

# Combination treatment of gemcitabine and sorafenib exerts a synergistic inhibitory effect on non-small cell lung cancer *in vitro* and *in vivo* via the epithelial-to-mesenchymal transition process

SHANSHAN JIANG, RONG WANG, XUAN ZHANG, FEIHUA WU, SHENGNAN LI and YONGFANG YUAN

Department of Pharmacy, Shanghai Ninth People's Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200011, P.R. China

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**Abstract.** Standard chemotherapy is commonly used in clinical practice for the treatment of non-small cell lung cancer (NSCLC). However, its therapeutic efficacy remains low. Combination therapy for cancer treatment has attracted attention in recent years. The present study aimed to investigate the antitumor effect of the combination treatment with gemcitabine and sorafenib on NSCLC *in vitro* and *in vivo*, and to determine its underlying molecular mechanisms. The anti-NSCLC effects of combination therapy were analyzed by flow cytometry analysis, MTT, western blotting, reverse transcription-quantitative PCR, wound healing and Transwell invasion assays. A549 cells subjected to combination treatment with gemcitabine and sorafenib demonstrated a more irregular cellular morphology and lower cell viability compared with the monotherapy groups. Combination of gemcitabine and sorafenib significantly induced cell cycle arrest and apoptosis in A549 cells. Additionally, combination therapy was demonstrated to restrain the migration and invasion of tumor cells by suppressing epithelial-to-mesenchymal transition (EMT) of A549 cells. *In vivo* analyses confirmed that co-treatment with gemcitabine and sorafenib decreased NSCLC tumor growth and tumor weight in nude mice. Taken together, the results of the present study suggested that combination treatment with gemcitabine and sorafenib exerted a synergistic inhibitory effect on NSCLC *in vitro* and *in vivo* via the EMT process.

## Introduction

Lung cancer notably contributes to tumor-associated mortality worldwide which is accounting for 18.4% of all such deaths and responsible for more than 1.8 million deaths each year (1,2). Non-small cell lung cancer (NSCLC) accounts for ~85% of histological subtypes among all types of lung cancer (3). Chemotherapy is the most commonly used therapeutic regimen for the majority of patients with NSCLC, and significant progress has been achieved with targeted or immunotherapeutic agents for the treatment of NSCLC (4). However, the 5-year survival rate of patients with NSCLC still remains very low (5). The high mortality and low survival rate of patients with NSCLC are largely attributed to the capability of NSCLC cells to invade normal tissues, resulting in metastasis to remote sites (6). Thus, the development of novel strategies on anti-invasion and antimetastatic treatment for NSCLC are urgently required. The complex pathogenesis of NSCLC indicates that the combination of two or more antitumor agents possessing different molecular mechanisms may be a therapeutic option for efficient treatment (7).

Gemcitabine is a deoxycytidine analogue that has been approved as a first-line therapy drug for NSCLC for almost a decade (8). Gemcitabine has also been approved for the treatment of ovarian, advanced lung and pancreatic cancer due to its profound antitumor activity (9-11). However, drug resistance and the adverse side effects (such as anemia and leukopenia) associated with gemcitabine limit its chemotherapeutic efficacy (12). Previous studies have identified potential agents that may be used in combination with gemcitabine as effective chemotherapeutic regimens (13,14). For example, combination of gemcitabine and carboplatin has been verified as an effective adjuvant chemotherapeutic strategy for patients with completely resected NSCLC in a phase II study (15). In addition, gemcitabine in combination with nab-paclitaxel has been used in patients with metastatic breast cancer (14).

Sorafenib is a non-selective multi-kinase inhibitor that has the ability to suppress tumor cell proliferation and angiogenesis, primarily by restraining the activities of targets in tumor cells, such as the RAF/mitogen-activated protein kinase 1 (MEK)/ERK signaling pathway, vascular endothelial growth factor receptor-3 and platelet-derived growth factor

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*Correspondence to:* Dr Yongfang Yuan, Department of Pharmacy, Shanghai Ninth People's Hospital, Shanghai Jiaotong University School of Medicine, 639 Zhizaoju Road, Shanghai 200011, P.R. China  
E-mail: nmxyyf@126.com

*Abbreviations:* NSCLC, non-small cell lung cancer; EMT, epithelial-to-mesenchymal transition

*Key words:* gemcitabine, sorafenib, synergistic effect, EMT, NSCLC

receptor  $\beta$  (16). Sorafenib has also been reported to inhibit transforming growth factor- $\beta$ 1-induced epithelial-to-mesenchymal transition (EMT) in A549 cells (17). The antitumor effect of sorafenib in preclinical NSCLC models has been frequently reported (18,19). Sorafenib treatment is associated with adverse side effects, including hand-foot skin diseases, hypertension and diarrhea (20), which results in implementation of low doses, thus effecting efficacy. Thus, development of novel strategies are required in order to relieve the side effects or enhance the antitumor activity of sorafenib (21,22).

Combination treatment with various agents is a potential strategy to improve the synergistic therapeutic efficacy, as different agents exhibit their therapeutic effects via different molecular mechanisms, leading to a synergistic anticancer response (23). In addition, combination therapy has been demonstrated to overcome the harmful side effects associated with high-dose drugs (24). For example, a randomized phase II study revealed that combination treatment with gemcitabine and sorafenib is well tolerated and was demonstrated to be efficient in patients with advanced NSCLC compared with other combination treatments (25). However, the molecular mechanisms underlying the combination treatment with gemcitabine and sorafenib in NSCLC remain largely unknown. The present study aimed to investigate the effect of combination therapy with gemcitabine and sorafenib for NSCLC *in vitro* and *in vivo*.

## Materials and methods

**Materials and antibodies.** Sorafenib was purchased from Dalian Meilun Biotech Co., Ltd., and gemcitabine was purchased from Shanghai Yuanye Biotech Co., Ltd. MTT was supplied by Sigma-Aldrich; Merck KGaA. Primary antibodies against N-cadherin, E-cadherin and Twist-1 were purchased from Cell Signaling Technology, Inc., whereas the antibody against GAPDH and horseradish peroxidase-conjugated secondary antibodies against rabbit or mouse IgG were purchased from Weiao Biotechnology Co., Ltd.

**Cell culture.** A549, H1975 and H1650 cell lines were purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. Cells were maintained in RPMI-1640 medium (Corning Inc.) supplemented with 10% heat-inactivated FBS (Gibco; Thermo Fisher Scientific, Inc.), and 100  $\mu$ g/ml penicillin and 100 U/ml streptomycin (Beyotime Institute of Biotechnology) at 37°C with 5% CO<sub>2</sub>.

**MTT assay.** A549, H1975 and H1650 cells were seeded into 96-well plates at a density of 4x10<sup>3</sup> cells/well and treated with 0-100  $\mu$ g/ml gemcitabine or 0-50  $\mu$ M sorafenib at 37°C for 48 h. Subsequently, the cells were incubated with 0.5 mg/ml MTT at 37°C for 4 h to determine cell viability. Following the MTT incubation, the purple formazan crystals were dissolved in 150  $\mu$ l DMSO, and cell viability was subsequently analyzed using a microplate reader (BioTek Instruments, Inc.) at a wavelength of 570 nm.

**Morphological observation.** A549 cells were incubated with 10  $\mu$ g/ml gemcitabine or 10  $\mu$ M sorafenib at 37°C for 48 h. The morphology of the tumor cells was observed using an inverted microscope (Nikon, Japan). Magnification, x400.

**Flow cytometric analysis of the cell cycle.** Following treatment with 10  $\mu$ g/ml gemcitabine and 10  $\mu$ M sorafenib at 37°C for 48 h, A549 cells were collected and subsequently fixed with 70% ethanol at 4°C for 12 h. The samples were washed with pre-cooled PBS and incubated with 100  $\mu$ g/ml of RNaseA (Nanjing KeyGen Biotech Co., Ltd.) at 37°C for 30 min. The cells were subsequently stained with 50  $\mu$ g/ml of propidium iodide (Nanjing KeyGen Biotech Co., Ltd.) at 37°C for 15 min and cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences). The results were analyzed using Flow Jo software version 7.6.1 (TreeStar).

**Flow cytometric analysis of apoptosis.** Following treatment with 10  $\mu$ g/ml gemcitabine and 10  $\mu$ M sorafenib at 37°C for 48 h, A549 cells were collected using centrifugation at 800 x g at 4°C for 10 min. Then, cells were stained with annexin V-fluorescein isothiocyanate (FITC) and PI using the Annexin V-FITC Apoptosis Detection kit (Nanjing KeyGen Biotech Co., Ltd.). Briefly, the cells were incubated with Annexin V-FITC/PI at 37°C for 20 min, and apoptotic cells were subsequently analyzed using a FACSCalibur flow cytometer (BD Biosciences); Annexin V<sup>+</sup>/PI<sup>+</sup> and Annexin V<sup>+</sup>/PI<sup>-</sup> cells were considered to be apoptotic. A549 cells treated with 100 nM docetaxel at 37°C for 48 h were used as positive control. The data was analyzed using CellQuest software version 3.1 (BD Biosciences).

**Western blotting.** Total protein was extracted from A549 cells and tumor tissue homogenates using RIPA cell lysis buffer (Nanjing KeyGen Biotech Co., Ltd.), and quantified using the BCA Protein Quantitation kit (Nanjing KeyGen Biotech Co., Ltd.). Equal amounts of total protein (20  $\mu$ g) were separated by 12% SDS-PAGE and subsequently transferred to polyvinylidene difluoride membranes. Membrane blocking and incubation with the primary and secondary antibodies were performed as previously described (26). Protein bands were visualized using the Enhanced Chemiluminescent Detection kit (Pierce; Thermo Fisher Scientific, Inc.). The intensity of resulting bands were quantified using ImageJ version 1.47 (National Institutes of Health).

**Reverse-transcription quantitative (RT-qPCR).** Total RNA was extracted from A549 cells using TRIzol<sup>®</sup> reagent (Invitrogen; Thermo Fisher Scientific, Inc.), reverse transcribed into cDNA using the SuperScript First Strand Synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc.) at 42°C for 50 min according to the manufacturer's protocol. qPCR was detected using the BeyoFast<sup>™</sup> SYBR Green qPCR Mix (Beyotime Institute of Biotechnology; cat. no. D7260) on Applied Biosystems<sup>®</sup> 7500 Real-Time PCR Systems (Thermo Fisher Scientific, Inc.). The thermocycling conditions were used as follows: 95°C for 20 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. The following primers sequences were used for qPCR: E-cadherin forward, 5'-TGCGCGTGAAGTTT GCCAGT-3' and reverse, 5'-ACGTTGTCCCGGGTGTCA TCCT-3'; N-cadherin forward, 5'-CATCATCATCTGCT TATCCTTGT-3' and reverse, 5'-GGTCTTCTTCTCCTC CACCTTCT-3'; Twist-1 forward, 5'-GCATGCATTCTCAAG AGGT-3' and reverse, 5'-CAGTTTGATCCCAGCGTTTT-3'; and GAPDH forward, 5'-GGACCTGACCTGCCGTCTAG-3'

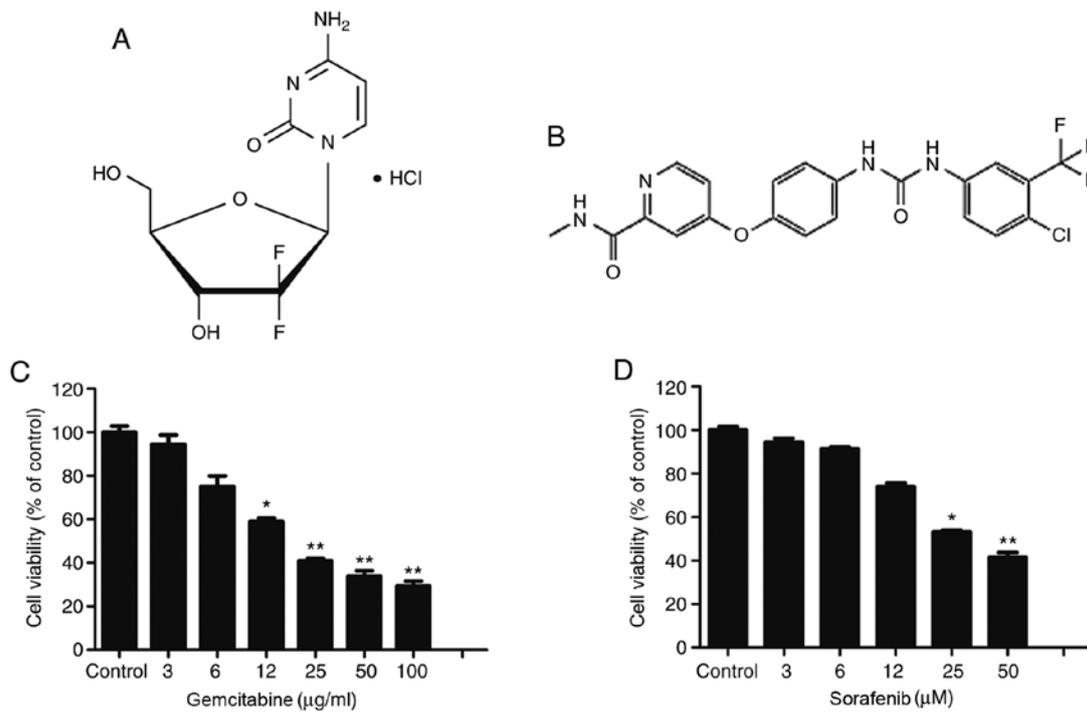


Figure 1. Dose-dependent cytotoxic effect of gemcitabine and sorafenib on A549 cells. (A and B) The molecular structure of (A) gemcitabine and (B) sorafenib. (C and D) A549 cells were treated with (C) 0-100 µg/ml of gemcitabine and (D) 0-100 µM of sorafenib for 48 h, and cell viability was determined by an MTT assay. \*P<0.05 vs. Control, \*\*P<0.01 vs. Control.

and reverse, 5'-GTAGCCAGGATGCCCTTGA-3'. Relative expression levels were quantified using the  $2^{-\Delta\Delta C_q}$  method (27) and normalized to the internal reference gene GAPDH.

**Wound healing assay.** Cell migration was assessed using the wound healing assay. Briefly, A549 cells were cultured with 1640 medium (containing 10% FBS) in a 12-well plates until they reached a confluent monolayer, and the cell monolayers were subsequently scratched using a 10 µl pipette tip. Culture medium was replaced with fresh culture medium (no FBS) containing 10 µg/ml gemcitabine or 10 µM sorafenib, followed by incubation for 0, 12, 24 and 48 h at 37°C. Migratory cells were counted in five randomly selected fields using an inverted light microscope at x200 magnification (Nikon).

**Invasion assay.** Chamber inserts (8-µm pore; Corning, Inc.) were precoated with 300 µg/ml Matrigel for 30 min at 37°C according to the manufacturer's instructions. A total of  $\sim 5 \times 10^3$  A549 cells were plated in the upper chambers of Transwell plates in RPMI-1640 supplemented with 10% FBS and incubated in the presence of 10 µg/ml gemcitabine or 10 µM sorafenib. A total of 500 µl RPMI-1640 medium supplemented with 20% FBS was placed in the lower chambers. Following incubation at 37°C for 48 h, invasive cells on the lower chambers were stained with 0.1% crystal violet at 37°C for 15 min. Stained cells were washed twice with PBS and counted in randomly selected 5 fields using an inverted microscope (Nikon), magnification, x400. Subsequently, crystal violet was dissolved using 33% acetic acid, and the invasive ability of the cells was measured at a wavelength of 590 nm using a microplate reader (BioTek Instruments, Inc.).

**Tumor xenograft experiments.** All experimental protocols involving animals were approved by the Institutional Animal Committee of Shanghai Ninth People's Hospital (Shanghai, China). A549 cells ( $1 \times 10^6$  cells/ml in 1 ml PBS) were subcutaneously injected into the right thigh of BALB/c nude mice (4-6 weeks old; 18-22 g, Charles River Laboratories, Inc.). The mice were housed at 24-26°C with 50-70% humidity and 12-h day/night cycle and provided with free access to food and water. The mice were randomized into four groups (6 mice/group) as follows: i) Vehicle (methylcellulose/Tween 80, intraperitoneal injection); ii) sorafenib (40 mg/kg/day, intraperitoneal injection); iii) gemcitabine (50 mg/kg/day, intraperitoneal injection); and iv) sorafenib (40 mg/kg/day, intraperitoneal injection) + gemcitabine (50 mg/kg/day, intraperitoneal injection). The body weights of the mice was measured twice/week, the tumor size was measured every other day using calipers, and tumor volumes were determined using the following formula:  $\text{Volume} = \text{length} \times (\text{width})^2 / 2$ . The mice were sacrificed before the end of the experiment if the maximum tumor diameter reached 2 cm, and all mice were sacrificed after 28 days using CO<sub>2</sub>.

**Statistical analysis.** Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, Inc.). Combination index (CI) of gemcitabine and sorafenib was calculated by CalcuSyn 2.0 software (Biosoft). CI values <1, =1 and >1 indicated synergism, additive effect and antagonism, respectively. Data are presented as the mean ± standard deviation. Differences between two groups were analyzed using unpaired Student's t-test (2-tailed), whereas one-way analysis of variance followed by Tukey's post hoc test was used to compare differences among multiple groups. P<0.05 was considered to indicate a statistically significant difference.

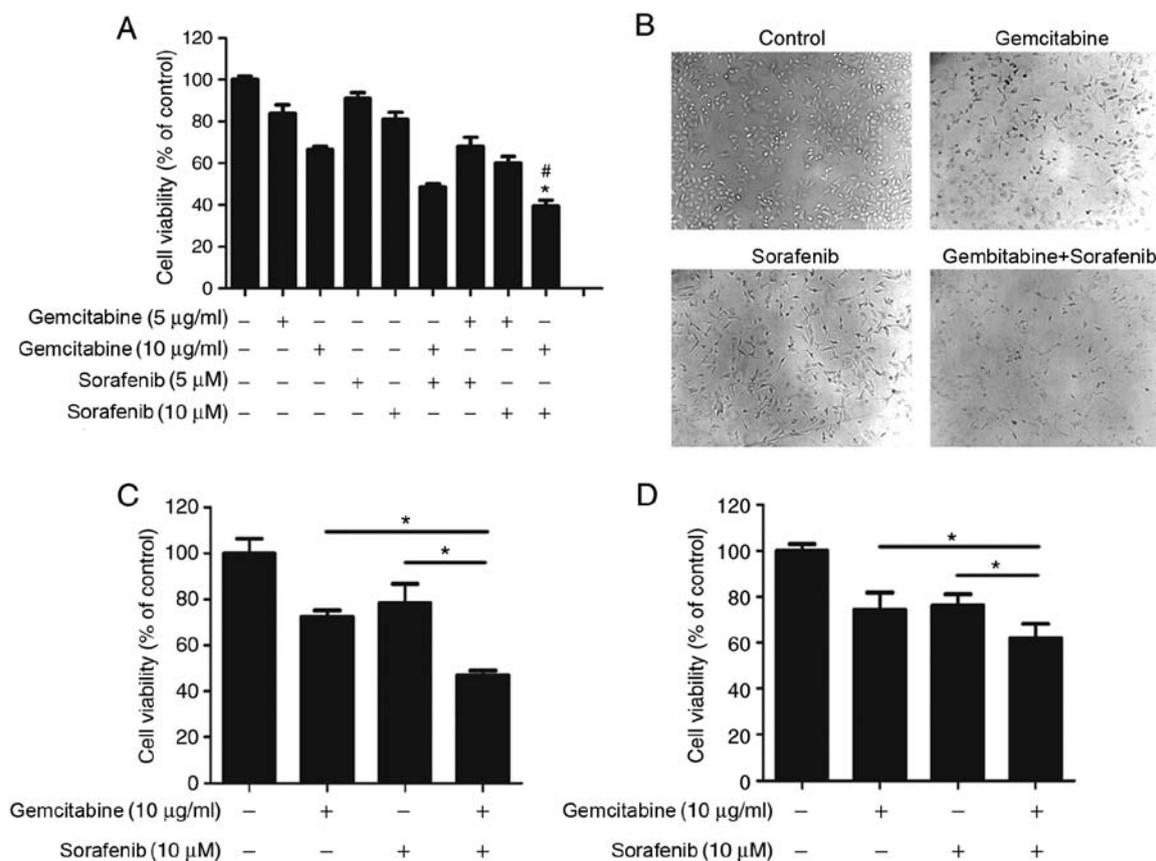


Figure 2. Synergistic antitumor effects of gemcitabine and sorafenib on A549 cells. (A) A549 cells were treated with 5 or 10  $\mu$ g/ml gemcitabine and 5 or 10  $\mu$ M sorafenib, either alone or in combination for 48 h, and cell viability was determined by an MTT assay. (B) A549 cells were treated with a combination of 10  $\mu$ g/ml gemcitabine and 10  $\mu$ M sorafenib for 48 h, and cell morphology was subsequently observed using an inverted microscope. (C) H1975 and (D) H1650 cells were treated with 10  $\mu$ g/ml gemcitabine and 10  $\mu$ M sorafenib, either alone or in combination for 48 h, and cell viability was determined by an MTT assay. \* $P$ <0.05 compared vs. gemcitabine group; # $P$ <0.05 compared vs. sorafenib group.

## Results

**Dose-dependent cytotoxicity of gemcitabine and sorafenib to A549 cells.** Fig. 1A and B illustrate the molecular structures of gemcitabine and sorafenib, respectively. Gemcitabine is a deoxycytidine analogue that has been approved as a first-line therapy drug for NSCLC. Conversely, sorafenib is a non-selective multi-kinase inhibitor, which has been approved for the treatment of hepatocellular carcinoma. The present study assessed the cytotoxic effects of gemcitabine and sorafenib as single agents on A549 cells using the MTT assay. The results demonstrated that treatment of A549 cells with varying concentrations of gemcitabine (0-100  $\mu$ g/ml) or sorafenib (0-100  $\mu$ M) for 48 h inhibited the viability of tumor cells in a dose-dependent manner ( $P$ <0.05), and the half maximal inhibitory concentration values of gemcitabine and sorafenib in A549 cells were 19.2  $\mu$ g/ml and 44.8  $\mu$ M, respectively (Fig. 1C and D).

**Synergistic antitumor effects of gemcitabine and sorafenib on A549 cells.** To determine whether a coadjutant antitumor effect existed between gemcitabine and sorafenib, A549 cells were treated with different concentrations of gemcitabine (5  $\mu$ g/ml or 10  $\mu$ g/ml) and sorafenib (5  $\mu$ M or 10  $\mu$ M) either individually or in combination. The results demonstrated

that combination treatment significantly inhibited A549 cell viability compared with gemcitabine or sorafenib treatment alone ( $P$ <0.05, Fig. 2A), and the CI value calculated by Calcsyn 2.0 software was 0.65 with 10  $\mu$ g/ml gemcitabine and 10  $\mu$ M sorafenib. Subsequently, cell morphology was assessed, which demonstrated regular spindle-shaped cells in the control group, whereas irregular shapes were observed in cells treated with gemcitabine or sorafenib alone. Cells in the combination treatment group displayed multiple changes compared with the control group, including decreased volume and irregular shapes (Fig. 2B). The effect of the combination treatment was also assessed in NSCLC H1650 and H1975 cell lines. The results demonstrated stronger cytotoxicity in H1650 and H1975 cells in the combination treatment group than sorafenib and gemcitabine treated groups ( $P$ <0.05, Fig. 2C and D). Overall, cell viability analysis indicated that gemcitabine and sorafenib exerted a synergistic effect on NSCLC cells.

**Combination of gemcitabine and sorafenib induces cell cycle arrest and apoptosis in A549 cells.** Previous studies have reported that both gemcitabine and sorafenib disturb the cell cycle of tumor cells (21,28). Thus, the present study investigated the combined effects of gemcitabine and sorafenib on the cell cycle. The results demonstrated that co-treatment induced a significant increase in the number

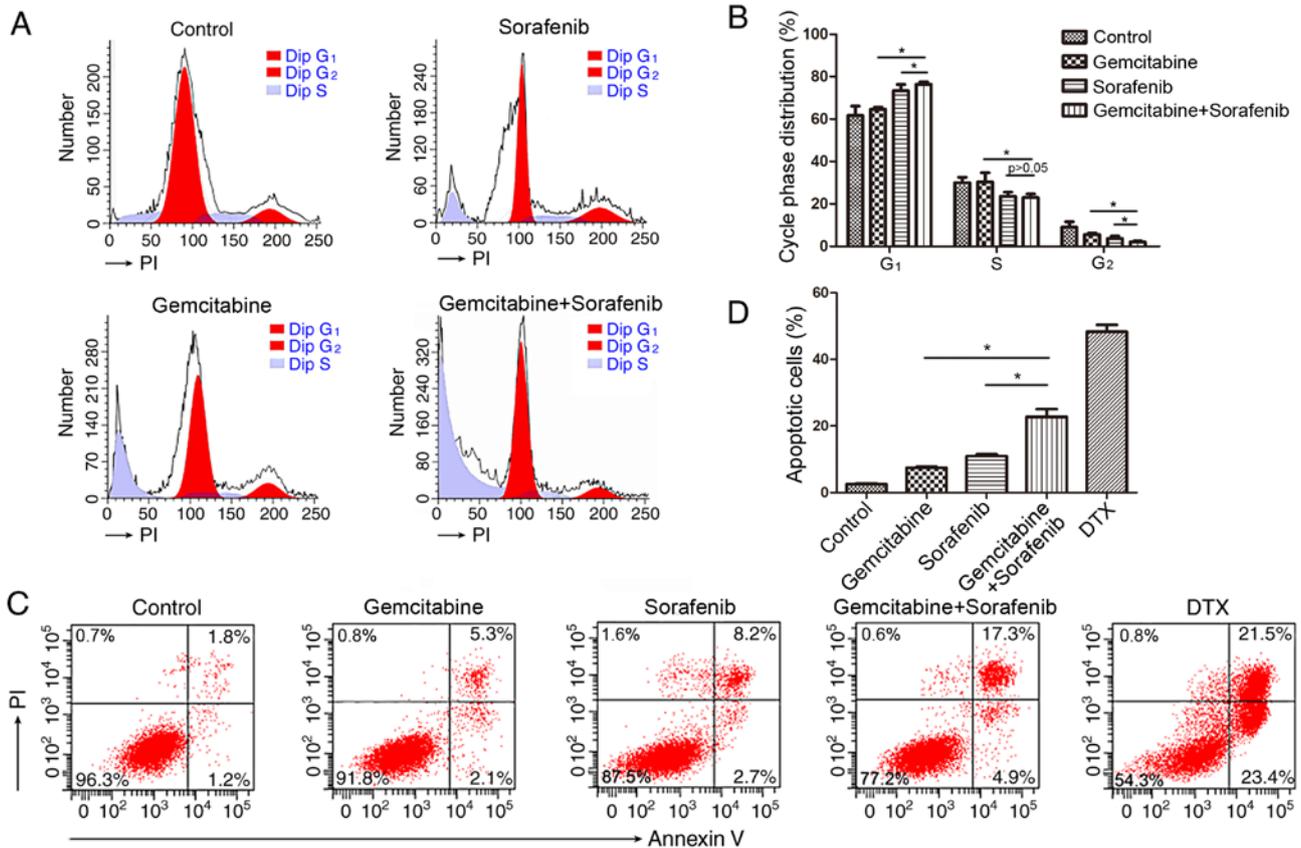


Figure 3. Co-treatment with gemcitabine and sorafenib induces apoptosis and cell cycle arrest in A549 cells. A549 cells were treated with 10  $\mu$ g/ml gemcitabine and 10  $\mu$ M sorafenib, either alone or in combination for 48 h. (A) Cell cycle distribution was determined by flow cytometry analysis. (B) The quantification of cells in different phases of the cell cycle is presented in bar charts. (C) The percentage of apoptotic cells was determined by flow cytometry analysis. PI, propidium iodide. (D) The percentage of A549 cells positive for Annexin V staining was presented in bar charts. \* $P < 0.05$ .

of cells in the G<sub>1</sub> phase, while decreasing the number of cells in the S phase compared with the control group, indicating that a G<sub>1</sub>-phase arrest was induced in A549 cells by the combination treatment ( $P < 0.05$ , Fig. 3A and B). Furthermore, a higher proportion of apoptotic cells (22.2%) was detected in the combination treatment group compared with gemcitabine (7.4%) or sorafenib (10.9%) monotherapy ( $P < 0.05$ , Fig. 3C-D).

*Effects of combination treatment on A549 cell migration.*

The wound healing assay was performed to detect the effects of combination treatment on the migratory ability of A549 cells. The results demonstrated that the scratch of cell monolayers in the negative control group was almost healed, and that of monotherapy groups was gradually closed following treatment for 48 h (Fig. 4A). However, the scratch of the combination treatment group still remained wide apart at 48 h ( $P < 0.05$ , Fig. 4B). Taken together, the results indicated that the migratory ability of A549 cells was notably inhibited following co-treatment with gemcitabine and sorafenib.

*Effects of combination treatment on the invasive ability of A549 cells.* The effect of combination treatment on cell invasion was subsequently investigated. The results demonstrated that the number of invasive A549 cells in the combination treatment

group was significantly decreased compared with the control and monotherapy groups ( $P < 0.05$ , Fig. 5A and B). These results suggested that combination treatment with gemcitabine and sorafenib may inhibit the invasion of A549 cells.

*Combination treatment suppresses the EMT of A549 cells.*

In order to further investigate the molecular mechanisms underlying the effects of combination treatment on the migration and invasion of A549 cells, expression levels of EMT-associated proteins were assessed by western blotting. The results demonstrated that co-treatment with gemcitabine and sorafenib upregulated the expression of the epithelial marker E-cadherin, whereas the expression levels of the mesenchymal markers N-cadherin and Twist-1 were downregulated compared with the basal level of the monotherapy and the negative control groups ( $P < 0.05$ , Fig. 6A-D). Furthermore, RT-qPCR analysis indicated that N-cadherin and Twist-1 mRNA expression levels were significantly lower in the combination treatment group compared with the control and monotherapy groups, whereas the expression of E-cadherin was increased ( $P < 0.05$ , Fig. 6E-G).

*Synergistic antitumor effects of combined treatment in NSCLC xenograft model.*

To further determine whether gemcitabine and sorafenib exerted synergistic anti-NSCLC effects *in vivo*, nude mice were subcutaneously injected with A549 cells to establish a subcutaneous xenotransplanted tumor model. The

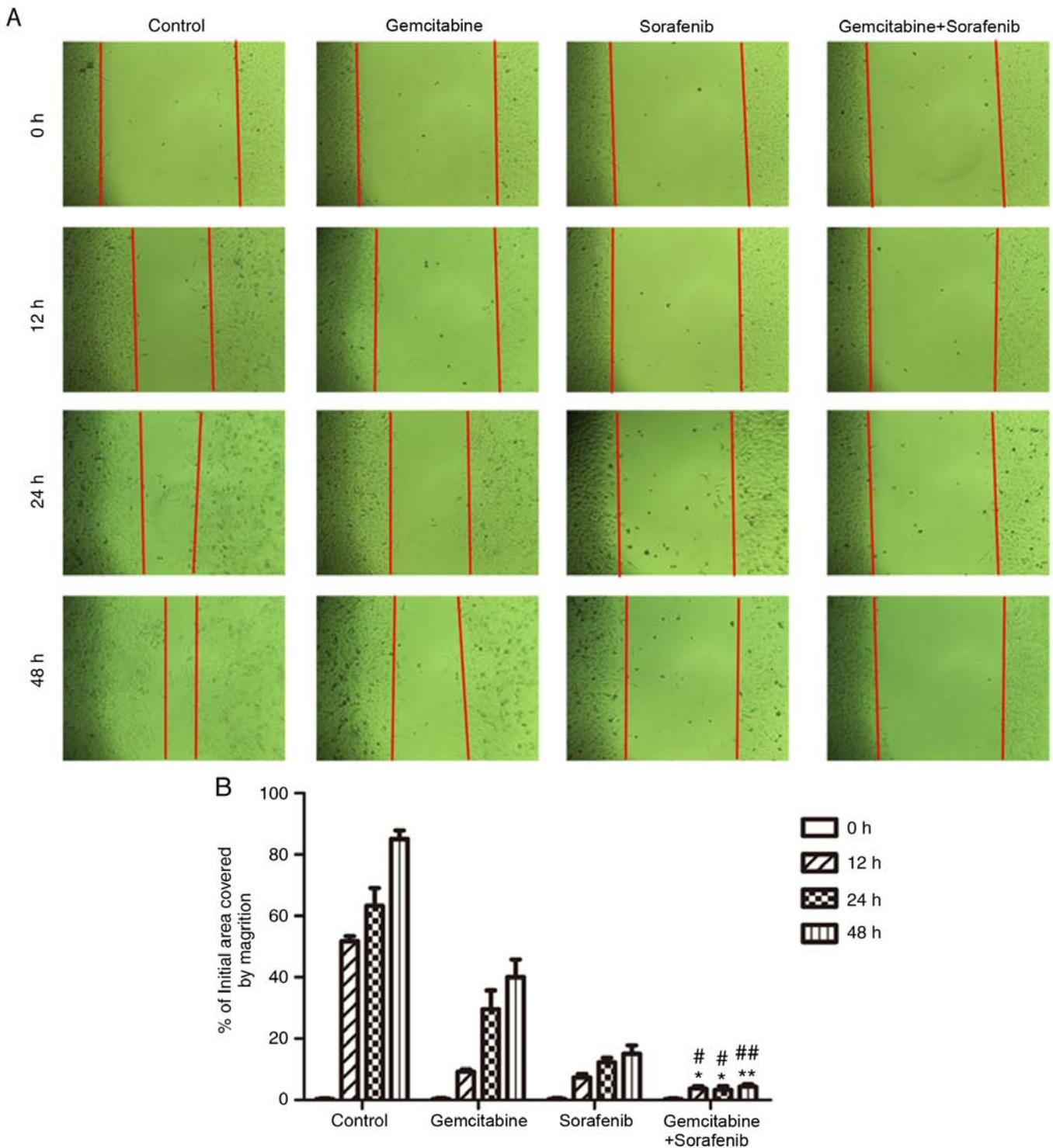


Figure 4. Effects of co-treatment with gemcitabine and sorafenib on migration of A549 cells. (A) A549 cells were treated with a combination of 10  $\mu\text{g}/\text{ml}$  gemcitabine and 10  $\mu\text{M}$  sorafenib for 48 h, and cell migration was determined by a wound healing assay using an inverted microscope (magnification,  $\times 200$ ). (B) Quantification of A549 migratory cells. \* $P < 0.05$  vs. gemcitabine group; \*\* $P < 0.01$  vs. gemcitabine group; # $P < 0.05$  vs. sorafenib group; ## $P < 0.01$  vs. sorafenib group.

results demonstrated that co-treatment with gemcitabine and sorafenib significantly inhibited tumor growth, as the average tumor volumes were decreased by 54.1 and 41.8% compared with gemcitabine or sorafenib alone, respectively ( $P < 0.05$ , Fig. 7A and B). Furthermore, the combination treatment group exhibited significantly decreased tumor weight compared with the monotherapy groups ( $P < 0.05$ , Fig. 7C), whereas no

significant differences were observed regarding the body weight ( $P > 0.05$ , Fig. 7D). The protein expression levels of N-cadherin and Twist-1 in the tumor tissues of the combination treatment group were significantly decreased in the co-treatment group compared with the monotherapy groups, whereas E-cadherin expression increased ( $P < 0.05$ , Fig. 7E-H). Taken together, these results suggested that combination

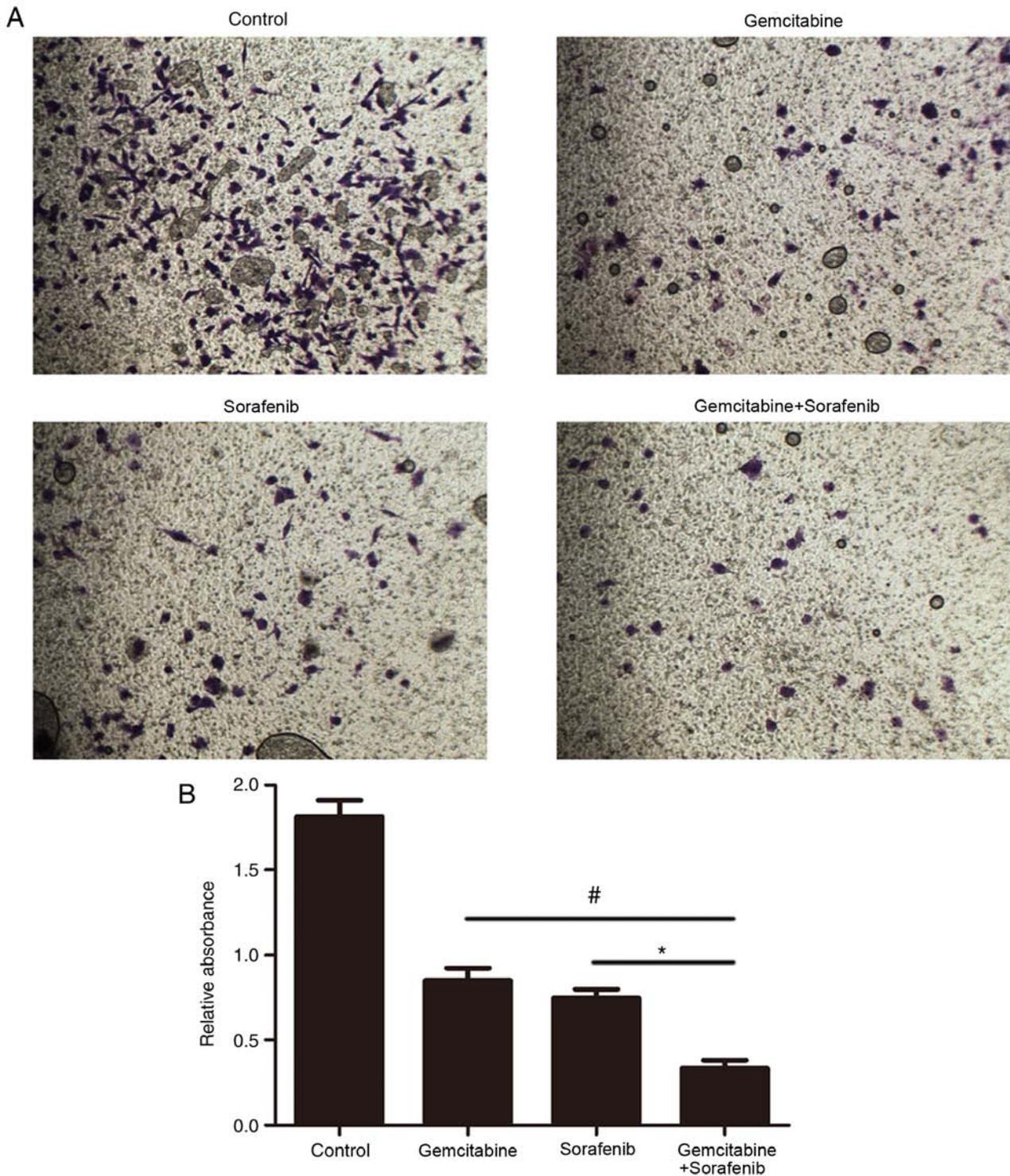


Figure 5. Effects of co-treatment with gemcitabine and sorafenib on invasion of A549 cells. (A) The result of cell invasion. (B) Quantification of A549 invasive cells. \*P<0.05, #P<0.05.

treatment with gemcitabine and sorafenib exerted antitumor effects via the EMT process *in vivo*.

**Discussion**

Invasion and metastasis greatly contribute to the poor prognosis of patients with NSCLC. The majority of cases of relapsed NSCLC can be attributed to intractable late-stage metastatic disease (29). Distant metastases are confirmed in

almost half of all clinically diagnosed NSCLC cases (30). Multilevel cross-reactions among the different targets in the metastatic progression of lung cancer cells have been identified, and suppression of one target allows others to act as immune escape molecular mechanisms for tumor cells (31). Combination treatment consisting of two or more anticancer agents is considered to be more effective in inhibiting tumor progression compared with single-targeted agents (32). A previous study has reported that combination treatment of

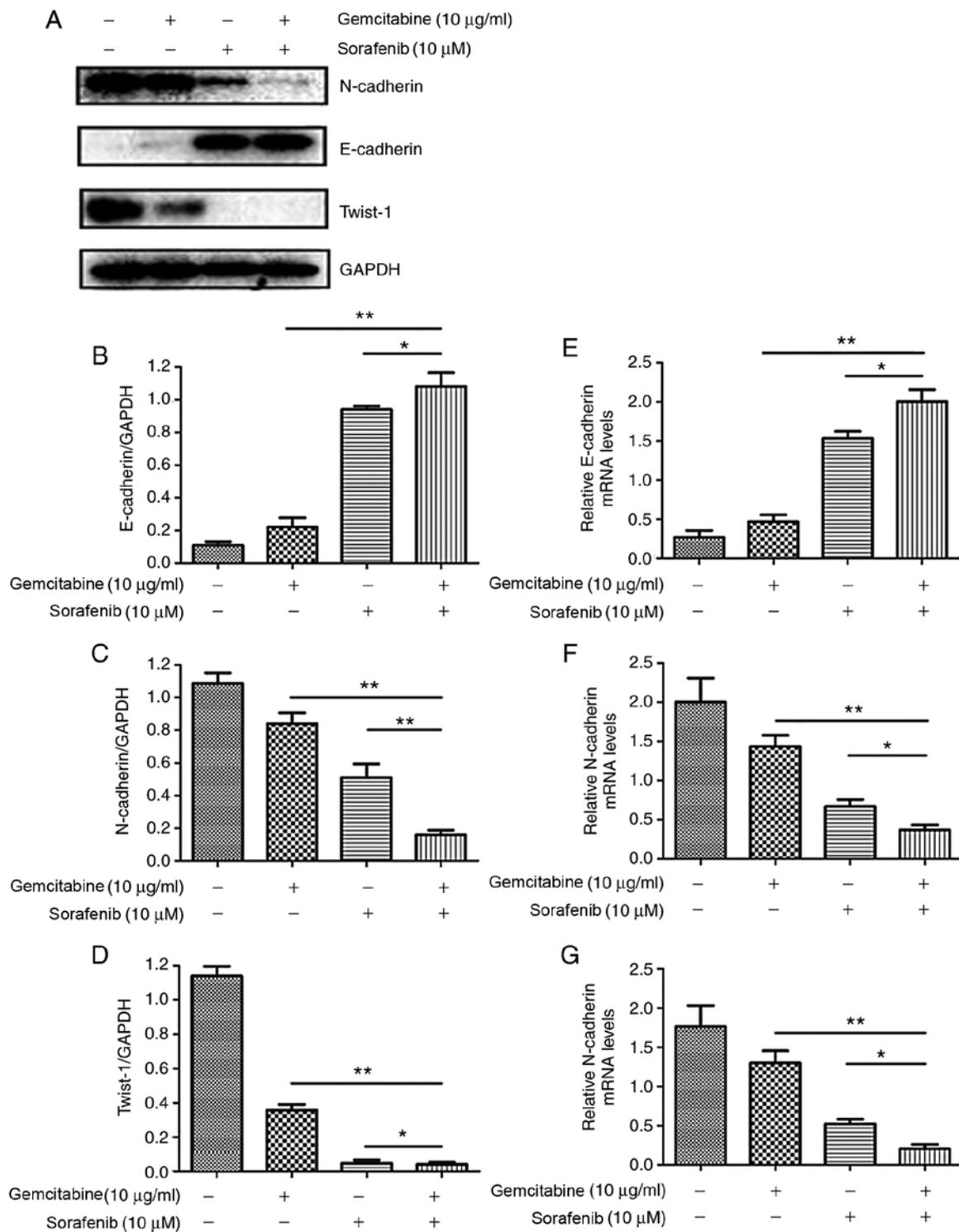


Figure 6. Combination treatment with gemcitabine and sorafenib induces EMT inhibition of A549 cells. A549 cells were treated with a combination of 10  $\mu$ g/ml gemcitabine and 10  $\mu$ M sorafenib for 48 h. (A) Protein expression levels of EMT-associated markers N-cadherin, E-cadherin and Twist-1 were assessed by western blot analysis. (B-D) Quantitative analysis of (B) E-cadherin, (C) N-cadherin and (D) Twist-1. (E-G) mRNA expression levels of (E) E-cadherin, (F) N-cadherin and (G) Twist-1. \* $P$ <0.05, \*\* $P$ <0.01. EMT, epithelial-to-mesenchymal transition.

C2-ceramide and sorafenib has a synergistic interaction in Bel-7402 cells via the PI3K/AKT/mTOR and ERK signaling pathways (31). Similarly, Li *et al* (33) have demonstrated that the combination of gemcitabine and sorafenib exhibits a synergistic effect in A549 cells by inhibiting epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitor (TKI)-sensitive and EGFR-TKI-resistant cells. In addition,

gemcitabine inhibits micrometastasis of NSCLC by targeting epithelial cellular adhesion molecule-positive circulating tumor cells via the HGF/cMET signaling pathway (34). Previous studies have reported that sorafenib suppresses cell migration and invasion by inhibiting the MET and MEK/ERK signaling pathways (35,36). Li *et al* (37) have confirmed that sorafenib and gemcitabine or pemetrexed have synergistic

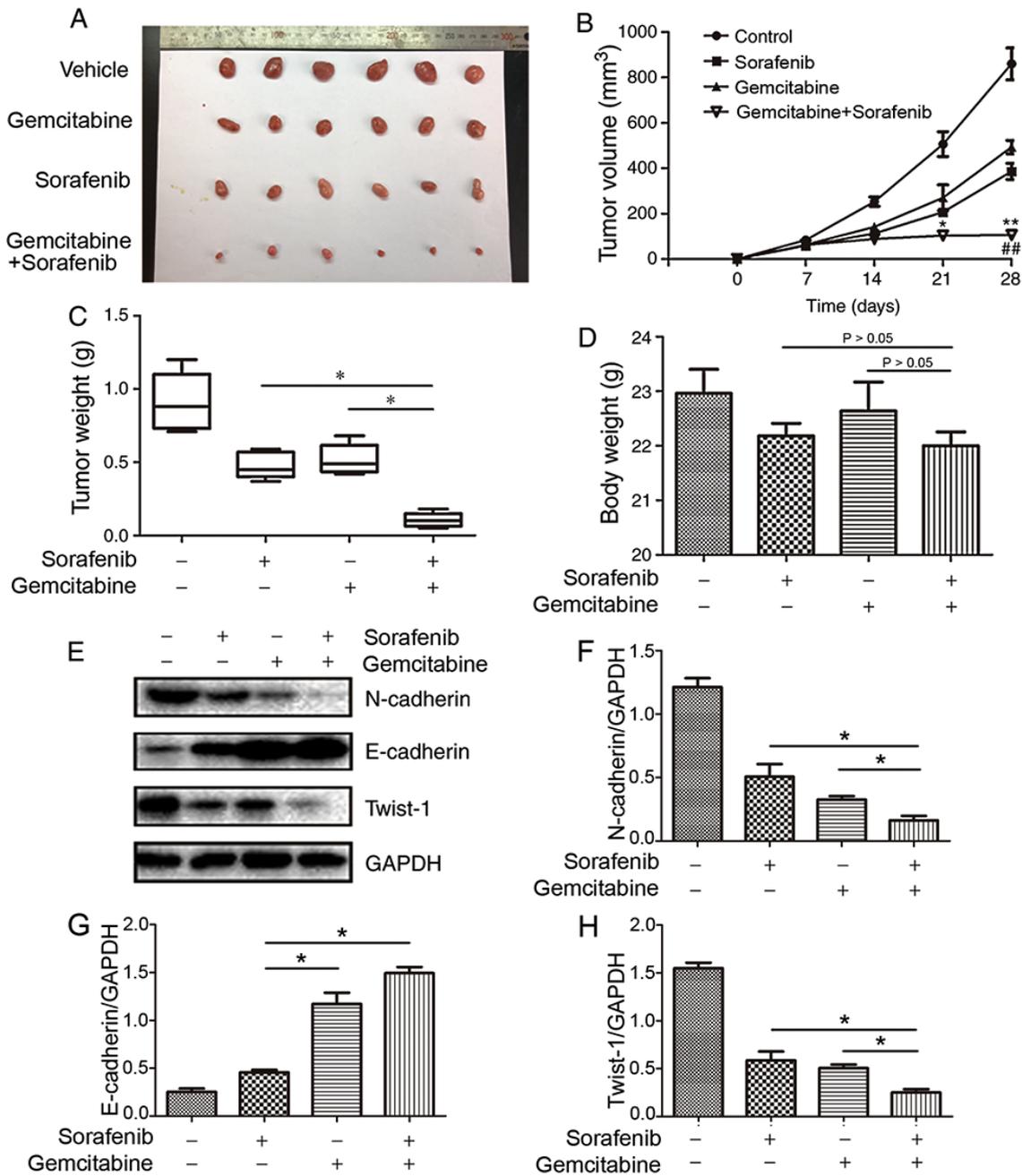


Figure 7. Synergistic antitumor effect of co-treatment with gemcitabine and sorafenib in non-small cell lung cancer xenograft model. (A) The images of tumor diameters of the different groups. (B) Tumor volume (\*P<0.05 compared vs. gemcitabine group; \*\*P<0.01 compared vs. gemcitabine group; ##P<0.01 compared vs. sorafenib group). (C) Tumor weight (\*P<0.05) and (D) body weight of the different groups (P>0.05). (E) Protein expression levels of N-cadherin, E-cadherin and Twist-1 in tumor tissues. (F-H) Quantitative analysis of (F) N-cadherin, (G) E-cadherin and (H) Twist-1. \*P<0.05.

effects by inducing apoptosis and cell cycle arrest, and inhibiting proliferation of lung cancer cells. However, the underlying molecular mechanisms of gemcitabine in combination with sorafenib on cell migration and invasion in NSCLC remain unclear. The present study investigated whether gemcitabine and sorafenib exerted a synergistic inhibitory effect on NSCLC *in vitro* and *in vivo*, and aimed to determine the potential molecular mechanisms underlying these interactions. The results demonstrated that co-treatment with gemcitabine and sorafenib markedly induced the cytotoxicity and cell cycle arrest in A549 cells. In addition, the combination treatment was demonstrated to significantly inhibit the invasion and

migration of NSCLC cells by Transwell invasion and wound healing assays. Taken together, the results of the present study suggested that combination treatment with gemcitabine and sorafenib inhibited the metastatic capability of NSCLC cells, indicating its potential as a novel treatment option for NSCLC.

The EMT process serves an important role in cancer metastasis and invasion (38). During cancer progression, cells lose the cell-matrix contact, cell-cell connections and normal epithelial polarity, while simultaneously adopting mesenchymal characteristics that allow migration into the surrounding matrices (39,40). During EMT, the expression levels of mesenchymal markers such as N-cadherin

are upregulated, whereas the epithelial cell markers such as E-cadherin are downregulated (41). Twist-1 is a transcriptional factor that belongs to the basic helix-loop-helix family; previous studies have reported that Twist-1 is an EMT inducer in the progression of NSCLC (42,43). In the present study, inhibition of EMT was observed *in vitro* and *in vivo* following combination treatment, as demonstrated by the notably down-regulated expression of N-cadherin and Twist-1, as well the markedly upregulated expression of E-cadherin, at both the mRNA and protein levels.

In conclusion, the results of the present study demonstrated that combination treatment with gemcitabine and sorafenib exerted antimetastatic and anti-invasive effects by inhibiting EMT in A549 cells. In addition, combination treatment demonstrated synergistic cytotoxicity to NSCLC cells *in vitro* and *in vivo*. Thus, combination treatment with gemcitabine and sorafenib may provide valuable insights into the development of synergetic anticancer agents.

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### Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

### Authors' contributions

SSJ and YFY conceived and designed the present study. SSJ, RW, FHW, XZ and SNL performed the experiments. SSJ wrote the paper. RW, XZ, SNL, FHW and YFY reviewed and edited the manuscript. All authors read and approved the final manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

### Ethics approval and consent to participate

All experimental protocols involving animals were approved by the Institutional Animal Committee of Shanghai Ninth People's Hospital (Shanghai, China) (approval no. SH9H-2019-A296-1).

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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