

Silencing Smad4 attenuates sensitivity of colorectal cancer cells to cetuximab by promoting epithelial-mesenchymal transition

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Abstract. The aberrant expression of tumor suppressor Smad4 often occurs in colorectal cancer (CRC), and this phenomenon is believed to be associated with drug resistance. The present study aimed to investigate the effects of Smad4 on the sensitivity of CRC cells to cetuximab, and the possible mechanism underlying such an effect. A total of 629 colorectal adenocarcinoma cases were downloaded from The Cancer Genome Atlas (TCGA) database, and a Smad4 mutation rate of ~21% was demonstrated among the cases. Low expression of Smad4 was present in CRC tissues analyzed by TCGA and in four CRC cell lines, as determined by reverse transcription-quantitative PCR (RT-qPCR) and western blot analysis. Cell Counting kit-8 (CCK-8) was used to measure the effects of different concentrations of cetuximab on SW480 cell viability at 24 and 48 h. The results demonstrated that treatment of SW480 cells with 20 μ g/ml cetuximab for 48 h markedly reduced cell viability. In addition, plasmids were transfected into SW480 cells to induce Smad4 silencing or overexpression. Silencing Smad4 attenuated the sensitivity of SW480 CRC cells to cetuximab; this effect was reflected in increased cell viability and slightly increased migration and invasion, as determined by CCK-8, wound scratch and Transwell analyses. RT-qPCR and western blotting was performed to assess the expression levels of apoptosis- and epithelial-mesenchymal transition (EMT)-related genes. Silencing Smad4 partly reversed the effects of cetuximab on the mRNA and protein expression levels of vimentin, Bax/Bcl-2 and E-cadherin. However, Smad4 overexpression enhanced SW480 cell sensitivity to cetuximab. In conclusion, Smad4 may serve a vital role in the sensitivity of CRC cells to chemotherapeutic drugs by promoting EMT.

Introduction

Colorectal cancer (CRC) is one of the most common types of cancer worldwide, second to lung or breast cancer. In addition, CRC is the third most common type of cancer among men and the second among women worldwide. Annually, 30,000 new cases of CRC are identified and there are 90,000 cases of CRC-associated mortality globally (1). In addition, CRC is the third leading cause of morbidity and mortality among all malignant tumors in the United States (2). Despite continuing innovations in cancer treatment, the survival rate of patients with advanced CRC is low. The possibility of metastasis and recurrence is significantly increased once the tumor enters the middle and late stage, which is the main cause of mortality (3). In CRC, the 5-year survival rate for cancer *in situ* is >65%; however, the 5-year survival rate is between 25 and 60% if lymph node metastasis develops, and the 5-year survival rate remains <7% once tumor cells have metastasized to distal organs (4). Conventional chemotherapeutic drugs, including irinotecan, oxaliplatin and fluorouracil, can improve the efficacy of metastatic CRC (mCRC) treatment; however, the median survival of patients remains <2 years (5,6). The target epidermal growth factor receptor (EGFR) monoclonal antibody cetuximab, as a single drug therapy or as part of combination therapy, is the main method used to treat late mCRC (7). However, a number of patients are still resistant to cetuximab following treatment (8,9).

The tumor suppressor gene Smad4 is an important transcriptional factor in the transforming growth factor β signaling pathway. Gene aberration, including chromosome fragment loss, gene mutation and abnormal gene expression, often occurs in CRC and other gastrointestinal tumors (10-13). Smad4 is a member of the Smads protein family, and is located on chromosome 18q21 (14). Clinical studies have demonstrated that the risk of Smad4 deletion is increased in patients with advanced CRC with liver metastasis, and leads to poor prognosis (15-17). By contrast, the median survival time of CRC patients with high Smad4 expression is significantly longer compared with in those with low Smad4 expression (14).

Previous studies have demonstrated that tumor cells undergo epithelial-mesenchymal transition (EMT) with increased drug resistance (18,19). EMT is a biological process in which epithelial cells gradually transform into cells with an interstitial phenotype through a specific procedure; this

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process may be involved in numerous biological behaviors, including wound healing and tumor metastasis (20-22). Its main characteristics are decreased cell adhesion molecule expression, transformation of the cytoskeleton from a cytokeratin to vimentin phenotype, and morphological characteristics of mesenchymal cells (22,23). From classic morphological observations of CRC, it has been identified that reversible morphological alterations occur during the process of tumor invasion and metastasis. Therefore, EMT is considered to serve an important role in CRC metastasis (24,25).

Although several studies have reported that mutation or loss of Smad4 in CRC is closely associated with chemoresistance, these previous studies have mainly focused on conventional chemotherapeutic drugs, including 5-fluorouracil and oxaliplatin, and classic pathways including Akt and PI3K signaling (26-29). The present study aimed to investigate the effects of Smad4 on the sensitivity of CRC cells to cetuximab, which is an EGFR monoclonal antibody, and whether the effects were implicated in EMT.

Materials and methods

The Cancer Genome Atlas (TCGA) database analysis. A total of 629 colorectal adenocarcinoma cases were downloaded from TCGA database (<http://www.cbiportal.org/>). The mutations of Smad4, and the expression of Smad4 in CRC and matched normal tissues were analyzed.

Cell culture. Normal human colon epithelial cells (CCD 841 CoN cells) and four CRC cell lines (SW480, LoVo, SW620 and LS174T) were obtained from Invitrogen; Thermo Fisher Scientific, Inc. The cells were cultured in RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 0.03% glutamine and 100 µg/ml streptomycin at 37°C in an incubator with 5% CO₂. The cells were digested and subcultured with 0.25% trypsin when confluence reached 80-90%.

Cell transfection and grouping. The Smad4 silencing plasmid (siSmad4) (Forward: 5'-CGAAUACACCAACAAGUAATT-3', Reverse: 5'-UUACUUGUUGGUGUAUUAUCGTA-3'), Smad4 overexpression plasmid and empty control plasmid (negative control, NC) were purchased from Invitrogen; Thermo Fisher Scientific, Inc. SW480 cells were seeded in a 6-well plate (1.0x10⁵ cells/well) for 24 h before transfection and divided into three groups: i) the control (0.1% PBS) subgroup, siNC subgroup, siSmad4 subgroup, NC subgroup and Smad4 overexpression plasmid subgroup; ii) the control (0.1% PBS) subgroup, cetuximab (20 µg/ml; Merck KGaA) subgroup, NC + cetuximab (20 µg/ml) subgroup, siSmad4 + cetuximab (20 µg/ml) subgroup and siSmad4 subgroup; iii) the control (0.1% PBS) subgroup, NC + cetuximab (20 µg/ml) subgroup and Smad4 overexpression plasmid + cetuximab (20 µg/ml) subgroup. Transient transfection was effected by Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. A total of 20 µM NC, siSmad4 or Smad4 overexpression plasmid, and 5 µl Lipofectamine[®] 2000 were added to Opti-MEM and incubated at 25°C for 10 min. Lipofectamine[®] 2000 was

then mixed into each well and the cells were cultured in Opti-MEM. After 6 h of culturing, the medium was replaced with RPMI-1640 containing 10% FBS. After 24 h, the cells were used to perform the subsequent experiments.

Cell Counting kit-8 (CCK-8) viability assay. In the present study, the CCK-8 assay was conducted in triplicate. Cells were plated into 96-well plates at a seeding density of 1x10⁴ cells/well for 24 h. Initially, fresh medium containing 0.1, 1, 10, 30, 50 or 100 µg/ml Cetuximab was used to treat the cells for 24 or 48 h at 37°C, after which the CCK-8 assay was performed. In addition, the CCK-8 assay was performed to explore the effects of silencing or overexpressing Smad4 on the viability of cetuximab-treated cells. Briefly, 10 µl CCK-8 solution was added to the cells for 2 h at 37°C. Optical density (OD) was measured at 450 nm (Thermo Fisher Scientific, Inc.).

Cell wound scratch assay. Cells were seeded in 6-well plates (6x10⁴ cells/well) and incubated at 37°C for 24 h. A sterile 100-µl pipette tip was used to generate a wound in the center of the plate. PBS was used to gently wash the cells three times, and serum-free medium was added to the cells. Cell migration was observed using an inverted light microscope at 24 h. The wound area was measured using ImageJ software version 1.49 (National Institutes of Health).

Cell invasion assay. Cell invasion was detected using a Matrigel-coated Transwell assay. Briefly, 100 µl serum-free medium containing 10⁵ cells was added into the upper chamber (Corning, Inc.) and 600 µl culture medium containing 10% fetal bovine serum was added to the lower chamber. The cells were incubated for 24-48 h at 37°C in order to detect invasion into the lower membrane. Subsequently, the cells were fixed with 95% ethanol for 10 min at 25°C and stained with 0.5% crystal violet solution for 5 min at room temperature after the Matrigel on the filter membrane was removed.

Flow cytometry. Cell apoptosis was assessed by flow cytometry. Cells were harvested, collected and washed with cold PBS twice. The suspension (5x10⁵ cells/well) in binding buffer was cultured with Annexin V-APC/7-AAD apoptosis kit [cat. no. AP105, Hangzhou Multi Sciences (Lianke) Biotech Co., Ltd.] in the dark at 25°C for 20 min. Binding buffer was then added to each well and the samples were analyzed by flow cytometry within 1 h using BD CellQuest Pro Software version 1.2; BD Biosciences).

Reverse transcription-quantitative PCR (RT-qPCR). Smad4, apoptosis-related genes (Bax and Bcl-2), E-cadherin and vimentin were detected by RT-qPCR in the different groups. Total RNA was extracted from cultured cells using TRIzol[®] (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Total RNA was reverse transcribed with the PrimeScript[™] RT reagent kit (Takara Bio, Inc.) at 37°C for 45 min and then at 85°C for 5 min. cDNA was amplified using SYBR Fast qPCR Mix (Invitrogen; Thermo Fisher Scientific, Inc.). For quantitative real-time PCR, the PCR reaction was set at 95°C for 5 min, followed by 33 cycles at 94°C for 30 sec, 55°C for 30 sec, 72°C for 45 sec and 72°C extension for 10 min. The primer sequences used are listed in Table I.

Table I. Primers used in reverse transcription-quantitative PCR.

Gene	Sequence
Smad4	F: 5'-CTGAAGTGTGTTGACCTCTGGGCC ATATTGC-3' R: 5'-CAAATTTCTGAAGAGTAGGTGATC CGGGTGGAG-3'
Bcl-2	F: 5'-ATGGCGCACGCTGGGAGAAC-3' R: 5'-CTGGCGGAGGGTCAGGTGGA-3'
Bax	F: 5'-GCCGCCGTGGACACAGACTC-3' R: 5'-CCGCTCCCGGAGGAAGTCCA-3'
E-cadherin	F: 5'-ATCCAAAGCCTCAGGTCATA-3' R: 5'-CAGCAAGAGCAGCAGAAT-3'
Vimentin	F: 5'-GAACTTTGCCGTTGAAGCTG-3' R: 5'-TCTCAATGTCAAGGGCCATC-3'
GAPDH	F: 5'-ACCACAGTCCATGCCATCAC-3' R: 5'-TCCACCACCCTGTTGCTGTA-3'

F, forward; R, reverse.

mRNA expression was quantified using the $2^{-\Delta\Delta C_q}$ method (30). GAPDH served as an internal control.

Western blotting. Total protein was extracted from cultured cells using lysis buffer; RIPA buffer (Thermo Fisher Scientific, Inc.) was added to each well and cells were centrifuged at 14,000 x g for 15 min at 4°C. Bicinchoninic acid protein quantification (Thermo Fisher Scientific, Inc.) was used to detect protein concentration. Proteins (20 µg/lane) were separated by 10% SDS-PAGE and were then transferred onto polyvinylidene difluoride membranes, which were blocked in TBS-0.1% Tween-20 (TBST) containing 1% milk at room temperature for 2 h. The membranes were then incubated with the following primary antibodies: Rabbit anti-Smad4 (cat. no. ab40759, 1:5,000; Abcam), anti-Bcl-2 (cat. no. ab182858, 1:2,000; Abcam), anti-Bax (cat. no. ab32503, 1:1,500; Abcam), mouse anti-E-cadherin (cat. no. ab1416, 1:50; Abcam), anti-vimentin (cat. no. ab8978, 1:1,000; Abcam) and anti-GAPDH (cat. no. ab8245, 1:1,000; Abcam). The membranes were washed with TBST, and were then incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:2,000; SA00001-2; ProteinTech Group, Inc.) and with horseradish peroxidase-conjugated goat anti-mouse IgG (1:2,000; sc-516102; Santa Cruz Biotechnology, Inc.) as secondary antibodies. The blots were visualized by enhanced chemiluminescence (ECL; Thermo Fisher Scientific, Inc.). An ECL system (Amersham; GE Healthcare) was used to detect the bands. The density of the blots was measured using Quantity One software version 2.4 (Bio-Rad Laboratories, Inc.).

Statistical analysis. Statistical analysis was performed using Prism GraphPad version 6.0 software (GraphPad Software, Inc.). All data are presented as the means ± standard deviation. Differences were analyzed using one-way analysis of variance

following Tukey's multiple comparison post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Low expression of Smad4 in CRC tissues and CRC cell lines. TCGA data (Fig. 1A and B) demonstrated that Smad4 was mutated in 131 (21%) out of 629 sequenced cases/patients with colorectal adenocarcinoma. The majority of the mutations were deletions. In addition, mRNA and protein expression levels of Smad4 in cancer tissues were lower than those in normal tissues in the cases downloaded from TCGA (Fig. 1C). The expression levels of Smad4 in normal colonic epithelial cells and four CRC cell lines were determined by RT-qPCR and western blot analysis. The results demonstrated that expression of Smad4 in CRC cell lines (SW480, LoVo, SW620 and LS174T) was significantly decreased compared with in CCD 841 CoN cells at the mRNA and protein levels ($P < 0.01$; Fig. 1D and E). The SW480 cell line was selected as the subject of subsequent experiments as it had the lowest expression of Smad4 at the mRNA and protein levels.

Silencing Smad4 attenuates cell sensitivity to cetuximab in SW480 CRC cells. SW480 cells were treated with or without different concentrations (0.1, 1, 10, 30, 50 and 100 µg/ml) of cetuximab. There was a difference in OD 450 nm with 0 µg/ml Cetuximab since the cells proliferated after 24 and 48 h. Cell viability was increased in response to treatment with different concentrations of cetuximab for 24 and 48 h compared to 0 h ($P < 0.05$; Fig. 2A). The results indicated that certain cells already had drug resistance, and therefore, the inhibitory effect of cetuximab was not significant at 24 h. However, at 48 h, cell viability was downregulated in a dose-dependent manner until the cells were treated with 20 µg/ml cetuximab. Therefore, 20 µg/ml cetuximab (48 h) was selected as an effective concentration in subsequent experiments. The transfection efficiency of siSmad4 was demonstrated in Fig. 2B and C; the expression levels of Smad4 were significantly decreased post-transfection with siSmad4. In addition, treatment with 20 µg/ml cetuximab ($P < 0.05$) or siSmad4 ($P < 0.01$) significantly inhibited Smad4 protein (Fig. 2D) and mRNA (Fig. 2E) expression. When cetuximab was used in combination with siSmad4, the inhibitory effect on Smad4 expression was at its most significant ($P < 0.01$). Furthermore, cetuximab treatment alone and NC + cetuximab markedly downregulated SW480 cell viability ($P < 0.01$; Fig. 2F). However, the effect of cetuximab treatment was clearly inhibited when cetuximab was used in combination with siSmad4, compared with single cetuximab or NC + cetuximab ($P < 0.01$). In addition, compared with groups treated with cetuximab, siSmad4 alone significantly enhanced cell viability ($P < 0.01$). Silencing Smad4 not only increased SW480 cell viability, but also partly reversed the cell viability decrease induced by cetuximab, indicating that silencing Smad4 may attenuate cell sensitivity to cetuximab in the SW480 CRC cell line.

Silencing Smad4 partly reverses the decrease in cell migration and invasion, and the increase in cell apoptosis induced by cetuximab. Cell migration was detected using a scratch assay. The results revealed that cetuximab and NC + cetuximab

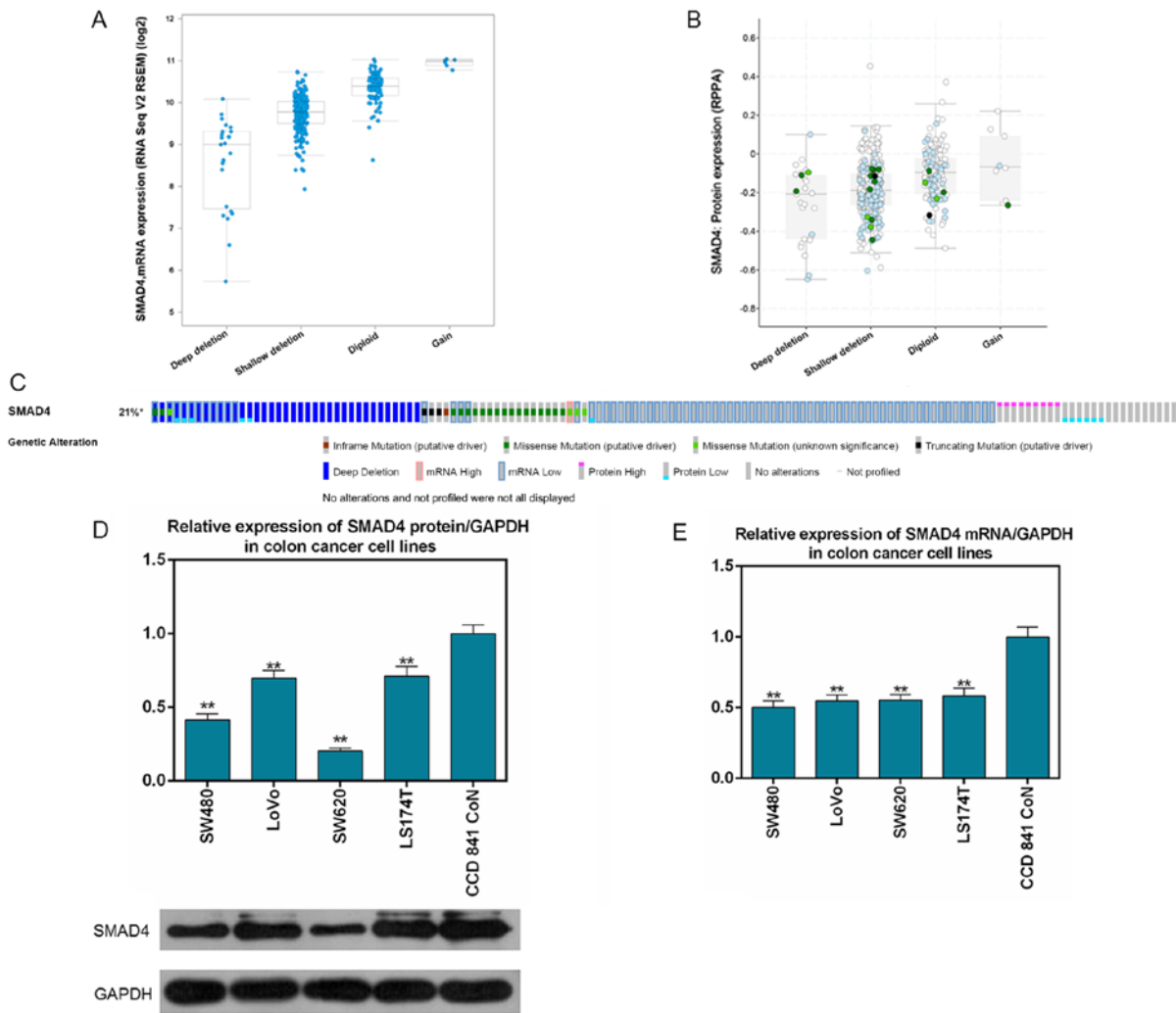


Figure 1. mRNA and protein expression levels of Smad4 in samples from patients with CRC and several cell lines. (A) The Cancer Genome Atlas database analysis of Smad4 mRNA sequences in 629 patients with CRC. (B) The Cancer Genome Atlas database analysis of Smad4 protein sequences in patients with CRC. (C) The Cancer Genome Atlas database analysis of Smad4 on Genetic alteration. (D) Protein expression levels of Smad4 in colonic epithelial cells and CRC cell lines were detected using western blotting. (E) mRNA expression levels of Smad4 in normal colonic epithelial cells (CCD 841 CoN cells) and CRC cell lines (SW480, LoVo, SW620 and LS174T) were detected by reverse transcription-quantitative polymerase chain reaction. GAPDH was used as an internal control. ** $P < 0.01$ vs. normal colonic epithelial cells. CRC, colorectal cancer.

treatment significantly increased the open wound area, compared with the control ($P < 0.01$; Fig. 3A). Silencing Smad4 markedly reduced the open wound area ($P < 0.01$; Fig. 3A). The open wound area was decreased by siSmad4 in combination with cetuximab compared with siSmad4 treatment alone; however, the decreased effect was not significant compared with cetuximab or cetuximab + NC. A Transwell assay was used to determine cell invasion and the result demonstrated that the invasion rate of SW480 cells was markedly decreased in groups treated with cetuximab ($P < 0.01$; Fig. 3B). Conversely, invasion of cells transfected with siSmad4 was significantly increased ($P < 0.01$; Fig. 3B). However, silencing Smad4 only partly increased the cell invasion attenuated by cetuximab. The effects of silencing Smad4 on the apoptosis of SW480 cells with or without cetuximab were detected by flow cytometric analysis. A noticeable increase in apoptosis ($P < 0.01$; Fig. 3C) was identified in the cetuximab (17.47%), NC + cetuximab (18.4%) and siSmad4 + cetuximab (9.18%) groups, compared with in the control group (4.48%). Silencing Smad4 (4.17%) significantly downregulated apoptosis rate ($P < 0.01$) compared

with in the cetuximab (17.47%) or siSmad4 + cetuximab (9.18%) groups. In addition, silencing Smad4 partly reversed the increase in cell apoptosis induced by cetuximab ($P < 0.01$).

Effects of silencing Smad4 on apoptosis- and EMT-related gene expression in SW480 cells with or without cetuximab treatment. The expression levels of the apoptosis-related genes Bax and Bcl-2 were detected by RT-qPCR and western blot analysis. The results also demonstrated that cetuximab down-regulated Bcl-2 ($P < 0.01$; Fig. 4A, B and F) and upregulated Bax protein ($P < 0.01$; Fig. 4A and C) and mRNA expression ($P < 0.01$; Fig. 4G) compared with control. Silencing Smad4 partly reversed the decreased expression of Bcl-2 and the increased expression of Bax (protein, $P < 0.01$) by cetuximab. Silencing Smad4 treatment alone could attenuate Bax expression in comparison with siSmad4 + cetuximab (protein, $P < 0.05$; mRNA, $P < 0.01$). To further determine how silencing Smad4 could affect EMT in SW480 CRC cells with or without cetuximab treatment, the expression of EMT-associated genes (E-cadherin and vimentin) was detected using RT-qPCR

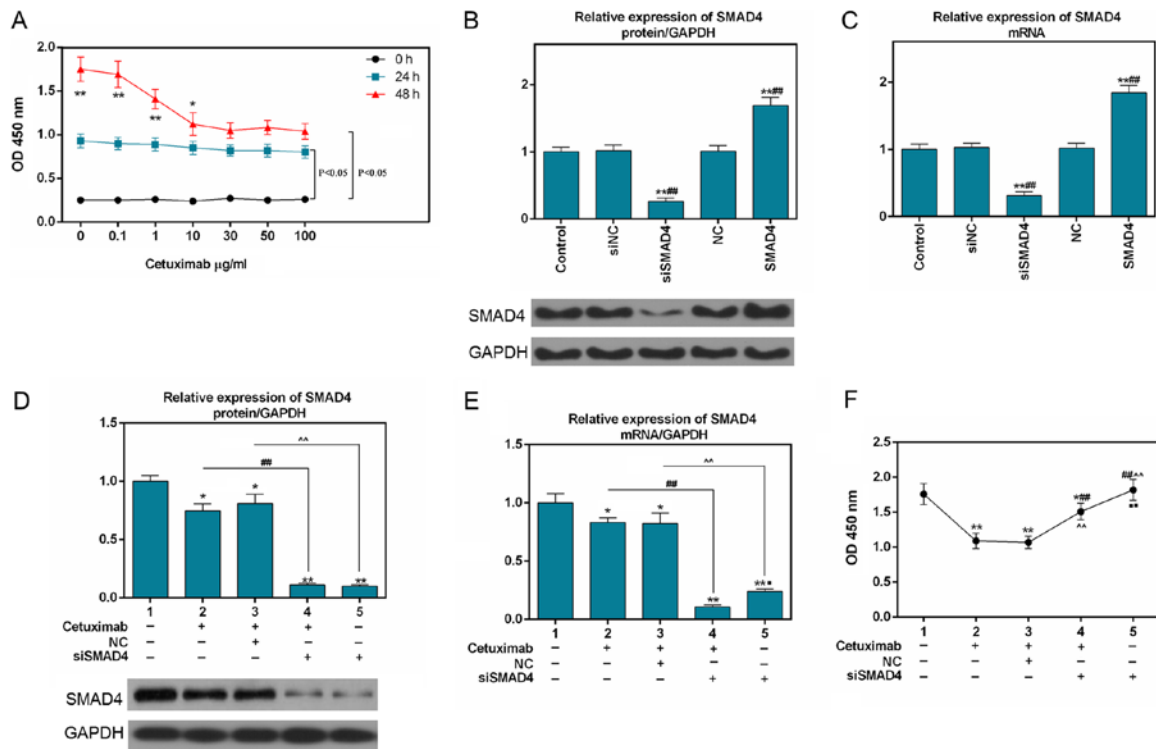


Figure 2. mRNA and protein expression levels of Smad4, and cell viability analysis in SW480 colorectal cancer cells with or without cetuximab treatment. (A) Different concentrations (0.1, 1, 10, 30, 50 and 100 $\mu\text{g/ml}$) of cetuximab were used to treat SW480 cells for 0, 24 or 48 h, and cell viability was detected by CCK-8 assay. * $P < 0.05$ and ** $P < 0.01$ vs. 0 h. (B) Protein expression levels of Smad4 were assessed post-transfection with plasmids silencing or overexpressing Smad4 by western blotting in SW480 cells. (C) mRNA expression levels of Smad4 in SW480 cells post-transfection with plasmids silencing and overexpressing Smad4 by reverse transcription-quantitative PCR. GAPDH served as an internal control. Data are expressed as the means \pm standard deviation from three independent experiments. ** $P < 0.01$ vs. control; ## $P < 0.01$ vs. NC. (D) Protein expression levels of Smad4 were assessed post-transfection with a plasmid silencing Smad4 using western blot analysis in SW480 cells. * $P < 0.05$ and ** $P < 0.01$ vs. control; ## $P < 0.01$ vs. cetuximab; ^^ $P < 0.01$ vs. NC + cetuximab. (E) mRNA expression levels of Smad4 were assessed post-transfection by reverse transcription-quantitative PCR in SW480 cells. GAPDH served as an internal control. (F) Viability of SW480 CRC cells was assessed by CCK-8 assay after 48 h. Data are expressed as the means \pm standard deviation from three independent experiments. * $P < 0.05$ and ** $P < 0.01$ vs. control; * $P < 0.05$ and ** $P < 0.01$ vs. siSmad4 + cetuximab; ## $P < 0.01$ vs. cetuximab; ^^ $P < 0.01$ vs. NC + cetuximab. CCK-8, Cell Counting kit-8; CRC, colorectal cancer; NC, negative control; OD, optical density; siSmad4, small interfering RNA-Smad4.

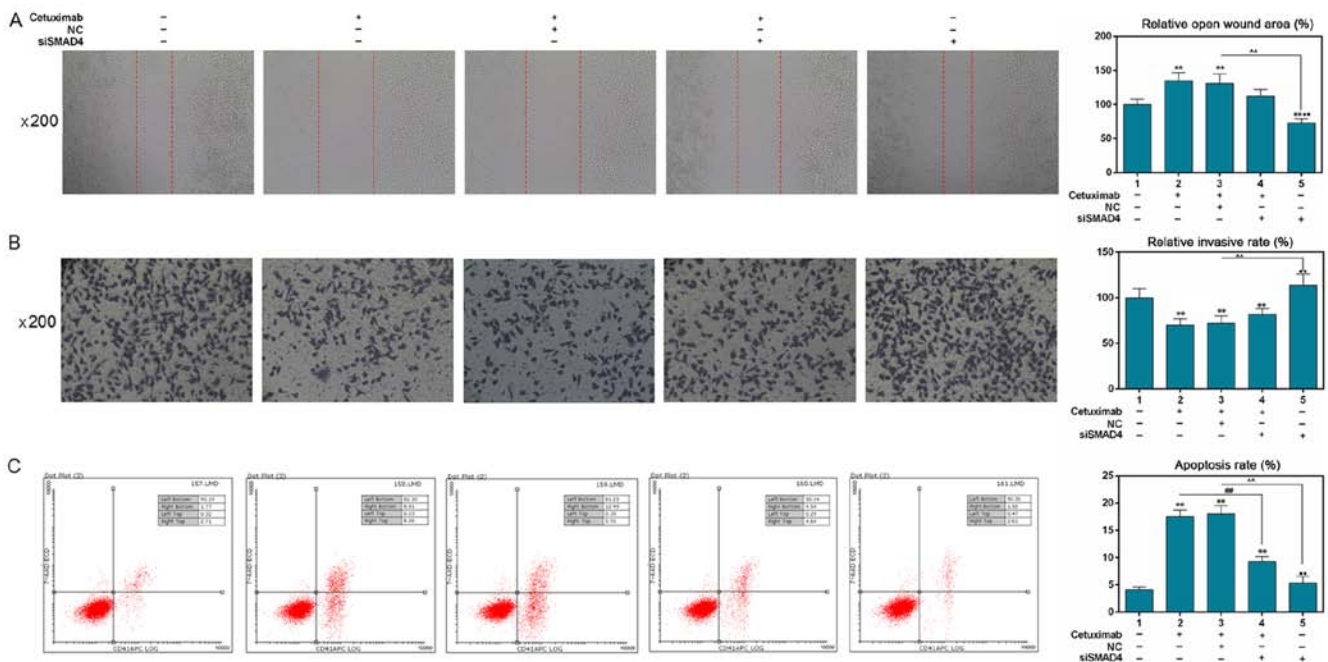


Figure 3. siSmad4 affects cell migration, invasion and apoptosis of SW480 cells with or without cetuximab treatment. (A) Cell migration was detected using a scratch assay, (x200 magnification; light microscopy). (B) Transwell assay was used to determine cell invasion (x200 magnification; light microscopy). (C) Apoptosis was detected by flow cytometry. ** $P < 0.01$ vs. control; ## $P < 0.01$ vs. siSmad4 + cetuximab; ## $P < 0.01$ vs. cetuximab; ^^ $P < 0.01$ vs. NC + cetuximab. NC, negative control; siSmad4, small interfering RNA-Smad4.

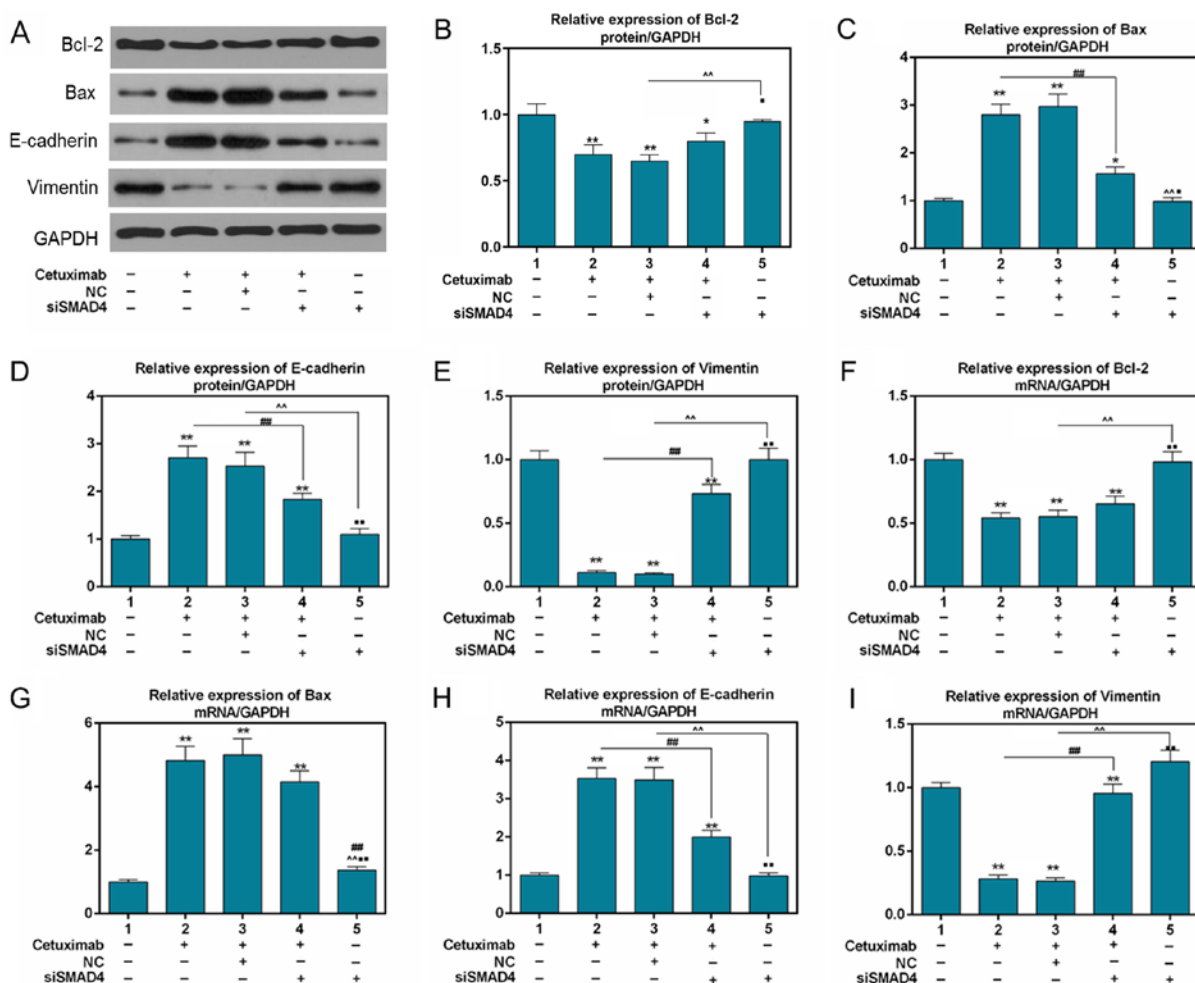


Figure 4. Silencing Smad4 affects the expression of apoptosis- and epithelial-mesenchymal transition-associated genes in SW480 cells with or without cetuximab treatment. (A) Protein expression levels of Bcl-2, Bax, E-cadherin and vimentin were assessed using western blot analysis in SW480 cells with or without cetuximab treatment. Relative protein expression levels of (B) Bcl-2, (C) Bax, (D) E-cadherin and (E) vimentin were presented as histograms. mRNA expression levels of (F) Bcl-2, (G) Bax, (H) E-cadherin and (I) vimentin were assessed using reverse transcription-quantitative PCR in SW480 cells with or without cetuximab treatment. GAPDH served as an internal control. Data are expressed as the means \pm standard deviation from three independent experiments. * $P < 0.05$ and ** $P < 0.01$ vs. control; # $P < 0.05$ and ## $P < 0.01$ vs. siSmad4 + cetuximab; ^^ $P < 0.01$ vs. cetuximab; ^^ $P < 0.01$ vs. NC + cetuximab. NC, negative control; siSmad4, small interfering RNA-Smad4.

and western blot analysis. The results demonstrated that both protein (Fig. 4A and D) and mRNA levels (Fig. 4H) of E-cadherin were increased in the cetuximab and NC + cetuximab groups compared with in the control group ($P < 0.01$). Conversely, the mRNA and protein expression levels of E-cadherin were significantly downregulated in the siSmad4 and siSmad4 + cetuximab groups compared with in the cetuximab or NC + cetuximab groups ($P < 0.01$). The effects of siSmad4 on vimentin expression demonstrated opposite results to those on E-cadherin at the mRNA and protein levels ($P < 0.01$; Fig. 4A, E and I). These results indicated that silencing Smad4 partly reversed the effects of cetuximab on the expression of apoptosis- and EMT-related genes.

Overexpression of Smad4 enhances the sensitivity of SW480 cells to cetuximab. The transfection efficiency of the Smad4 overexpression plasmid was demonstrated in Fig. 2B and C; Smad4 expression was significantly increased post-transfection with the overexpression plasmid. As shown in Fig. 5A-C, cetuximab significantly inhibited Smad4 protein ($P < 0.05$) and mRNA expression ($P < 0.01$). Smad4 overexpression partly

reversed the expression of Smad4 decreased by cetuximab ($P < 0.01$). Cell viability was detected using the CCK-8 assay; the results demonstrated that NC + cetuximab significantly decreased cell viability ($P < 0.01$; Fig. 5D). Cetuximab in combination with Smad4 overexpression further decreased cell viability compared with the control group ($P < 0.01$). In addition, cell viability was attenuated in the Smad4 overexpression + cetuximab group compared with in the NC + cetuximab group ($P < 0.05$), suggesting that overexpressing Smad4 may enhance the sensitivity of SW480 cells to cetuximab.

Overexpression of Smad4 enhances the effects of cetuximab on invasion, migration and apoptosis of SW480 cells. As shown in Fig. 6A, NC + cetuximab slightly increased the wound area compared with in the control group ($P < 0.05$). Overexpression of Smad4 had no evident effect on the inhibition of migration induced by cetuximab ($P > 0.05$). The Transwell assay demonstrated that NC + cetuximab and Smad4 overexpression + cetuximab significantly downregulated invasion rate ($P < 0.01$; Fig. 6B). For cell apoptosis, the results demonstrated that cetuximab treatment alone (18.79%), and also Smad4

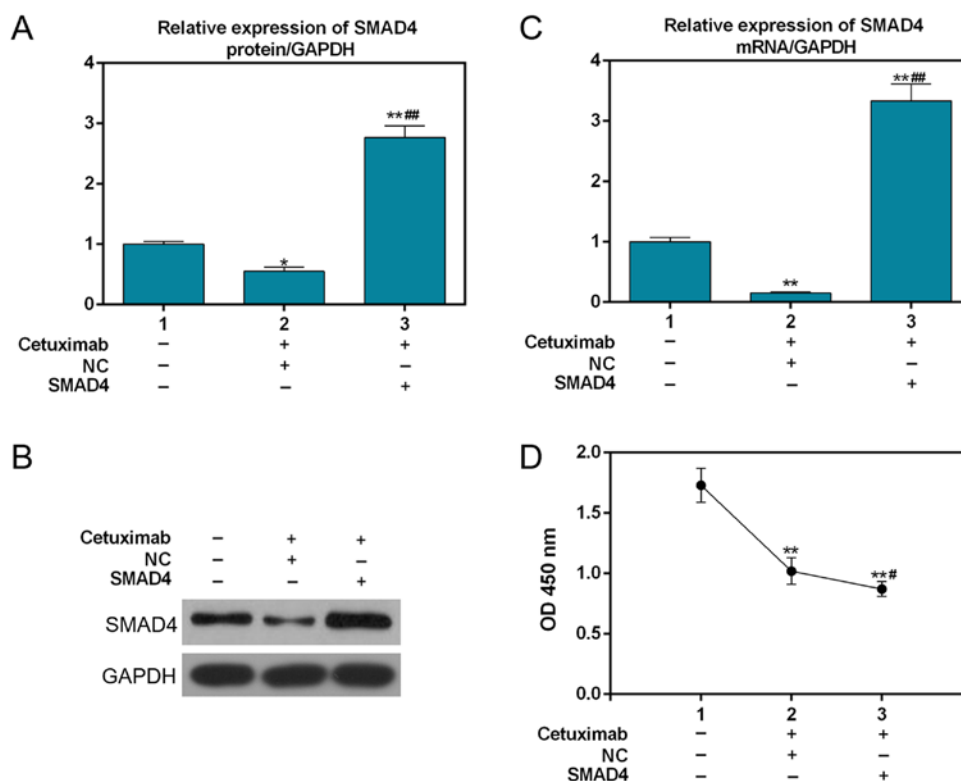


Figure 5. mRNA and protein expression levels of Smad4, and cell viability analysis of SW480 cells overexpressing Smad4 and treated with cetuximab. (A and B) Protein expression levels of Smad4 were detected by western blotting and presented as histograms. (C) mRNA expression levels of Smad4 were assessed by reverse transcription-quantitative PCR; GAPDH served as an internal control. (D) Effects of Smad4 overexpression on the viability of SW480 colorectal cancer cells treated with cetuximab was assessed by the Cell Counting kit-8 assay after 48 h. Data are expressed as the means \pm standard deviation from three independent experiments. * $P < 0.05$ and ** $P < 0.01$ vs. control; # $P < 0.05$ and ## $P < 0.01$ vs. NC + cetuximab. NC, negative control; OD, optical density; siSmad4, small interfering RNA-Smad4.

overexpression + cetuximab (25.25%) significantly upregulated the apoptosis rate compared with in the control group (4.64%, $P < 0.01$; Fig. 6C). Additionally, the apoptotic effect of Smad4 overexpression + cetuximab was significantly different compared with treatment with cetuximab alone ($P < 0.01$).

Effects of Smad4 overexpression on apoptosis- and EMT-related gene expression in SW480 cells treated with cetuximab. The results revealed that the anti-apoptosis gene Bcl-2 was markedly decreased in the Smad4 overexpression + cetuximab group compared with in the control and NC + cetuximab groups, at the mRNA and protein levels ($P < 0.01$; Fig. 7A, B and F). At the protein level, the expression of Bax was significantly increased in the Smad4 overexpression + cetuximab and NC + cetuximab groups compared with in the control group ($P < 0.01$; Fig. 7A and C). Notably, the increase in Bax expression was more noticeable in the Smad4 overexpression + cetuximab group compared with in the NC + cetuximab group. The effects of treatments on the mRNA expression levels of Bax were consistent with those on protein expression ($P < 0.01$; Fig. 7G). Furthermore, the protein and mRNA expression levels of E-cadherin were significantly upregulated in the Smad4 overexpression + cetuximab group compared with in the control and NC + cetuximab groups ($P < 0.01$; Fig. 7A, D and H). Conversely, the protein expression levels of vimentin were significantly decreased in the Smad4 overexpression + cetuximab group compared with in the control and NC + cetuximab groups ($P < 0.01$; Fig. 7A and E). At the mRNA level, the Smad4 overexpression + cetuximab group

exhibited no clear difference in vimentin expression compared with the NC + cetuximab group ($P > 0.05$; Fig. 7I). However, the mRNA expression levels of vimentin were markedly downregulated compared with in the control group ($P < 0.01$; Fig. 7I).

Discussion

Smad4 is absent or mutated in numerous tumor types, including pancreatic, colon, gastric and liver cancer, and particularly in digestive system tumors. Therefore, it is thought to function as a tumor suppressor gene (31-34). One of the major causes of CRC progression is chromosomal instability, including APC, p53 and DCC/Smad4 deficiency (35-37). Therefore, the roles of Smad4 cannot be neglected in the development of CRC (38,39). Surgery is the main method for treating early CRC; however, recurrence and metastasis often occur following surgery. With the increasing use of adjuvant therapy using chemicals such as cetuximab, drug resistance of tumor cells is a pivotal cause of poor prognosis among patients with CRC (6).

The present study revealed that cetuximab treatment alone inhibited SW480 cell viability, migration and invasion, and promoted cell apoptosis. Although Mei *et al* (40) reported that Smad4 and neurofibromin 1 mutations may be potential biomarkers for poor responses to cetuximab-based therapy, related molecular mechanisms have not been studied. The present study suggested that silencing Smad4 in combination with cetuximab treatment enhanced SW480 cell viability, migration and invasion, and inhibited cell apoptosis.

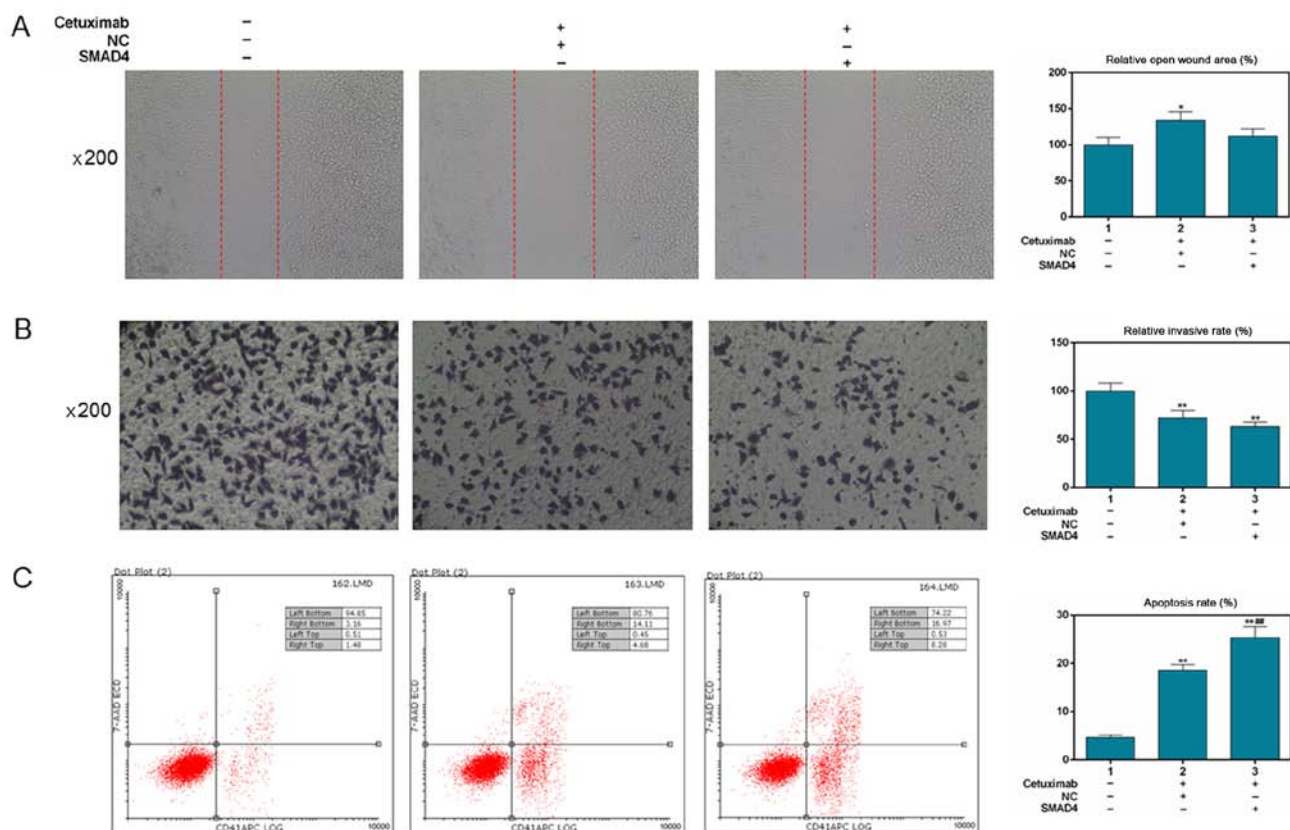


Figure 6. Smad4 overexpression affects migration, invasion and apoptosis of SW480 cells treated with cetuximab. (A) Cell migration was detected using scratch assay (x200 magnification; light microscopy). (B) Transwell assay was used to determine cell invasion (magnification, x200; light microscopy). (C) Apoptosis was detected by flow cytometry. * $P < 0.05$ and ** $P < 0.01$ vs. control; ## $P < 0.01$ vs. NC + cetuximab. NC, negative control.

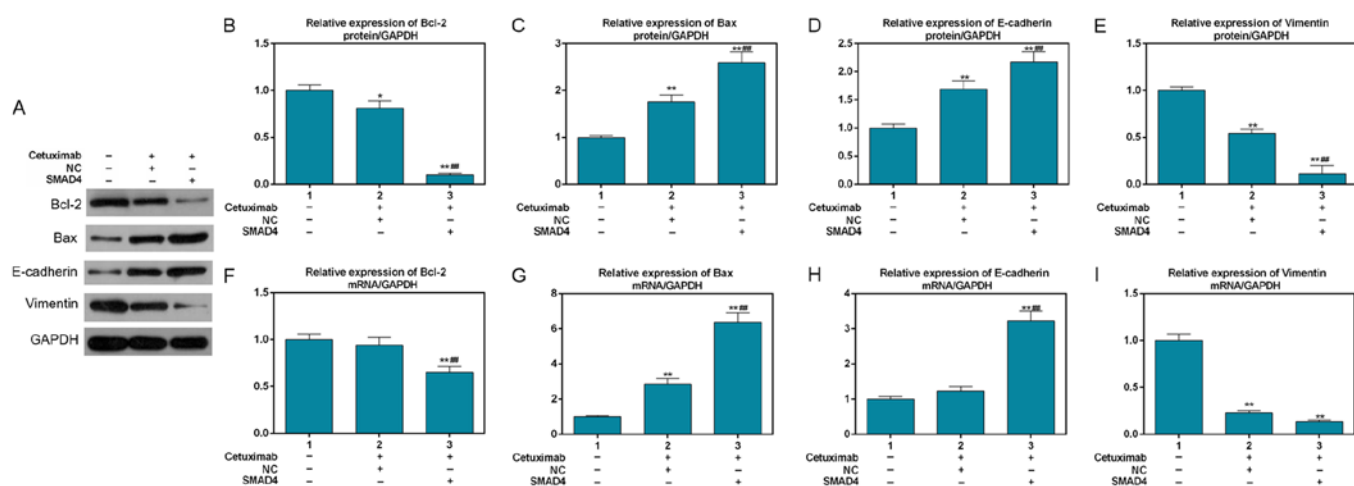


Figure 7. Smad4 overexpression affects the expression of apoptosis- and epithelial-mesenchymal transition-associated genes in SW480 cells treated with cetuximab. (A) Protein expression levels of Bcl-2, Bax, E-cadherin and vimentin were assessed using western blotting in SW480 cells with cetuximab treatment. Relative protein expression levels of (B) Bcl-2, (C) Bax, (D) E-cadherin and (E) vimentin were presented as histograms. mRNA expression levels of (F) Bcl-2, (G) Bax, (H) E-cadherin and (I) vimentin were assessed using reverse transcription-quantitative PCR in SW480 cells with cetuximab treatment; GAPDH served as an internal control. Data are expressed as the means \pm standard deviation from three independent experiments. * $P < 0.05$ and ** $P < 0.01$ vs. control; ## $P < 0.01$ vs. NC + cetuximab. NC, negative control.

Conversely, the sensitivity of SW480 cells to cetuximab was enhanced when SW480 cells were transfected with a plasmid overexpressing Smad4. The results indicated that the expression of Smad4 may serve a role in the response of SW480 cells to cetuximab. Previous studies have reported that downregulation of Smad4 is associated with a poor prognosis in response

to 5-fluorouracil chemotherapy among patients with advanced CRC (15,41). Evidence also demonstrated that Smad4 is associated with chemotherapy and radiotherapy resistance in cancer. For example, Smad4 knockdown promotes cetuximab resistance in head and neck squamous cell carcinoma by inducing mitogen-activated protein kinases/JNK activation (42,43).

Wang *et al* (44) suggested that Smad4 mutations are responsible for the resistance of pancreatic cancer to radiotherapy. Sun *et al* (45) also reported that microRNA-574 promotes doxorubicin resistance of MCF-7 cells by downregulating Smad4 in breast cancer cells.

A number of studies have reported that low expression of Smad4 is associated with EMT and poor prognosis of colon cancer (46-48). Therefore it was speculated that this may be the underlying mechanism by which Smad4 affects the sensitivity of SW480 CRC cells to cetuximab. In the present study, silencing Smad4 partly reversed the expression of E-cadherin increased by cetuximab and the expression of vimentin decreased by cetuximab. EMT commonly occurs in the heart or palate, and is associated with placentation (49-53). In recent years, EMT has been proposed to be involved in the invasion and metastasis of tumor cells (49). Accordingly, fibroblastoid or 'spindle-cell' tumors of epithelial origin have been characterized as highly malignant and invasive (54-56). E-cadherin is an important component of epithelial intercellular junctions and vimentin is an interstitial cell-specific protein; the two proteins are typical characteristic markers of EMT (57-60). Fuchs *et al* (61) suggested that EMT serves an important role in cetuximab resistance to hepatocellular carcinoma. Liu *et al* has also demonstrated that Smad4 is implicated with cancer cell resistance caused by EMT (62).

Studies of the definite mechanism underlying the effect rendered by Smad4 may also be beneficial. Various factors, including Wnt, Hedgehog and Notch signaling, may regulate the association between EMT and cancer cell resistance (63-65). However, there remain a number of shortcomings in the present study, including the lack of *in vivo* experiments. We aim to conduct a further detailed investigation into the association of Smad4 protein levels with E-cadherin or vimentin protein levels in CRC tissues or matched normal tissues, and also to perform validation experiments in one more cell line and *in vivo* mice models.

In conclusion, Smad4 may serve a vital role in the sensitivity of CRC cells to chemotherapy via EMT. Furthermore, a high expression of Smad4 may clinically benefit cetuximab-based treatment.

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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 on reasonable request.

Authors' contributions

ZL made substantial contributions to conception and design; LZ, JZho and JZhe performed data acquisition, data analysis

and interpretation. All authors drafted the article or critically revised it for important intellectual content and All authors approved the final version to be published. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work were appropriately investigated and resolved:

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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