was found that HepG2 cells treated with psi-ADAM10 and SOR alone or in combination significantly induced cell apoptosis compared with the control and psi-Scramble groups (Fig. 3A). Treatment with the combination of psi-ADAM10 and SOR resulted in a marked increase in apoptotic cells compare to the single drug treatment group (P<0.01) (Fig. 3A).

To determine the possible mechanism of induction of cell apoptosis of the combination with psi-ADAM10 and SOR, caspase-8 activity was detected using ELISA. The results showed that caspase-8 activity was significantly increased in psi-ADAM10 and SOR alone or in the combination treatment groups compared to the control and psi-Scramble groups (P<0.05; Fig. 3B). Compared to the psi-ADAM10 and SOR groups, the combination treatment significantly increased caspase-8 activity (P<0.05; Fig. 3B). In addition, expression patterns of survivin and Bcl-2 were determined by western

blotting. The results showed that the combination with psi-ADAM10 and SOR significantly decreased the expression of apoptosis inhibiting gene survivin and Bcl-2 in HepG2 cells compared to the single drug treatment, and control and psi-Scramble groups (Fig. 3C and D, P<0.05).

Effects of SOR and psi-ADAM10 alone or in combination on HepG2 cell migration and invasion. To ascertain the inhibitory effect of psi-ADAM10 and SOR alone or in combination on HCC cell motility *in vitro*, a wound-healing assay was performed. As shown in Fig. 4A, cell migration in the psi-ADAM10 and SOR alone or the combination groups was significantly reduced compared to the control and psi-Scramble groups when HepG2 cells were treated with the indicated drug treatment for 48 h (P<0.05). In addition, compared to the SOR and psi-ADAM10 groups, migration was significantly reduced in SOR in combination with the psi-ADAM10 group.

To determine whether psi-ADAM10 and SOR alone or their combination affected the invasiveness of human HCC HepG2 cells, the cells were treated with the indicated drug for 48 h. As shown in Fig. 4B, the result of the cell invasiveness assay showed that there was no significant difference in the number of cells that had passed through the simulated basement membrane between the control and psi-Scramble groups. However, the number of cells that had passed through the simulated basement membrane in the psi-ADAM10 and SOR alone or combination group were significantly reduced
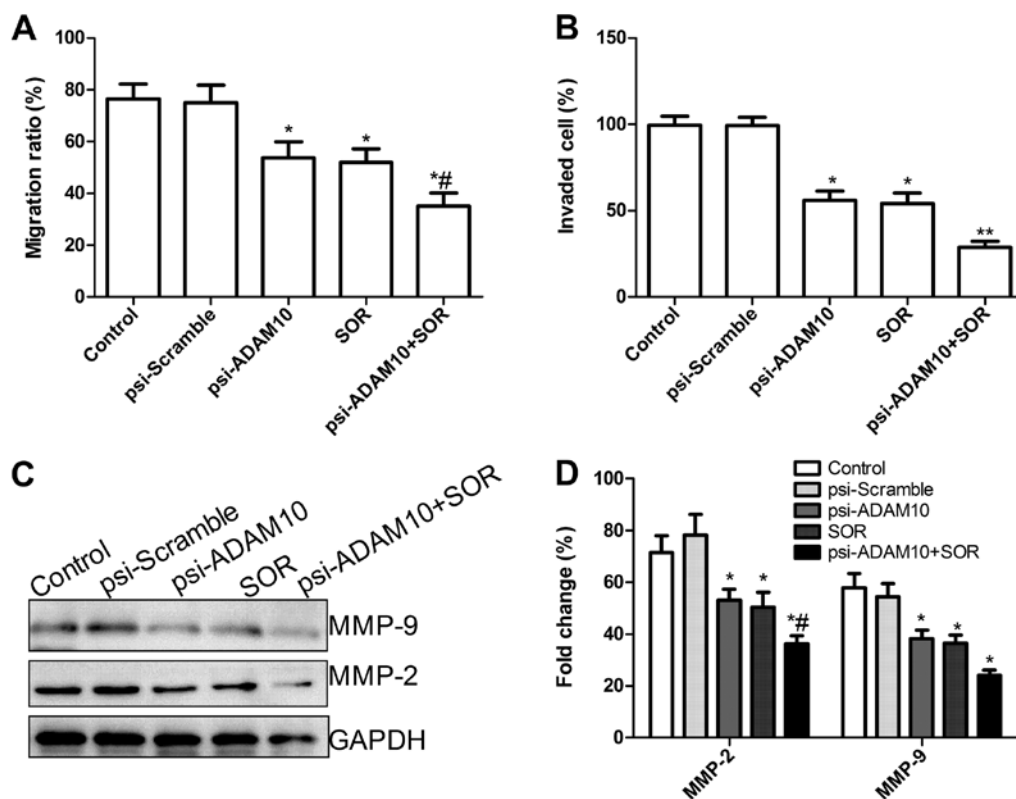
Figure 4. Effect of SOR and psi-ADAM10 alone and in combination on cell migration and invasion in HepG2 cells. (A) Cell migration was determined 48 h following treatment with SOR and psi-ADAM10 alone or in combination. (B) SOR and psi-ADAM10 alone or combination effect of HepG2 cell invasion through Matrigel. (C and D) Expression of MMP-2 and MMP-9 in HepG2 cells was determined by western blotting. Data are presented as the means ± SD. $^*P<0.05$ vs. control, $^\#P<0.05$ vs. SOR alone. SOR, sorafenib; ADAM10, a disintegrin and metalloproteinases 10.

when compared with the control and psi-Scramble groups (all P<0.05). Compared to the single treatment group, the cell invasion number was significantly reduced (P<0.05, Fig. 4B).

To determine the potential mechanism of SOR in combination with psi-ADAM10 inhibition of cell invasion *in vitro*, the MMP-2 and MMP-9 protein expression was determined by western blot analysis. Western blot analysis revealed a significant decrease in MMP-2 and MMP-9 proteins in the psi-ADAM10 and SOR alone or in the combination group compared to the control and psi-Scramble groups alone (P<0.05, Fig. 4C and D). Compared to the single treatment group, psi-ADAM10 in combination with the SOR group decreased the protein expression of MMP-2 and MMP-9 (P<0.05, Fig. 4C and D).

ADAM10 silencing sensitizes HCC cells to SOR treatment. To determine whether the downregulation of ADAM10 by siRNA affected the sensitivity of HCC cells to SOR, we stably transfected plasmid psi-ADAM10 and psi-Scramble into HepG2 cells, respectively, and then added SOR (20 μM) to HepG2 for further treatment. After 48 h, cell proliferation and apoptosis were determined. The results of the MTT assay showed that silencing ADAM10 significantly inhibited cell proliferation in the presence of SOR compared to the control and psi-Scramble groups in the presence of SOR (Fig. 5A). In addition, silencing of ADAM10 significantly induced cell apoptosis in the presence of SOR (Fig. 5B). Consistent with the increased apoptosis, the amount of cleaved caspase-8 was

profoundly elevated in ADAM10-silenced HCC cells in the presence of SOR (Fig. 5C). Moreover, ADAM10-deficient cells exhibited a reduced expression of the anti-apoptotic factor survivin and Bcl-2 (Fig. 5D).

PI3K/Akt pathway is involved in ADAM10-mediated chemoresistance to SOR. The PI3K/Akt pathway plays an important role in regulating the chemoresistance of cancer cells (27-29). We examined whether this signaling pathway mediated ADAM10-dependent chemoresistance in HCC cells. Measurements of the phosphorylation/activation pattern of PI3K and Akt was performed by western blotting. Our results showed that the downregulation of ADAM10 expression in HepG2 cells resulted in a marked reduction of phosphorylated PI3K and Akt relative to mock cells, without altering the total protein levels of PI3K or Akt in the presence of SOR (Fig. 6).

Effects of SOR and psi-ADAM10 alone or in combination on tumor growth in a murine model. The *in vivo* therapeutic efficacy of psi-ADAM10 and SOR alone or their combination on female BALB mice-bearing HepG2 tumor cells was assessed. The results showed that the tumor weight of psi-ADAM10 and SOR alone or the combination group was lower than that of the control and psi-Scramble groups (P<0.05, Fig. 7A). Compared with the results with either agent alone, the combination of psi-ADAM10 and SOR greatly inhibited tumor growth (Fig. 7A). In addition, we found that tumor volume after treatment with psi-ADAM10 and SOR was
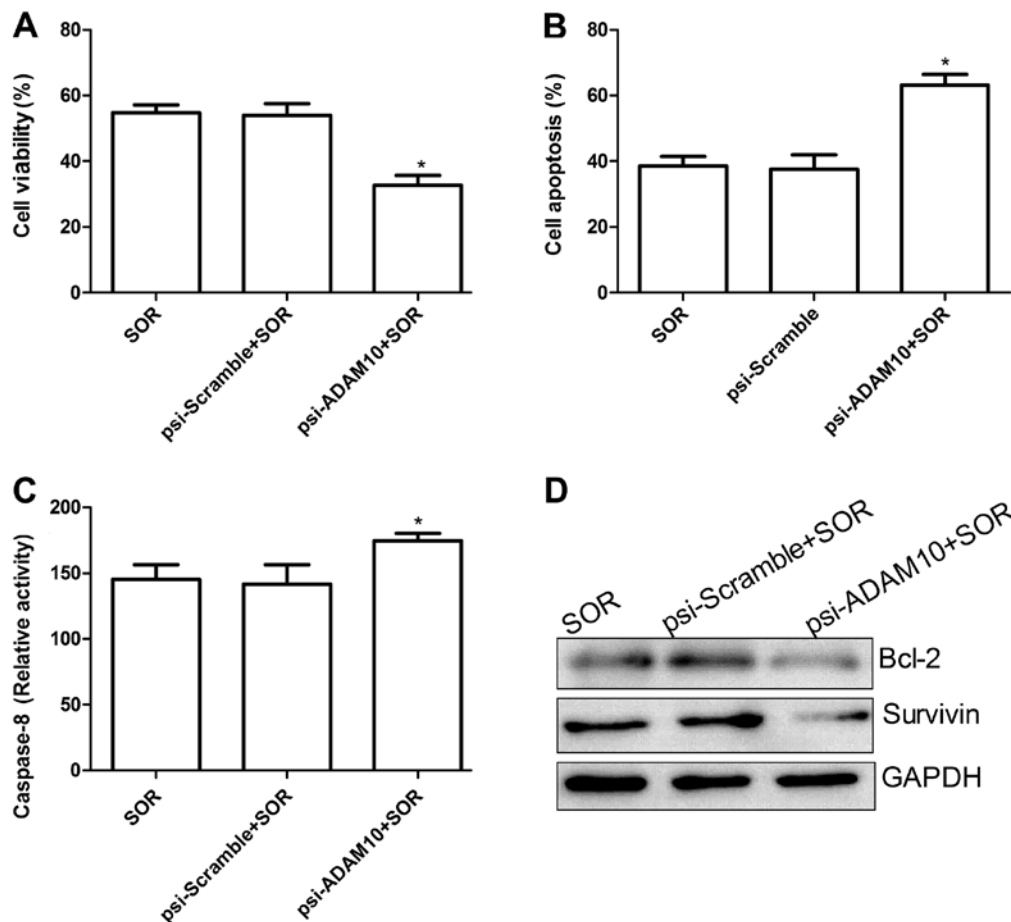
Figure 5. Downregulation of endogenous ADAM10 sensitizes HCC cells to sorafenib. (A) Cell proliferation and (B) apoptosis were measured 48 h after sorafenib was added to HepG2 cells transfected with psi-scrambled or psi-ADMA10. (C) Caspase-8 activity was determined after sorafenib was added to HepG2 cells transfected with psi-scrambled or psi-ADMA10. (D) Expression of survivin and BCl-2 in HepG2 cells was determined after sorafenib was added to HepG2 cells transfected with psi-scrambled or psi-ADMA10. Data are expressed as the means ± SD. *P<0.05 vs. SOR alone. ADAM10, a disintegrin and metalloproteinases 10; SOR, sorafenib.
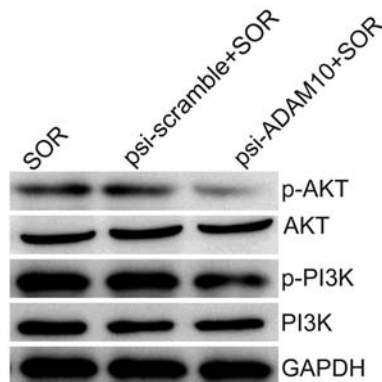

Figure 6. Measurements of the phosphorylation/activation pattern of PI3K and Akt was performed by western blotting. Downregulation of ADAM10 by RNAi expression in HepG2 cells resulted in a marked reduction of phosphorylated PI3K and Akt relative to mock cells, without altering the total protein levels of PI3K or Akt in the presence of SOR. ADAM10, a disintegrin and metalloproteinases 10; SOR, sorafenib.

significantly reduced for the HepG2 tumor cells compared with the control and psi-Scramble groups at different time points (P<0.05, Fig. 7B). Treatment with the combination of psi-ADAM10 and SOR resulted in marked inhibition of the

tumor volume compared to the psi-ADAM10 and SOR groups alone (P<0.05, Fig. 7B).

To assess the efficacy of psi-ADAM10 and SOR alone or combination in modulating splenocyte proliferation, MTT assay were performed. As shown in Fig. 7C, the inhibitory rates of psi-ADAM10 and SOR alone or the combination group were higher than those of the control and psi-Scramble groups (P<0.05). Treatment with the combination of psi-ADAM10 and SOR led to marked inhibition of cell proliferation compared to the psi-ADAM10 and SOR groups alone. In addition, we determined tumor tissue cell apoptosis *in vivo* by TUNEL. The results showed that psi-ADAM10 and SOR alone or the combination group significantly induced cell apoptosis compared to the control and psi-Scramble groups (P<0.05, Fig. 7D). Compared with the results with either agent alone, the combination of psi-ADAM10 and SOR greatly induced tumor cell apoptosis *in vivo*. These results demonstrated that the combination of psi-ADAM10 and SOR suppressed the tumor growth of HCC *in vivo*.

Discussion

Sorafenib (SOR) is currently the standard of care for hepatocellular carcinoma (HCC) patients with preserved liver
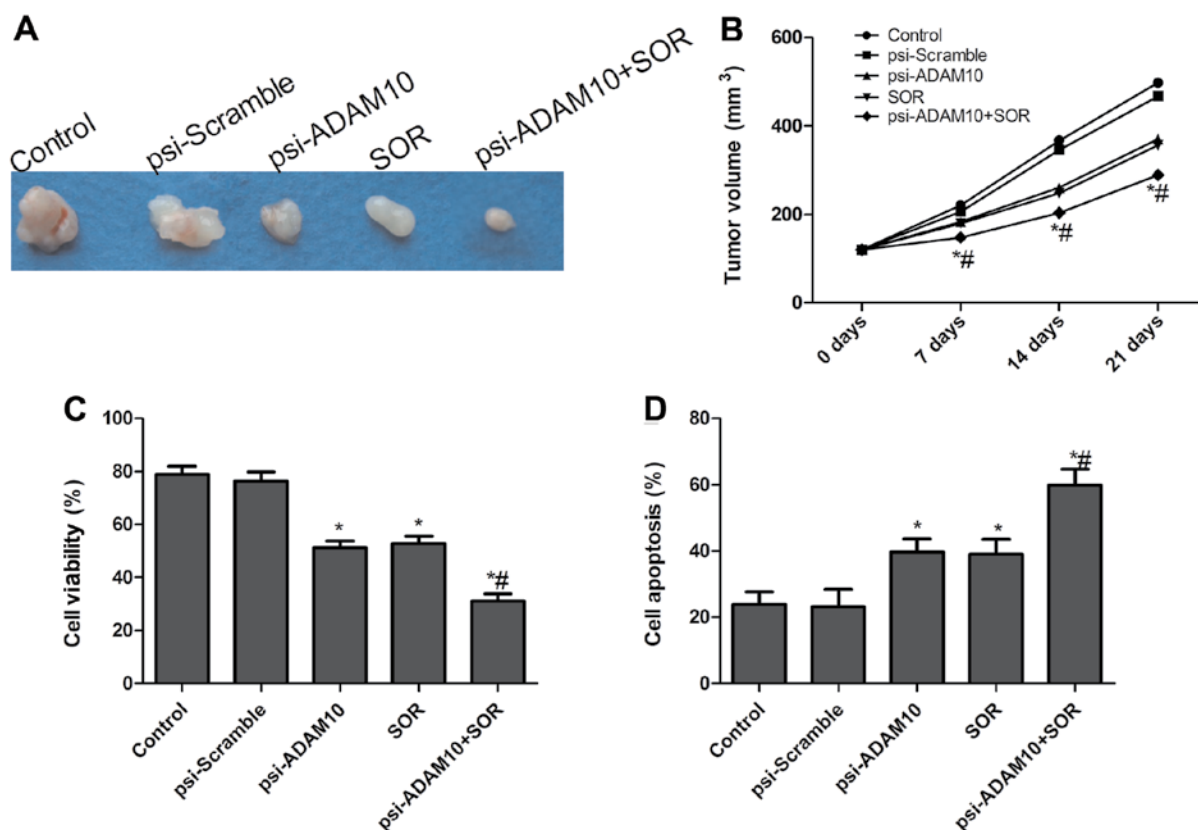
Figure 7. Antitumor activity of SOR and psi-ADAM10 alone or in combination in BALB/c mice bearing HepG2 cells. (A) Tumor weight in treated and untreated mice for 21 days. (B) The tumor volume in treated and untreated mice at the 7th, 14th and 21st days. (C) MTT assay of proliferation of splenocytes from mice. (D) TUNEL assay of cell apoptosis *in vivo*. Data are presented as the means ± SD. *$P<0.05$ vs. control, #$P<0.05$ vs. SOR alone. SOR, sorafenib; ADAM10, a disintegrin and metalloproteinases 10.

function who have metastatic or unresectable disease not amenable to liver transplantation (30). In two randomized controlled phase III trials, SOR significantly improved overall survival (OS) and time to progression compared with patients administered a placebo. However, the median OS in the SOR arms of the two studies was moderately increased (8,9). As a result, there is a need for new and effective HCC treatments to improve patient outcome.

It has been reported that ADAM is involved in various cell processes such as proliferation, differentiation, migration and invasion (31,32). A large number of studies suggest that ADAMs is important in cancer cell survival (20-24,31,32). ADAM10, a member of the ADAM family, has been shown to be upregulated in various types of cancer including HCC (22). Accumulating evidence have demonstrated that ADAM10 is involved in cancer cell progression and metastasis (21-23,31-35). For example, Klein *et al* (31) reported that ADAM10 can activate Notch signaling by suppressing ectodomain shedding of δ-1, which subsequently leads to a strong inhibitory effect on tumor cell proliferation and apoptosis (33). Endres *et al* showed that ADAM10 can cleave collagen type IV in the basement membrane, which is relevant to tumor metastasis and proliferation (34). In addition, it has been shown that the combined therapy with shRNA combination anticancer drug may achieve better antitumor activity (35). The study by Emdad *et al* demonstrated that when melanoma differentiation-associated gene-7 (mda-7) through a replication-incompetent adenovirus (Ad.mda-7)

and gefitinib are used in combination, they may provide an effective therapeutic approach for non-small cell lung cancer (NSCLC) since these agents target various cell survival pathways and are equally effective against NSCLC cells (36). Lu *et al* showed that lentivirus-carrying COX-2 gene combination with tamoxifen (TAM) in breast cancer cells significantly suppressed tumor growth *in vitro* and *in vivo* (26). Consistent with the abovementioned results, results of this study show that plasmid psi-ADAM10 in combination with SOR treatment in HCC cancer cells significantly suppressed proliferation, migration and invasion, and induced tumor apoptosis *in vitro* and suppressed tumor growth *in vivo*.

Resistance to chemotherapy poses a challenge for the successful treatment of HCC patients. It has been reported that ADAM10 decreases resistance to chemotherapeutic drugs (24,37). Yang *et al* demonstrated that ADAM10 plays an important role in modulating the chemosensitivity of HCC cells to doxorubicin (24). Bai *et al* found that the expression of microRNA-122, as a negative regulator of ADAM10, sensitizes HCC cells to SOR against HCC (37). Consistent with their results, results of the present study have shown that ADAM10 was involved in the modulation of the chemosensitivity of HCC cells to SOR against HCC and that the downregulation ADAM10 by siRNA sensitizes HCC cells to SOR against HCC.

The mediation of drug resistance depends on cellular changes, such as increased repair of DNA damage, alterations in the cell cycle and/or reduction of apoptosis by the activation of anti-apoptotic pathways (38). In this regard, activation of

the PI3K/AKT pathway is an important requirement of cancer cells in order to escape cell death following exposure to toxic stimuli. It has been shown that the phosphorylation of AKT was significantly higher among patients who received chemotherapy and this increase was associated with poor prognosis in various types of cancer including HCC (39). Furthermore, accumulating evidence has demonstrated that the activated PI3K/AKT signalling cascade which promotes resistance against several chemotherapeutic drugs was identified in various cell culture model systems including the HCC cell line (24,40-42). In the present study, the results have shown that ADAM10 is important in modulating the chemosensitivity of HCC cells to SOR against HCC, which, at least partially, involves the activation of the PI3K/Akt pathway, a finding that is consistent with results of a previous study (24).

In conclusion, our *in vitro* studies have demonstrated that when plasmid psi-ADAM10 and SOR are used in combination they may provide an effective therapeutic approach for HCC since this combination may significantly suppress proliferation, migration and invasion and induce tumor apoptosis *in vitro*. Additionally, studies with *in vivo* murine models confirmed that psi-ADAM10 combination with SOR may suppress HCC tumor growth. Therefore, it is of note to consider their combination as a novel therapeutic strategy for further evaluation in clinical trials for the treatment of HCC.

Acknowledgements

References

1. Venook AP, Papandreou C, Furuse J and de Guevara LL: The incidence and epidemiology of hepatocellular carcinoma: a global and regional perspective. Oncologist 15 (Suppl 4): 5-13, 2010.
2. Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. CA Cancer J Clin 61: 69-90, 2011.
3. Thorgeirsson SS and Grisham JW: Molecular pathogenesis of human hepatocellular carcinoma. Nat Genet 31: 339-346, 2002.
4. El-Serag HB and Rudolph KL: Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology 132: 2557-2576, 2007.
5. Wilhelm SM, Carter C, Tang L, et al: BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. Cancer Res 64: 7099-7109, 2004.
6. Liu L, Cao Y, Chen C, et al: Sorafenib blocks the RAF/MEK/ERK pathway, inhibits tumor angiogenesis, and induces tumor cell apoptosis in hepatocellular carcinoma model PLC/PRF/5. Cancer Res 66: 11851-11858, 2006.
7. Gu FM, Li QL, Gao Q, et al: Sorafenib inhibits growth and metastasis of hepatocellular carcinoma by blocking STAT3. World J Gastroenterol 17: 3922-3932, 2011.
8. Llovet JM, Ricci S, Mazzaferro V, et al: Sorafenib in advanced hepatocellular carcinoma. N Engl J Med 359: 378-390, 2008.
9. 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.
10. Roy M, Luo YH, Ye M and Liu J: Nonsmall cell lung cancer therapy: insight into multitargeted small-molecule growth factor receptor inhibitors. Biomed Res Int 2013: 964743, 2013.
11. Nojiri K, Sugimoto K, Shiraki K, et al: Sorafenib and TRAIL have synergistic effect on hepatocellular carcinoma. Int J Oncol 42: 101-108, 2013.
12. Schmieder R, Puehler F, Neuhaus R, et al: Allosteric MEK1/2 inhibitor refametinib (BAY 86-9766) in combination with sorafenib exhibits antitumor activity in preclinical murine and rat models of hepatocellular carcinoma. Neoplasia 15: 1161-1171, 2013.
13. Hoffmann K, Glimm H, Radeleff B, et al: Prospective, randomized, double-blind, multi-center, phase III clinical study on transarterial chemoembolization (TACE) combined with Sorafenib versus TACE plus placebo in patients with hepatocellular cancer before liver transplantation - HeiLivCa [ISRCTN24081794]. BMC Cancer 8: 349, 2008.
14. Abou-Alfa GK, Johnson P, Knox JJ, et al: Doxorubicin plus sorafenib vs doxorubicin alone in patients with advanced hepatocellular carcinoma: a randomized trial. JAMA 304: 2154-2160, 2010.
15. Hsu CH, Shen YC, Lin ZZ, et al: Phase II study of combining sorafenib with metronomic tegafur/uracil for advanced hepatocellular carcinoma. J Hepatol 53: 126-131, 2010.
16. Pawlik TM, Reyes DK, Cosgrove D, Kamel IR, Bhagat N and Geschwind JF: Phase II trial of sorafenib combined with concurrent transarterial chemoembolization with drug-eluting beads for hepatocellular carcinoma. J Clin Oncol 29: 3960-3967, 2011.
17. Cabrera R, Pannu DS, Caridi J, et al: The combination of sorafenib with transarterial chemoembolisation for hepatocellular carcinoma. Aliment Pharmacol Ther 34: 205-213, 2011.
18. Petrini I, Lencioni M, Ricasoli M, et al: Phase II trial of sorafenib in combination with 5-fluorouracil infusion in advanced hepatocellular carcinoma. Cancer Chemother Pharmacol 69: 773-780, 2012.
19. Mochizuki S and Okada Y: ADAMs in cancer cell proliferation and progression. Cancer Sci 98: 621-628, 2007.
20. Wang YY, Ye ZY, Li L, Zhao ZS, Shao QS and Tao HQ: ADAM 10 is associated with gastric cancer progression and prognosis of patients. J Surg Oncol 103: 116-123, 2011.
21. Lee SB, Schramme A, Doberstein K, et al: ADAM10 is upregulated in melanoma metastasis compared with primary melanoma. J Invest Dermatol 130: 763-773, 2010.
22. Zhang W, Liu S, Liu K, et al: A disintegrin and metalloprotease (ADAM)10 is highly expressed in hepatocellular carcinoma and is associated with tumour progression. J Int Med Res 42: 611-618, 2014.
23. Yue Y, Shao Y, Luo Q, Shi L and Wang Z: Downregulation of ADAM10 expression inhibits metastasis and invasiveness of human hepatocellular carcinoma HepG2 cells. Biomed Res Int 2013: 434561, 2013.
24. Yang CL, Jiang FQ, Xu F and Jiang GX: ADAM10 overexpression confers resistance to doxorubicin-induced apoptosis in hepatocellular carcinoma. Tumour Biol 33: 1535-1541, 2012.
25. Brummelkamp TR, Bernards R and Agami R: A system for stable expression of short interfering RNAs in mammalian cells. Science 296: 550-553, 2002.
26. Zhang H, Li Z and Wang K: Combining sorafenib with celecoxib synergistically inhibits tumor growth of non-small cell lung cancer cells in vitro and in vivo. Oncol Rep 31: 1954-1960, 2014.
27. Gagnon V, Van Themsche C, Turner S, Leblanc V and Asselin E: Akt and XIAP regulate the sensitivity of human uterine cancer cells to cisplatin, doxorubicin and taxol. Apoptosis 13: 259-271, 2008.
28. Chekenya M, Krakstad C, Svendsen A, et al: The progenitor cell marker NG2/MPG promotes chemoresistance by activation of integrin-dependent PI3K/Akt signaling. Oncogene 27: 5182-5194, 2008.
29. McDonald GT, Sullivan R, Paré GC and Graham CH: Inhibition of phosphatidylinositol 3-kinase promotes tumor cell resistance to chemotherapeutic agents via a mechanism involving delay in cell cycle progression. Exp Cell Res 316: 3197-3206, 2010.
30. Benson AB III, Abrams TA, Ben-Josef E, et al: NCCN clinical practice guidelines in oncology: hepatobiliary cancers. J Natl Compr Canc Netw 7: 350-391, 2009.
31. Klein T and Bischoff R: Active metalloproteases of the a disintegrin and metalloprotease (ADAM) family: biological function and structure. J Proteome Res 10: 17-33, 2011.
32. Seals DF and Courtneidge SA: The ADAMs family of metalloproteases: multidomain proteins with multiple functions. Genes Dev 17: 7-30, 2003.

33. Zhao H, Zhu J, Cui K, *et al*: Bioluminescence imaging reveals inhibition of tumor cell proliferation by Alzheimer's amyloid β protein. Cancer Cell Int 9: 15, 2009.
34. Endres K and Fahrenholz F: Upregulation of the α-secretase ADAM10 - risk or reason for hope? FEBS J 277: 1585-1596, 2010.
35. Lin T, Gu J, Zhang L, *et al*: Enhancing adenovirus-mediated gene transfer in vitro and in vivo by addition of protamine and hydrocortisone. J Gene Med 5: 868-875, 2003.
36. Emdad L, Lebedeva IV, Su ZZ, *et al*: Combinatorial treatment of non-small-cell lung cancers with gefitinib and Ad.*mda*-7 enhances apoptosis-induction and reverses resistance to a single therapy. J Cell Physiol 210: 549-559, 2007.
37. Bai S, Nasser MW, Wang B, *et al*: MicroRNA-122 inhibits tumorigenic properties of hepatocellular carcinoma cells and sensitizes these cells to sorafenib. J Biol Chem 284: 32015-32027, 2009.
38. McCubrey JA, Sokolosky ML, Lehmann BD, *et al*: Alteration of Akt activity increases chemotherapeutic drug and hormonal resistance in breast cancer yet confers an achilles heel by sensitization to targeted therapy. Adv Enzyme Regul 48: 113-135, 2008.
39. Kunter I, Erdal E, Nart D, *et al*: Active form of AKT controls cell proliferation and response to apoptosis in hepatocellular carcinoma. Oncol Rep 31: 573-580, 2014.
40. Chiablaem K, Lirdprapamongkol K, Keeratichamroen S, Surarit R and Svasti J: Curcumin suppresses vasculogenic mimicry capacity of hepatocellular carcinoma cells through STAT3 and PI3K/AKT inhibition. Anticancer Res 34: 1857-1864, 2014.
41. Xiu P, Xu Z, Liu F, *et al*: Downregulating sCLU enhances the sensitivity of hepatocellular carcinoma cells to gemcitabine by activating the intrinsic apoptosis pathway. Dig Dis Sci: Mar 27, 2014 (Epub ahead of print).
42. Edling CE, Selvaggi F, Ghonaim R, Maffucci T and Falasca M: Caffeine and the analog CGS 15943 inhibit cancer cell growth by targeting the phosphoinositide 3-kinase/Akt pathway. Cancer Biol Ther 15: 524-532 2014.