Gefitinib suppresses cervical cancer progression by inhibiting cell cycle progression and epithelial-mesenchymal transition

JIANYUN ZHENG[1], JIANXIN YU[2], MIN YANG[3] and LI TANG[3]

[1]Department of Pathology, The First Affiliated Hospital of Xi'an Medical University, School of General Medicine, Xi'an Medical University, Xi'an, Shaanxi 710077; [2]Department of Laboratory, Central Hospital of Shanxian County, Heze, Shandong 274399; [3]Department of Gynaecology and Obstetrics, Puyang Oil Field General Hospital, Puyang, Henan 457001, P.R. China

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Abstract. Cervical cancer (CC) is the second most common malignant cancer among women. Gefitinib was one of the first-generation epidermal growth factor receptor-tyrosine kinase inhibitors in clinical trials. However, the underlying mechanism of gefitinib in regulating CC progression remains unknown. In the current study, two CC cell lines, HeLa and Siha, were used to investigate the effects of gefitinib. Cell counting kit-8 assays demonstrated that treatment with gefitinib exerted strong cytotoxicity in HeLa and Siha cells. Flow cytometry was used to examine cell cycle progression and apoptosis. Treatment with gefitinib enhanced the number of cells in the G_0/G_1 phase and increased apoptosis in HeLa and Siha cells. Furthermore, treatment with gefitinib decreased the protein expression level of Bcl-2 and increased the protein expression level of Bax. Taken together, these results suggest that gefitinib may suppress CC cell proliferation and induce cell cycle arrest and apoptosis. The current study also demonstrated that treatment with gefitinib suppressed epithelial-mesenchymal transition (EMT) as the expression level of the epithelial marker, E-cadherin was increased, while the expression level of the mesenchymal marker, vimentin was decreased. The current study demonstrated that treatment with gefitinib decreased the protein expression levels of phosphorylated-GSK3β and β-catenin, which suggests that gefitinib may be a potential novel therapeutic strategy in CC by suppressing the Wnt/β-catenin signaling pathway and EMT to inhibit tumor metastasis in CC cells. In conclusion, gefitinib may suppress the EMT process during cell invasion and induce cell apoptosis and cell cycle arrest via inhibition of the Wnt/β-catenin signaling pathway.

Correspondence to: Dr Min Yang or Dr Li Tang, Department of Gynaecology and Obstetrics, Puyang Oil Field General Hospital, 341 Daqing Road, Puyang, Henan 457001, P.R. China
E-mail: zhongnanshan2018@yeah.net
E-mail: smd167725@yeah.net

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Introduction

Cervical cancer (CC) is the second most common malignant cancer among women (1). CC is characterized by high incidence and recurrence rates, as well as high resistance to systemic therapies (2). Therefore, prognosis for patients with CC remains relatively poor. Increasing evidence has demonstrated that the human papillomavirus (HPV) is correlated with the development of a high-grade precursor lesions and invasion in CC (3). HPV can infect epithelial cells, which remain active in cell-cycle progression and no longer undergo apoptosis (4).

Studies have demonstrated that the Wnt signaling pathway serves a key role in cell differentiation, proliferation, migration and polarity (5,6). In addition, the Wnt signaling pathway serves a key role in maintaining protein stability, subcellular localization and transcriptional activity (7). In the progression of tumors, the Wnt/β-catenin signaling pathway is an evolutionarily conserved and versatile pathway (8). Aberrant activation of the Wnt/β-catenin pathway can lead to abnormal accumulation of β-catenin in the nucleus, which accelerates the epithelial-mesenchymal transition (EMT) process (9,10). It is therefore important to maintain appropriate Wnt signaling.

Gefitinib was one of the first-generation epidermal growth factor receptor-tyrosine kinase inhibitors in clinical trials, and is now widely used for the treatment of several types of cancer (11,12). An increasing number of studies have been designed to identify the efficacy and toxicity of gefitinib (13,14). However, the underlying mechanism of gefitinib in regulating CC progression remains unknown. Therefore, the aim of the current study was to investigate the specific role and underlying mechanism of gefitinib in CC using human CC cell lines.

Materials and methods

Cell culture. Human cervical cancer cell lines HeLa and Siha were obtained from the Institute of Life Sciences Cell Resource Center (Shanghai, China). HeLa and Siha cells were cultured in

minimal essential medium (MEM, HyClone; GE Healthcare Life Sciences) supplemented with 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences), penicillin (100 U/ml)-streptomycin (100 U/ml) liquid (Thermo Fisher Scientific, Inc.) and 0.25 μg/ml amphotericin B (Ameresco, Inc.). Cells were cultured at 37˚C in a humidified incubator containing 5% CO_2.

Cell proliferation assay. HeLa and Siha cell proliferation was examined using the cell counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.), according to the manufacturer's protocol. Exponentially growing cells were seeded into 96-well plates at a density of 5×10^3 cells/well in a final volume of 100 μl MEM and cultured under normal conditions for 24 h at 37˚C in a 5% CO_2-humidified incubator. Subsequently, different concentrations (0.3125, 0.625, 1.25, 2.5, 5, 10, 20, 40 and 80 μmol/l) of gefitinib (cat. no. SML1657; Sigma-Aldrich; Merck KGaA; Darmstadt, Germany) were added to each well, with DMSO used as the vehicle control. After 48 h, 10 μl CCK-8 reagent was added to each well and incubated for 1 h at 37˚C. Cell proliferation was calculated by measuring the absorbance at a wavelength of 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.). Growth inhibition was calculated as a percentage of the untreated controls. All experiments were performed in triplicate and the data were expressed as the mean value ± standard deviation of five wells per treatment. For each cell line, the half maximal inhibitory concentration (IC_{50}) was determined using the four-parameter logistic model.

Cell cycle analysis. HeLa and Siha cells were seeded into six-well plates at the density of 10^6 cells/well. Following treatment with 10 μmol/l of gefitinib for 48 h, cells were harvested using trypsin without EDTA, washed three times with ice-cold PBS and fixed with 70% ethanol overnight at 4˚C. Cells were subsequently stained 25 μl propidium iodide with 10 μl RNase A at 37˚C for 30 min in dark using the Cell cycle and apoptosis analysis kit (cat. no. C1052; Beyotime Institute of Biotechnology, Haimen, China). Cell cycle analysis was performed using a BD FACSCalibur system and CellQuest pro software (version 2.0; BD Biosciences, Franklin Lakes, NJ, USA). All experiments were performed in triplicate.

Flow cytometry evaluation of apoptosis. Cell apoptosis was examined using the Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) Apoptosis Detection kit (BD Biosciences). Following treatment with 10 μmol/l of gefitinib for 48 h, cells were harvested using trypsin without EDTA and washed three times with ice-cold PBS. Cells were subsequently suspended at 1×10^4 cells/ml in Annexin V-Binding buffer and incubated with 5 μl Annexin V-FITC for 15 min at 37˚C in dark, followed by staining with 5 μl PI. Apoptotic cells were immediately analyzed using a BD FACSCalibur system and CellQuest pro software. All experiments were performed in triplicate.

Dual-luciferase reporter assay. HeLa and Siha cells were seeded into 24-well plates at a density of 1×10^5 cells/well and incubated overnight. After 24 h of culture, cells were co-transfected with 200 ng pTOP-Flash (Promega Corporation, Madison, WI, USA) or pFOP-Flash reporter plasmids (Promega Corporation) and 200 ng β-galactosidase (β-gal) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) to monitor for transfection efficiency. After transfection for 48 h, the activity was measured. The TCF-responsive TOP-Flash reporter contains three TCF binding sites, and the corresponding FOP-Flash contains three mutated TCF sites (15). Cells were treated with 10 μmol/l gefitinib for 4 h at 37˚C and luciferase activity was analyzed using a Dual-Luciferase Reporter assay system (Promega Corporation), according to the manufacturer's protocol. Luciferase activity was normalized for β-gal activity. The experiment was performed in triplicate.

Western blot analysis. Total cellular protein was extracted from cells on ice for 15 min using radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology) supplemented with fresh proteinase inhibitor cocktail and phosphatase inhibitor (Sigma-Aldrich; Merck KGaA). Samples were centrifuged at 11,000 x g for 20 min at 4˚C. Total protein was quantified using a bicinchoninic acid assay (Sigma-Aldrich; Merck KGaA) and 20 μg protein/well was separated via SDS-PAGE on a 10% gel. The fractionated proteins were transferred onto polyvinylidene difluoride membranes (EMD Millipore) and blocked for 1 h at room temperature with 5% non-fat skimmed milk. The membranes were incubated with primary antibodies, including E-cadherin (1:1,000; cat. no. ab1416), Vimentin (1:1,000; cat. no. ab8978), GSK3β (1:1,000; cat. no. ab93926), p-GSK3β (1:1,000; cat. no. ab131097), β-catenin (1:1,000; cat. no. ab32572) and GAPDH (1:5,000; cat. no. ab181602) overnight at 4˚C (all from Abcam, Cambridge, UK). Following primary antibody incubation, membranes were incubated with horseradish peroxidase-conjugated secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5,000; cat. no. ZB-2306; OriGne Technologies, Inc.), for 1 h at room temperature. Protein bands were visualized using a Western Lightning® Chemiluminescence Reagent Plus according to the manufacturer's protocol (cat. no. NEL105001EA; PerkinElmer, Inc., Waltham, MA, USA). Protein expression was quantified using ImageJ (version 1.8.0; National Institutes of Health, Bethesda, MD, USA).

Immunofluorescence. HeLa and Siha cells were cultured in a six-well plate with glass coverslips and following treatment with 10 μM gefitinib at 37˚C for 48 h, cells were fixed with 4% paraformaldehyde for 30 min at room temperature. Cells were washed three times with PBS for 5 min and blocked for 2 h at room temperature with 8% bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA). Subsequently, cells were incubated with primary antibodies against E-cadherin (1:50; cat. no. ab1416) and vimentin (1:50; cat. no. ab92547; both Abcam) in a humidified chamber overnight at 4˚C. Cells were washed three times with PBS. Following primary incubation, cells were incubated with tetramethylrhodamine-conjugated anti-rabbit IgG (1:500; cat. no. ZDR5209; OriGene Technologies, Inc.) at room temperature for 30 min. Cell nuclei were counterstained with DAPI (1:1,000; cat. no. C0060; Beijing Solarbio Science & Technology Co., Ltd.) for 20 min at room temperature. Cells were subsequently washed three times with PBS in the dark and the coverslips were mounted with mounting medium at room temperature for ~1 h in the dark. Fluorescence intensity was observed under

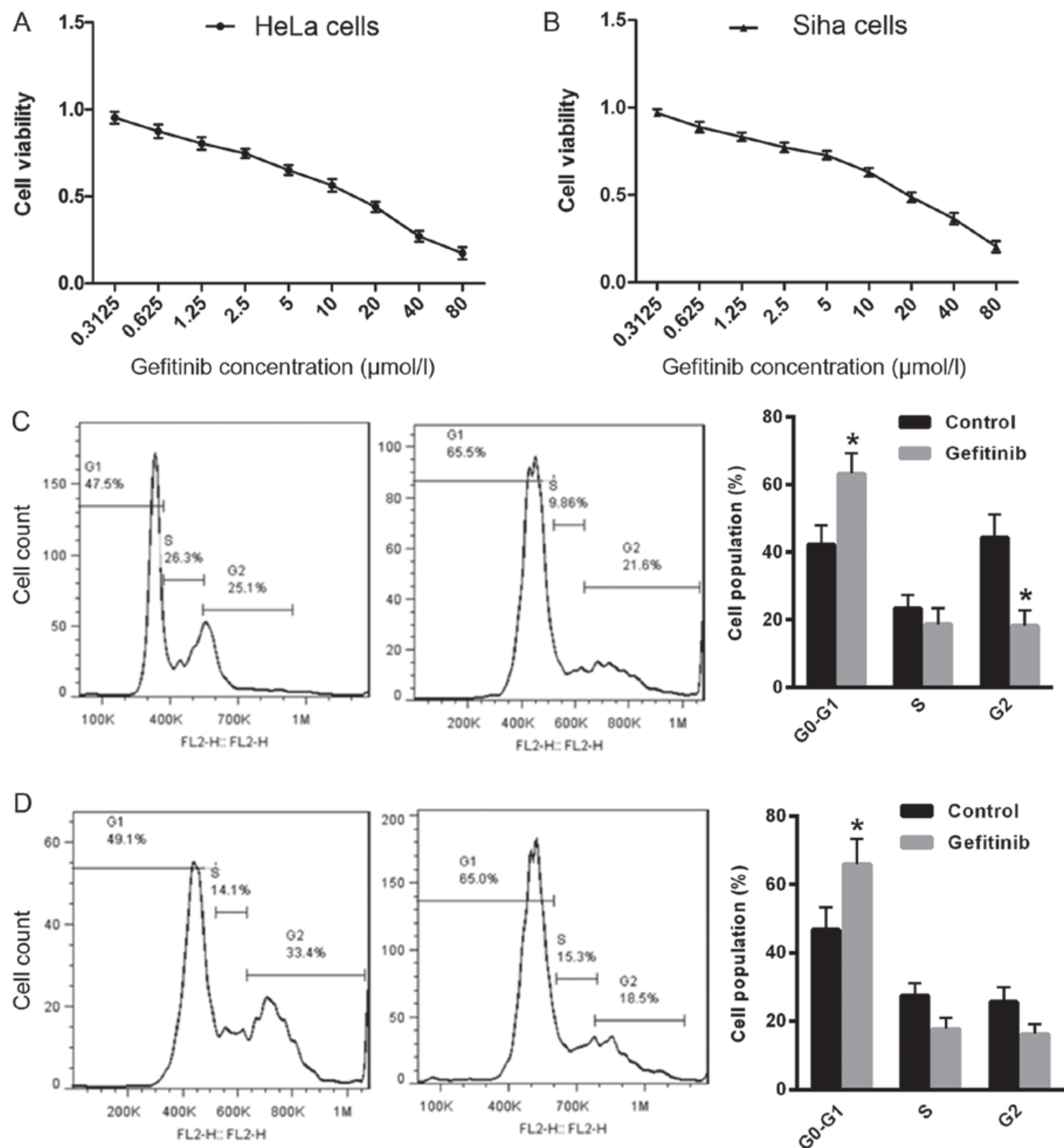

Figure 1. Gefitinib reduces cell proliferation and induces cell cycle arrest in HeLa and Siha cells. Cell proliferation was examined by CCK-8 assay in (A) HeLa and (B) Siha cells following treatment with gefitinib. Cell cycle analysis was determined by flow cytometry in (C) HeLa and (D) Siha cells following treatment with gefitinib. *P<0.05 vs. control. CC, cervical cancer; CCK-8, cell counting kit-8.

a fluorescence microscope (magnification, x40; XDS-500D; Shanghai Caikon Optical Instrument Co., Ltd.).

Statistical analysis. Data were presented as the mean ± standard error of the mean of at least three experiments. All statistical analyses were performed using GraphPad Prism software (version 5.0; GraphPad Software, Inc.). All experimental data were analyzed using the unpaired Student's t-test or one-way analysis of variance followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Gefitinib reduces proliferation and induces cell cycle arrest in CC cells. To determine the effect of gefitinib on CC cell growth, cell proliferation was examined in HeLa and Siha cells following treatment with gefitinib. Treatment with gefitinib exhibited strong cytotoxicity in HeLa cells [IC_{50}, 16.19±0.26 μmol/l; 95% confidence interval (CI): 1.077-1.341; Fig. 1A] and Siha cells (IC_{50}, 11.87±0.21 μmol/l, 95% CI: 1.003-1.146; Fig. 1B) compared with the control. Furthermore, the effect of gefitinib on cell cycle distribution was examined in HeLa and Siha cells. The number of cells in the G_0/G_1 phase was significantly increased in Hela and Siha cells following treatment with gefitinib compared with the control (Fig. 1C and D), which suggests that gefitinib may induce CC cell cycle arrest in the G_0/G_1 phase.

Gefitinib induces apoptosis in CC cells. To examine the effect of gefitinib on CC cell apoptosis, apoptosis was examined

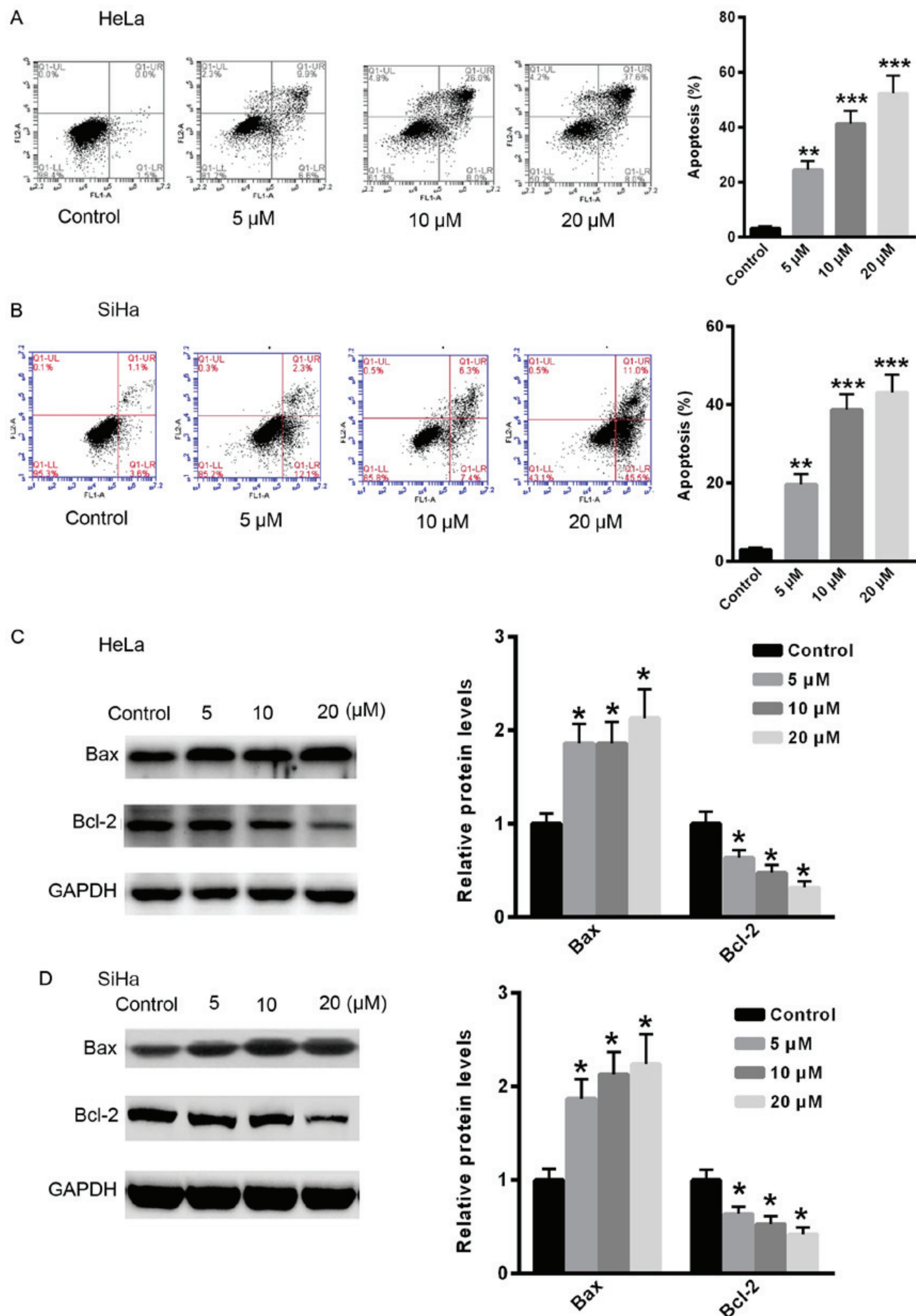

Figure 2. Gefitinib induces apoptosis in HeLa and Siha cells. Cell apoptosis was examined by flow cytometry in (A) HeLa and (B) Siha cells following treatment with gefitinib. The relative protein expression levels of Bcl-2 and Bax were determined by western blot analysis in (C) HeLa and (D) Siha cells following treatment with gefitinib. *P<0.05, **P<0.01 and ***P<0.001 vs. control. Con, control.

in HeLa and Siha cells following treatment with gefitinib. Treatment with gefitinib significantly induced apoptosis in CC cells in a dose-dependent manner compared with the control (Fig. 2A and B). Furthermore, the relative protein expression levels of apoptosis-related proteins, Bax and Bcl-2, were examined. Following treatment with gefitinib, the relative protein expression level of Bcl-2 was significantly reduced, whereas the protein expression level of Bax was significantly increased in HeLa and Siha cells compared with the control (Fig. 2C and D).

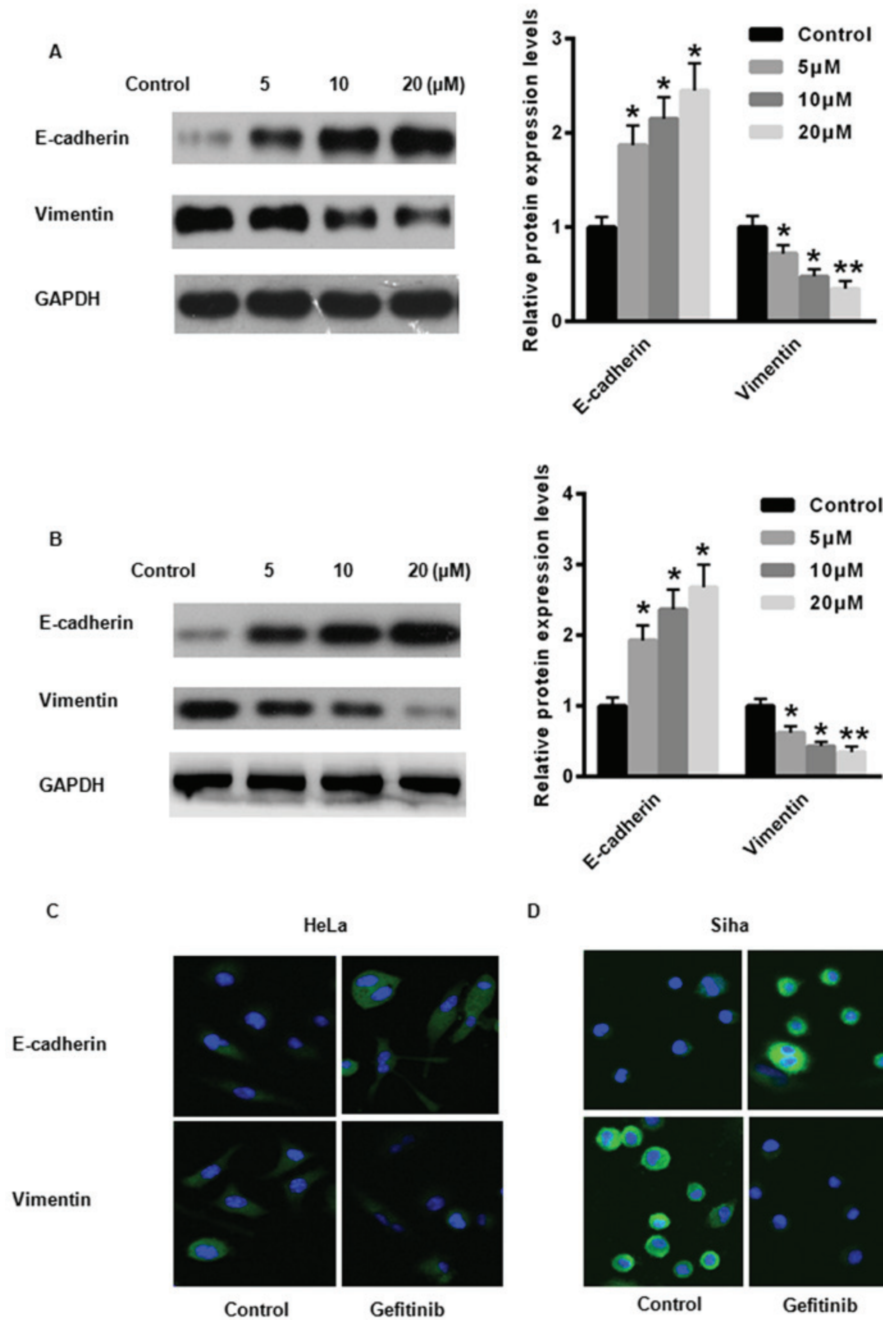

Figure 3. Gefitinib suppresses EMT in HeLa and Siha cells. The relative protein expression levels of E-cadherin and vimentin were determined by western blot analysis in (A) HeLa and (B) Siha cells following treatment with gefitinib. E-cadherin and vimentin immunostaining in (C) HeLa and (D) Siha cells following treatment with 20 μM gefitinib (magnification, x40). *P<0.05 and **P<0.01 vs. control. EMT, epithelial-mesenchymal transition.

Gefitinib suppresses EMT in CC cells. EMT is a key regulator of CC progression (16). Therefore, the expression levels of E-cadherin, an epithelial cell marker, and vimentin, a mesenchymal cell marker were examined in HeLa and Siha cells following treatment with gefitinib. Following treatment with gefitinib, the protein expression level of E-cadherin was significantly increased, whereas the protein expression level of vimentin was significantly reduced in HeLa and Siha cells compared with the control (Fig. 3A and B). Immunofluorescence demonstrated increased E-cadherin expression and reduced vimentin expression in HeLa and Siha cells following treatment with gefitinib compared with the control (Fig. 3C and D. These results suggest that gefitinib may suppress the EMT process in CC cells.

Gefitinib suppresses EMT via the Wnt/β-catenin signaling pathway in CC cells. The Wnt/β-catenin signaling pathway is a major contributor to CC tumorigenesis (17). GSK3β is a known negative regulator of β-catenin. Therefore, the phosphorylation of GSK3β at Ser9, an indicator of the activation state of GSK3β, was examined by western blot analysis in HeLa and Siha cells following treatment with gefitinib. The protein expression levels of p-GSK3β and β-catenin and were significantly reduced in HeLa and Siha cells following treatment with gefitinib compared with the control (Fig. 4A and B). Furthermore, luciferase reporter assays demonstrated that activation of the Wnt/β-catenin pathway was significantly suppressed following treatment with gefitinib in HeLa and

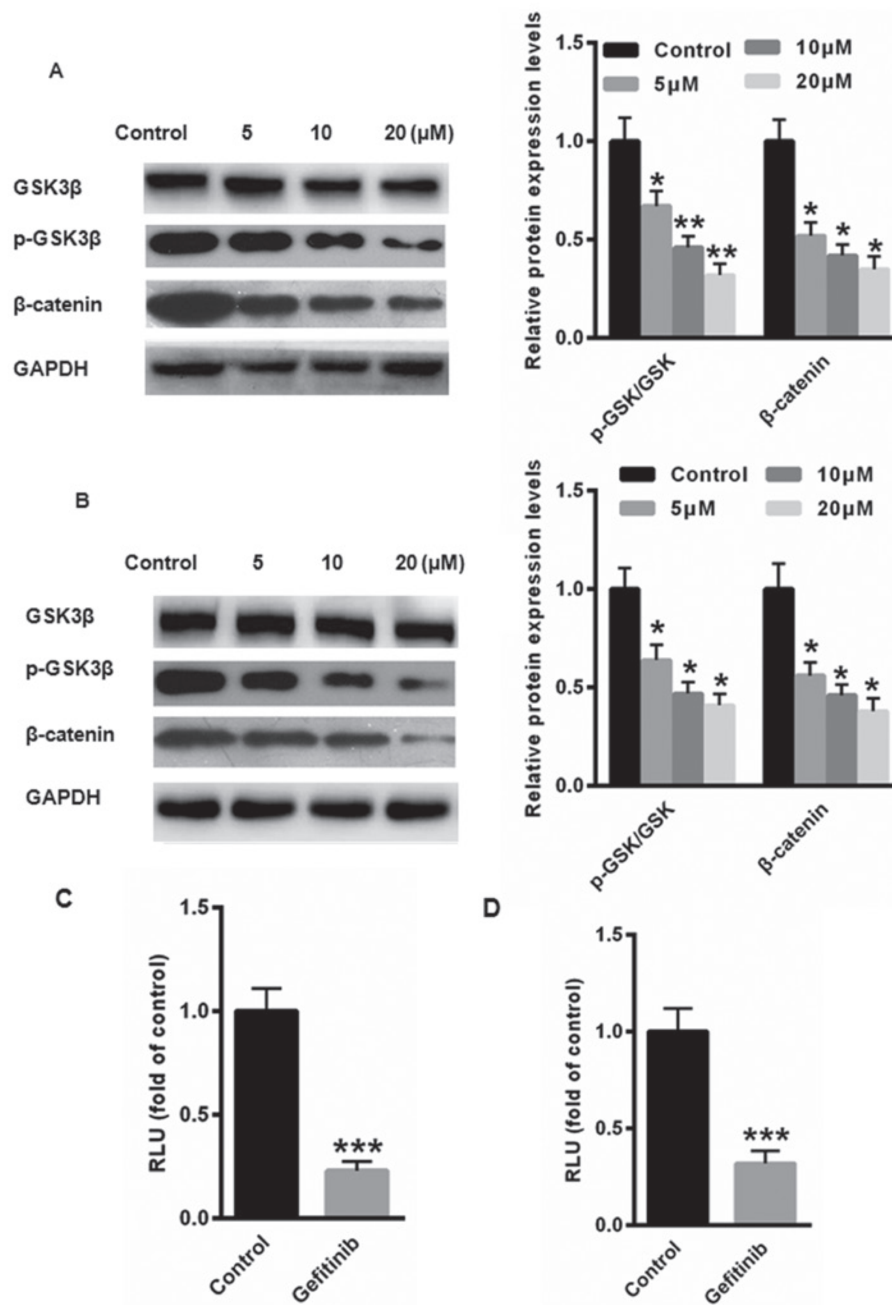

Figure 4. Gefitinib suppresses EMT via the Wnt/β-catenin signaling pathway in HeLa and Siha cells. The relative protein expression levels of GSK3β, p-GSK3β and β-catenin were determined by western blot analysis in (A) HeLa and (B) Siha cells following treatment with gefitinib. The activation of the Wnt/β-catenin pathway was determined by the luciferase reporter assay in (C) HeLa and (D) Siha cells following treatment with gefitinib. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ vs. control. EMT, epithelial-mesenchymal transition; p-, phosphorylated.

Siha cells compared with the control (Fig. 4C and D). Taken together, these results suggest that gefitinib may inactivate the Wnt/β-catenin signaling pathway to inhibit EMT in CC cells.

Discussion

In recent years, significant advances have been made in the diagnosis and treatment of CC, however, the overall 5-year survival rate remains poor (18). Currently, the most common treatment strategy for CC includes surgery with platinum-based chemotherapy (19). Unfortunately, patients with advanced CC relapse after primary treatment, and the majority of patients succumb to recurrence and metastasis (20). It is therefore necessary to identify novel therapeutic strategies for CC.

The *in vitro* and *in vivo* antitumor activity of gefitinib has been reported in several types of human cancer, including head and neck, colorectal, breast and lung cancer (21-23). However, the effect of gefitinib in CC remains unknown. In the current study, two CC cell lines, HeLa and Siha, were used to investigate the effects of gefitinib. CCK-8 assays demonstrated that gefitinib exerted strong cytotoxicity in HeLa and Siha cells. Flow cytometry was performed to examine cell cycle progression and apoptosis in CC following treatment with gefitinib. The current study demonstrated that treatment

with gefitinib enhanced the number of cells in the G_0/G_1 phase and increased apoptosis in HeLa and Siha cells. Furthermore, treatment with gefitinib reduced the protein expression level of Bcl-2, and enhanced the protein expression level of Bax. Taken together, these results suggest that gefitinib may suppress CC cell proliferation and induce cell cycle arrest and apoptosis.

To further investigate the underlying mechanism of gefitinib in regulating CC progression, the EMT process was examined in CC cells following treatment with gefitinib. In the progression of CC, EMT is a key regulator that promotes cancer cell proliferation and invasion (4). The current study demonstrated that treatment with gefitinib suppressed the EMT process by increasing the expression level of the epithelial marker, E-cadherin, and decreasing the expression level of the mesenchymal marker, vimentin. These results suggest that gefitinib may suppress the EMT process in CC.

The canonical Wnt/β-catenin signaling pathway serves an important role in EMT (25,26). Abnormal activation of Wnt/β-catenin signaling is reported to increase cancer cell proliferation, survival, differentiation and EMT (27,28). The current study examined the potential association between gefitinib and the Wnt/β-catenin signaling pathway in CC cells. The current study demonstrated that treatment with gefitinib decreased the protein expression levels of p-GSK3β and β-catenin, which suggests that gefitinib may be a potential novel therapeutic strategy in CC by suppressing the Wnt/β-catenin signaling pathway and EMT to inhibit tumor metastasis in CC cells.

In conclusion, the current study demonstrated that gefitinib may suppress EMT during cell invasion and induce apoptosis and cell cycle arrest by inhibiting the Wnt/β-catenin signaling pathway.

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Availability of data and materials

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

Authors' contributions

JZ performed the experiments and revised the manuscript for important intellectual content. JY performed the cell proliferation experiments and was involved in drafting the manuscript. MY and LT designed the experiments, analyzed the data and gave final approval of the version to be published. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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