CEACAM6 promotes cholangiocarcinoma migration and invasion by inducing epithelial-mesenchymal transition through inhibition of the SRC/PI3K/AKT signaling pathway

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Abstract. The immunoglobulin superfamily member carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) is overexpressed in a wide variety of human cancer types, and is associated with tumor invasion and migration. The aim of the present study was to determine the role of CEACAM6 in cholangiocarcinoma (CCA) invasion and migration *in vitro*. The results showed that CEACAM6 was highly expressed in CCA tissues, and that the expression level of CEACAM6 was negatively associated with the degree of differentiation of CCA. Silencing CEACAM6 inhibited cell viability, invasion and migration but promoted cell apoptosis in a human CCA cell line (RBE). In addition, CEACAM6 knockdown decreased the expression of an antiapoptotic protein (Bcl-2), an interstitial cell marker (N-cadherin), extracellular matrix proteins (MMP-2 and MMP-9), a transcription factor helix protein (Twist-related protein 1), an intermediate tumor cell scaffold marker (vimentin), a protein involved in tumor nutrient vascular formation (VEGFA) and a tumorigenesis factor (intercellular cell adhesion molecule-1), but increased the expression of pro-apoptotic proteins (Bax, and cleaved caspases-3, -8 and -9) and an epithelial cell marker protein (E-cadherin). Furthermore, CEACAM6-small interfering RNA reduced the expression of the SRC/PI3K/AKT signaling transduction pathway. Taken together, these results suggested that CEACAM6 may be an epithelial-mesenchymal transition biomarker and a potential therapeutic target in human CCA.

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Introduction

Cholangiocarcinoma (CCA) has been reported to be the second most common hepatic malignancy after hepatocellular carcinoma (HCC), and its incidence has gradually increased worldwide (1-2). CCA is a relatively infrequent malignancy arising from epithelial cells lining the biliary tree, and is classified anatomically as intrahepatic (iCCA), perihilar (pCCA) or distal (3). The incidence of iCCA has increased globally over the past few decades. According to the mortality rates reported in the World Health Organization database, the age-standardized mortality rates for iCCA have increased in almost all countries across all continents (4). Surgery is the preferred treatment option for a minority of patients with early-stage CCA (~35%). However, the currently available systemic therapies are of limited effectiveness for patients with advanced-stage or unresectable CCA, due to its absence of clinical symptoms and anatomical location of difficult access (5). Therefore, it is urgent to explore novel markers and molecules for CCA therapy.

Epithelial-mesenchymal transition (EMT) is associated with tumor invasion and migration, and is defined as a reversible dynamic process in which epithelial cells lose their phenotypic characteristics and adopt mesenchymal cell structural and functional features (6,7). These changes involve downregulation of E-cadherin and β-catenin, which are two main phenotypic characteristic components of epithelial cells, as well as upregulation of proteins involved in the mesenchymal phenotype (N-cadherin, α-smooth muscle actin, vimentin, fibronectin and MMPs), which occur via EMT-inducing transcription factors. The EMT-inducing transcription factor comprise three families: Snail, zinc finger E-box-binding homeobox and Twist-related proteins (TWIST) (8).

EMT is involved in human physiological and pathological processes, including embryonic and organ development, organ fibrosis, wound healing, and cancer progression (9,10), and is accompanied by marked changes in cellular morphology, enhanced migratory and invasive capabilities, as well as loss and remodeling of cell-cell interactions and cell-matrix adhesion (11). Thus, EMT may be an important mechanism involved

in the progression of CCA, and EMT biomarkers may provide new insights into the progression and migration of CCA. In addition, the activities of cellular (c)-SRC kinase, and PI3K and AKT, which are potentially located downstream of c-SRC, are hallmarks for malignant transformation and progression.

Carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6), a member of the CEACAM family, is normally expressed on the surface of myeloid and epithelial cells, while aberrant CEACAM6 expression leads to the development of human malignancies (12-14). Previous studies have reported that CEACAM6 is overexpressed in several epithelial carcinomas, including colon, breast and non-small cell lung cancer, as well as iCCA (15,16). CEACAM6 mediates cell-cell adhesion, which is required for guiding cells to their correct location during embryonic development, and for the integration of single cells into functional tissues and organs (17). However, overexpression of CEACAM6 has been shown to alter tissue architecture in several carcinoma cell lines (18). In addition, overexpression of CEACAM6 reduces cell apoptosis, indicating that CEACAM6 plays a key role in promoting the aberrant growth of adherent cells (19). Previous studies have shown that CEACAM6 induces EMT, and mediates invasion and migration in pancreatic and gastric cancer, and its clinical importance in colorectal cancer has also been reported (20,21). However, the physiological function of CEACAM6, and its potential involvement in tumor formation and progression in CCA have not been fully elucidated to date.

The present study demonstrated that CEACAM6 is an important regulator of CCA EMT, as well as of cell migration and invasion *in vitro*, which suggests that CEACAM6 may be an important target for the treatment of human CCA.

Materials and methods

Tissues and cell line. Primary CCA tissues and matched adjacent paracancerous tissues (distance, 1 cm) were surgically obtained from 27 patients with CCA 20 males and 7 females with an age range of 56-78 years (mean age, 66.4 years), including 9 cases of highly differentiated CCA and 18 cases of less differentiated CCA) at The Second Hospital of Hebei Medical University (Shijiazhuang, China) from January 2016 to December 2017. Ethics approval (approval no. 2014018) was obtained from The Second Hospital of Hebei Medical University and all samples were collected with written informed consent from the patients.

RBE cells (Qiao Xinzhou Biotechnology Co., Ltd.) were cultured at 37˚C with 5% CO_2 in DMEM (Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; Biological Industries; Sartorius AG) and 1% penicillin and streptomycin (Sigma-Aldrich; Merck KGaA). Cells in the logarithmic growth phase were used for experiments.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from CCA and paracancerous tissue and RBE cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and first-strand cDNA was synthesized using PrimeScript™ RT Reagent kit (Takara Biotechnology Co., Ltd.). qPCR was performed in triplicate with SYBRII qPCR Master Mix (Takara Biotechnology Co., Ltd.) according to the manufacturer's protocol using GAPDH as a control. RT was performed at 37˚C for 15 min and 85˚C for 5 min. Thermocycling conditions were as follows: Initial denaturation at 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 31 sec. The relative mRNA levels of the target genes were calculated with the $2^{-\Delta\Delta Cq}$ method (22). The primer sequences used in qPCR are listed in Table I.

Western blotting. Western blotting was performed as previously described (18). Briefly, total protein was extracted from RBE ($5x10^6$) cells, CCA tissues and paracancerous tissues with cold RIPA buffer containing protease and phosphatase inhibitors (all Beijing Solarbio Science and Technology Co., Ltd.), followed by quantification of protein concentration using the BCA assay. Protein samples (30 μg/lane) were heated to 100˚C for 10 min in SDS-PAGE loading buffer (Thermo Fisher Scientific, Inc.) and separated on 10% SDS-PAGE. The separated proteins were transferred at 200 mA to a PVDF membrane (MilliporeSigma) for 2 h at 4˚C. After blocking the membrane with TBS containing 5% skimmed milk for 1.5 h at room temperature, the membranes were incubated with primary antibodies at 4˚C overnight. The primary antibodies used were as follows: β-actin (rabbit monoclonal; 1:2,000; Abcam), Bax (rabbit monoclonal; 1:500; Abcam), Bcl2 (rabbit monoclonal antibody, dilution: 1:500; Abcam), cleaved caspase-3 (rabbit monoclonal antibody dilution: 1:500; Abcam), cleaved caspase-8 (rabbit polyclonal antibody, dilution: 1:500; Abcam), cleaved caspase-9 (rabbit polyclonal; 1:500; Abcam), MMP2 (rabbit polyclonal antibody, dilution: 1:500; Abcam), MMP9 (rabbit polyclonal antibody, dilution: 1:500; Abcam), ICAM-1 (rabbit polyclonal antibody, dilution: 1:500; Abcam); VEGFA (rabbit polyclonal; 1:500; Abcam); E-cadherin (rabbit polyclonal; 1:500; Abcam); N-cadherin (rabbit polyclonal antibody, dilution: 1:500; Abcam); TWIST (mouse monoclonal antibody, dilution: 1:500, Santa Cruz Biotechnology, Inc.); Vimentin (mouse monoclonal antibody, dilution: 1:500; Santa Cruz Biotechnology, Inc.); CSRC (rabbit monoclonal; 1:500; Abcam); p-CSRC (rabbit monoclonal antibody, dilution: 1:500; Abcam); PI3K, p-PI3K, AKT, p-AKT (rabbit monoclonal antibody, dilution: 1:500; Cell Signaling Technology, Inc.). Appropriate horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG, dilution: 1:5,000 and goat anti-mouse IgG, dilution: 1:3,000) at 25˚C for 1 h. The bands were detected using SuperECL Plus detection reagents (LI-COR Biosciences) and quantified with a Bio-Rad ChemiDoc imaging system (Bio-Rad Laboratories, Inc.) using β-actin as an internal control.

Cell transfection. RBE cells (30-40% confluence) were transfected with Lipofectamine® RNAiMAX Reagent (Thermo Fisher Scientific, Inc.) and 20 nM small interfering RNA (siRNA) (Guangzhou RiboBio Co., Ltd.) according to the manufacturer's instructions for 4 h at 37˚C. The sequences of all specific and control siRNAs are as follows: siRNA-1 CCUCUACAAGAGGUGGACAGAGAATT; siRNA-2 CCA UGGUGAGAAAUUGACGACUUCATT; siRNA-3 CAC CACUGCCAAGCUCACUAUUGAATT and control, CCU CUUACCUCAGUUACAAUUUAUATT. Three separate CEACAM6-specific siRNA sequences were used, and an unrelated control siRNA sequence was transfected using the same protocol, which had no effect on CEACAM6 expression.

Table I. Primer sequences for quantitative PCR.

Gene	Forward (5'-3')	Reverse (3'-5')
MMP-2	CCAACTACAACTTCTTCCCTCG	TCACATCGCTCCAGACTTG
MMP-9	ACGCAGACATCGTCATCCA	AGGGACCACAACTCGTCATC
ICAM-1	GTCATCATCACTGTGGTAGCAG	GGCTTGTGTGTTCGGTTTC
E-cadherin	GTGGTCAAAGAGCCCTTACTG	CGTTACGAGTCACTTCAGGC
N-cadherin	TCATTGCCATCCTGCTCTG	CATCCATACCACAAACATCAGC
Vimentin	AAATGGCTCGTCACCTTCG	AGAAATCCTGCTCTCCTCGC
TWIST1	GGAGTCCGCAGTCTTACGA	CTTGAGGGTCTGAATCTTGCT
Bcl-2	TGTGTGGAGAGCGTCAACC	TGGATCCAGGTGTGCAGGT
Bax	TTTCTGACGGCAACTTCAAC	AGTCCAATGTCCAGCCCAT
Caspase 3	ACTGGACTGTGGCATTGAGAC	TTGTCGGCATACTGTTTCAGC
Caspase 8	TGTTGGAGGAAAGCAATCTG	CTGCCTGGTGTCTGAAGTTC
Caspase 9	GCAGATTTGGCTTACATCCTG	ACGGCAGAAGTTCACATTGT
CEACAM6	CACTATTGAATCCACGCCG	TTGCCATCCACTCTTTCG
VEGFA	CTTGCCTTGCTGCTCTACCT	TGATGATTCTGCCCTCCTCCT
c-SRC	GAGGAGCCCATTTACATCG	CTTGAGAAAGTCCAGCAAACTC
PI3K	GCCTGCTCTGTAGTGGTAGATG	GGAGGTGTGTTGGTAATGTAGC
AKT	TACGAGATGATGTGCGGTC	TCTTGAGCAGCCCTGAAAG

ICAM-1, intercellular cell adhesion molecule-1; TWIST1, Twist-related protein 1; c-SRC, cellular SRC; CEACAM6, carcinoembryonic antigen-related cell adhesion molecule 6.

Non-transfected cells were also used as a negative control; these were treated with serum-free DMEM for 4 h at 37˚C. Knockdown of CEACAM6 expression was confirmed at 48 h by western blotting.

Cell viability determination. The viability of RBE cells was determined using a Cell Counting Kit (CCK)-8 assay (Abcam) according to the manufacturer's instructions. Briefly, transfected cells ($2x10^3$) in 96-well plates were incubated for 24 h at 37˚C and 5% CO_2. CCK-8 reagent (10 μl) was added for 1 h at 37˚C and optical density was measured at 460 nm.

Cell apoptosis analysis. RBE cells (80-90% confluence) transfected with siRNAs for 48 h were washed twice with PBS (both 30 sec at room temperature) and then resuspended in staining buffer containing 0.025 mg/ml Annexin V-FITC and 1 mg/ml propidium iodide (Shanghai Yisheng Biotechnology Co., Ltd.). Double staining was performed for 10 min at room temperature in the dark, and the number of apoptotic cells was then determined by flow cytometry using a BD FACSCanto™ II flow cytometer (BD Biosciences). The software used for data analysis is FlowJo (7.6.1; FlowJo, LLC.).

In vitro invasion assay. Cell invasion assays were performed using Transwell inserts (Corning, Inc.) coated with Matrigel (37˚C for 1 h) were performed as previously described (23). Briefly, RBE cells ($1x10^5$) in 0.2 ml serum-free DMEM were placed in the upper chamber, and the lower chamber was loaded with 0.5 ml medium containing 15% FBS. Cells that migrated to the lower surface of the filters were stained with 0.005% crystal violet solution for 40 min at room temperature, and cells in five fields of view were counted after 24 h of incubation at 37˚C and 5% CO_2 using light microscope. Three wells were examined for each cell type and condition, and the experiments were conducted in triplicate.

Wound healing assay. RBE cells ($6x10^5$) were seeded in 6-well plates. When cells reached 90% confluency, a 100-μl pipette tip was used to scratch the serum-starved cell monolayer (time 0 h). Images of migrating cells during the closure of the wounded region were captured at 0 and 24 h using light microscope (magnification, x10).

Statistical analysis. All statistical analyses were conducted using SPSS version 19.0 (IBM, Corp.). Data are presented as the mean ± standard error of the mean of three independent repeats. Comparisons of the means of different groups were performed by using two-way mixed ANOVA or one-way ANOVA. Pairwise comparisons with homogeneity of variance were performed using the post hoc Tukey's honestly significant difference and Student-Newman-Keuls tests. $P<0.05$ was considered to indicate a statistically significant difference.

Results

CEACAM6 is overexpressed in CCA tissues, and its expression level is negatively associated with tumor differentiation degree. As shown in Fig. 1, CEACAM6 was overexpressed in highly differentiated and less differentiated CCA tissues compared with its expression levels in highly differentiated and less differentiated paracancerous tissues ($P<0.05$). Furthermore, lesser differentiation degrees of CCA tissues resulted in higher CEACAM6 expression levels ($P<0.05$).

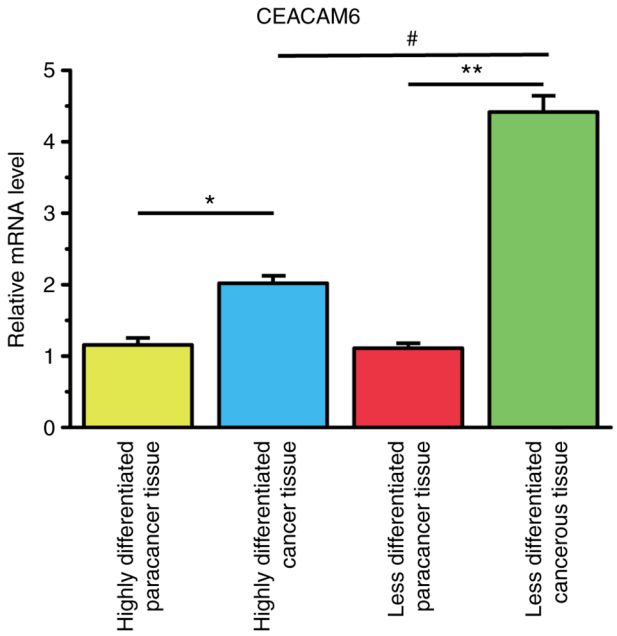

Figure 1. CEACAM6 expression in CCA and paracancerous tissues. Data are shown as the mean ± standard error of the mean (n=8), and were analyzed with two-way mixed ANOVA followed by Tukey's post hoc test. *P<0.05 highly differentiated paracancerous tissues vs. highly differentiated CCA tissues; **P<0.01 less differentiated paracancerous tissues vs. less differentiated CCA tissues; #P<0.05 highly differentiated CCA tissues vs. less differentiated CCA tissues. CEACAM6, carcinoembryonic antigen-related cell adhesion molecule 6; CCA, cholangiocarcinoma.

Silencing CEACAM6 inhibits cell and promotes cell apoptosis in RBE cells. After determining that CEACAM6 was highly expressed in CCA tissues, the role of CEACAM6 in the invasion and migration of RBE cells was next verified. siRNA transfection was used to silence the expression of CEACAM6. The results of Fig. 2A-D show that CEACAM6-siRNA-2 exhibited the best knockdown efficiency after 48 h. Thus, CEACAM6-siRNA-2 was utilized in subsequent experiments. As shown in Fig. 2E, CEACAM6-siRNA-2 significantly decreased RBE cell viability by ~50 compared with that of the control group (P<0.05). In addition, Fig. 2F shows that the percentage of apoptotic cells increased after transfection of CEACAM6-siRNA-2 (34.4%) compared with that of the scramble (scr)-siRNA group (13.8%; P<0.05).

CEACAM6 knockdown inhibits the invasion and migration of RBE cells. As shown in Fig. 3A and C, the invasion ability of RBE cells was decreased after transfection with CEACAM6-siRNA-2. Similar results were obtained regarding the migration ability of RBE cells, which was also declined upon CEACA-siRNA-2 transfection, as shown in Fig. 3B and D (P<0.05).

CEACAM6 silencing increases anti-apoptotic protein expression and decreases pro-apoptotic protein expression. Since the percentage of apoptotic cells increased after transfection with CEACAM6-siRNA-2, the present study next investigated whether the mRNA and protein expression levels of apoptosis-related proteins were consistent with the aforementioned findings. The results showed that the mRNA and protein levels of anti-apoptotic Bcl-2 decreased, while those of pro-apoptotic Bax increased after transfection with CEACAM6-siRNA-2.

The caspase family is composed of a class of proteolytic enzymes that mediate apoptosis and are activated during cell apoptosis. Caspases-3, -8 and -9 are major members of the caspase family. The results showed that the protein of cleaved caspases-3, -8 and -9 and mRNA levels of caspases-3, -8 and -9 were significantly upregulated after transfection with CEACAM6-siRNA-2 (Fig. 4A-G).

CEACAM6 silencing decreases EMT marker expression in RBE cells. There are four types of EMT markers (epithelial and interstitial cell, transcription factor and cytoskeleton); of them, the epithelial cell marker E-cadherin and the interstitial cell marker N-cadherin are the most studied. In the present study, E-cadherin expression was significantly increased, while N-cadherin expression was significantly decreased after CEACAM6-siRNA-2 transfection.

MMPs are a group of zinc ion (Zn^{2+})-dependent endopeptidases that degrade extracellular matrix (ECM) and then induce pathological processes, such as tumor cell invasion and migration. MMP-2 and MMP-9 are important MMPs, and the protein and mRNA expression levels of these endopeptidases were reduced after siRNA transfection.

The TWIST transcription factor enhances EMT by inhibiting E-cadherin expression and enhancing tumor cell migration and invasion. The protein and mRNA expression of TWIST decreased after transfection with CEACAM6-siRNA-2.

Vimentin is an intermediate filament protein in mesenchymal cells that regulates protein-protein interactions, such as those involving cytoskeletal proteins and cell adhesion molecules, which may participate in cell invasion, migration and signal transduction. After transfection with CEACAM6-siRNA-2, the expression level of this tumor-promoting molecule decreased.

Furthermore, tumor angiogenesis is associated with VEGFA overexpression in human iCCA and pCCA (24). In the present study, the protein and mRNA levels of VEGFA and tumorigenesis-promoting ICAM-1 decreased when CEACAM6-siRNA-2 was transfected into RBE cells. The aforementioned results are shown in Fig. 4H-R.

CEACAM6 silencing decreases the expression of members of the SRC/PI3K/AKT signal transduction pathway in RBE cells. The present study demonstrated that there was no significant difference in the relative mRNA levels of molecules involved in the SRC/PI3K/AKT signal transduction pathway when CEACAM6 was knocked down (Fig. 5A). However, when CEACAM6-siRNA-2 was added to the cells, the levels of phosphorylated molecules involved in the SRC/PI3K/AKT signal transduction pathway was reduced (Fig. 5B-E).

Discussion

CCA is the second most common type of primary liver cancer in human patients after HCC (5). The incidence and mortality rates of iCCA are increasing worldwide. Despite advances in the detection and treatment of metastatic CCA, mortality from

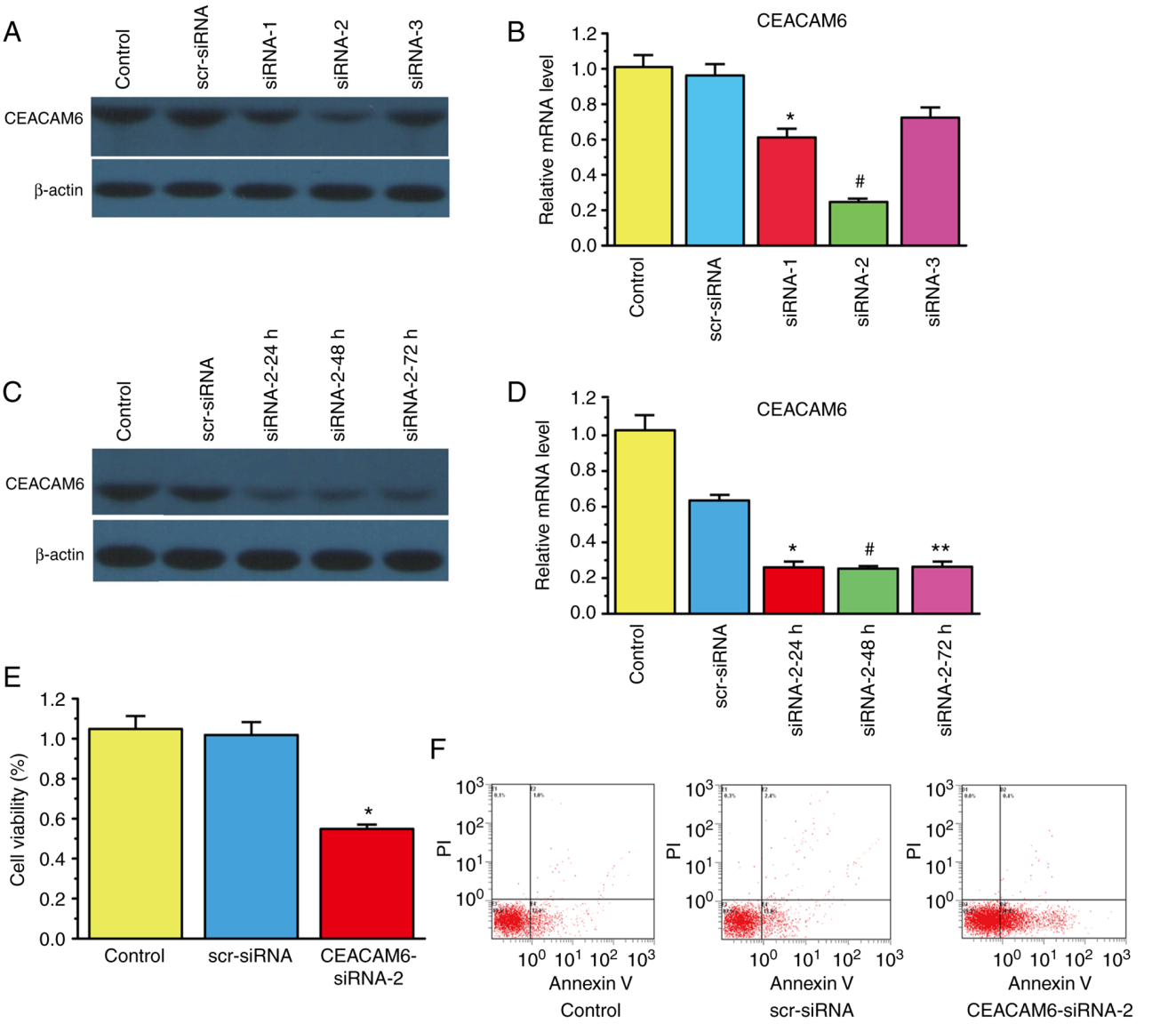

Figure 2. Effects of CEACAM6 silencing on RBE cell viability, apoptosis, invasion and migration *in vitro*. (A-D) Western blot and reverse transcription-quantitative PCR analyses were used to select siRNA and transfection time for CEACAM6 silencing. Data are shown as the mean ± standard error of the mean (n=8). Data were analyzed with one-way ANOVA followed by Tukey's (4 groups) or Student-Newman-Keuls (3 groups) post hoc tests. For part B: *P<0.05 scr-siRNA vs. siRNA-1; $^{\#}$P<0.05 scr-siRNA vs. siRNA-2. For part D: *P<0.05 scr-siRNA vs. siRNA-2-24 h; $^{\#}$P<0.05 scr-siRNA vs. siRNA-2-48 h; **P<0.05 scr-siRNA vs. siRNA-2-72 h. (E) Effects on cell viability of silencing CEACAM6 with CEACAM6-siRNA-2 after 48 h of transfection. *P<0.05 scr-siRNA vs. CEACAM6-siRNA. (F) Effects of silencing CEACAM6 by transfecting CEACAM6-siRNA-2 for 48 h on cell apoptosis. CEACAM6, carcinoembryonic antigen-related cell adhesion molecule 6; siRNA, small interfering RNA; scr, scramble.

this disease remains high due to its limited early-stage cytological and pathological diagnoses as well as limited effective treatments (1). Therefore, it is important to explore treatment methods based on specific markers and targets.

CEACAM6 is a member of the immunoglobulin superfamily, which is overexpressed in several human cancer types, including colorectal, pancreatic, lung and breast cancer (20,25,26). A previous report has revealed that CEACAM6 expression is associated with adverse pathological features and prognosis in pancreatic cancer (14). In agreement with those findings, the present study demonstrated that CEACAM6 was highly expressed in CCA tissues and was negatively associated with the differentiation degree of CCA, suggesting that CEACAM6 may be involved in the development and progression of CCA. The present study demonstrated that CEACAM6 was highly expressed in CCA tissue and was negatively associated with degree of differentiation.

CEACAM6 is oncogenic, as it inhibits cell differentiation, causes loss of cell polarity, and promotes cell adhesion, invasion and metastasis (27). These oncogenic properties are inhibited by an anti-CEACAM6 antibody in breast, pancreatic and colorectal cancer (28). Similarly, the present *in vitro* results revealed that the invasion and migration of RBE cells decreased after CEACAM6-siRNA-2 transfection.

Apoptosis, or programmed cell death, is an essential physiological process that plays a critical role in development and tissue homeostasis (29). The caspase cascade plays vital roles in the induction, transduction and amplification of intracellular

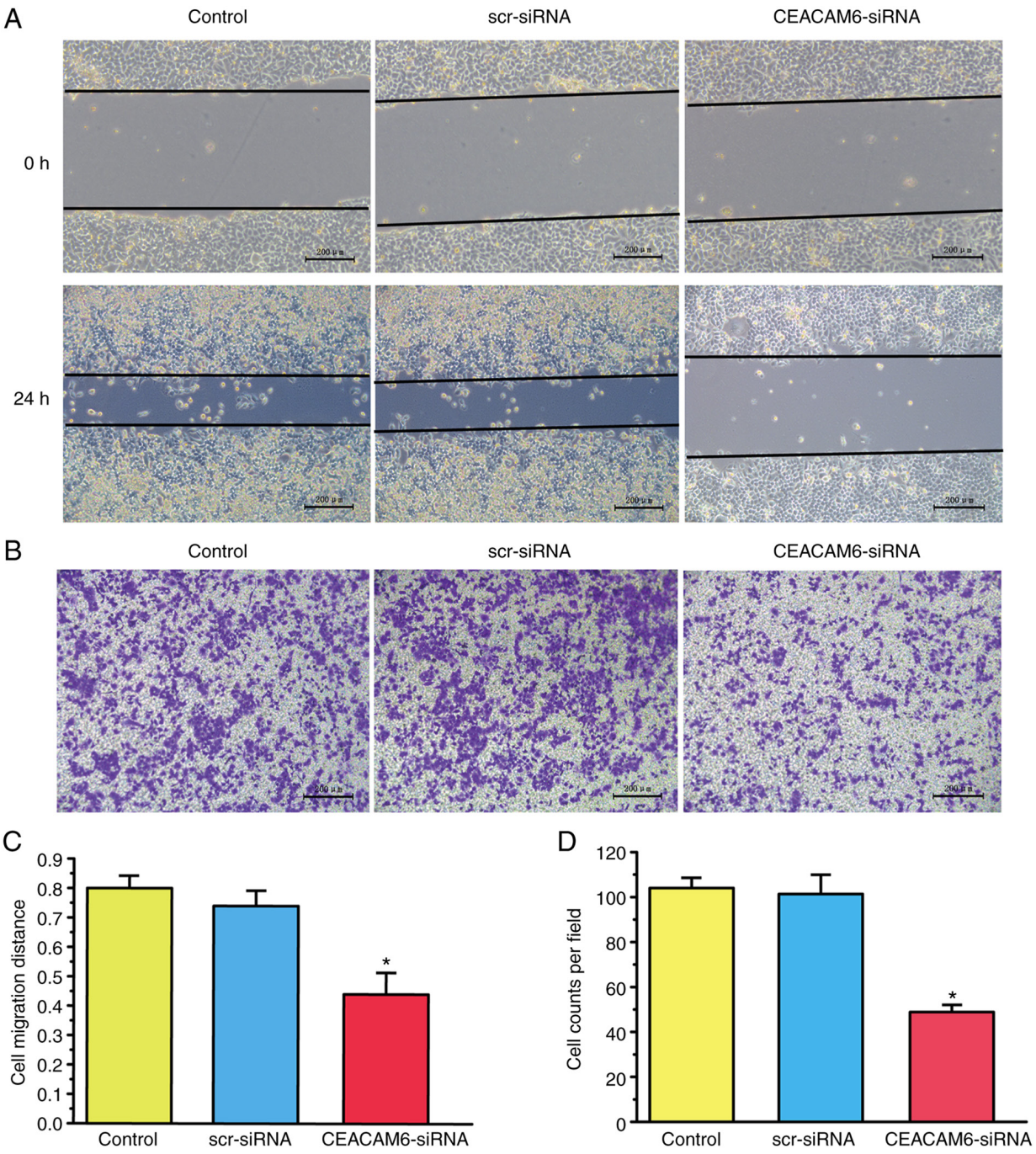

Figure 3. Effects CEACAM6 silencing on the invasion and migration of RBE cells *in vitro*. (A and C) Effects of silencing CEACAM6 via transfection of CEACAM6-siRNA-2 for 48 h on the wound healing and invasion abilities of RBE cells (magnification, x100; scale bar, 200 μm). (B and D) Effects of silencing CEACAM6 due to transfection of CEACAM6-siRNA-2 for 48 h on the invasion of RBE cells (magnification, x100; scale bar, 200 μm). Data are shown as the mean ± standard error of the mean (n=8), and were analyzed with one-way ANOVA followed by Student-Newman-Keuls post hoc test, *P<0.05, scr-siRNA vs. CEACAM6-siRNA. CEACAM6, carcinoembryonic antigen-related cell adhesion molecule 6; siRNA, small interfering RNA; scr, scramble.

apoptotic signals. The present results showed that the protein and mRNA expression levels of cleaved caspases-3, -8 and -9 increased after transfection with CEACAM6-siRNA-2.

EMT has gained increased attention in the past years regarding metastatic dissemination (9). The present study demonstrated that CEACAM6 was an important regulator of EMT biomarkers in CCA. However, there may be other mechanisms involved in CCA progression, such as the role played by circulating tumor cells or that of exosomes released by different tumor cell types (30,31), which suggests the need for further studies. Furthermore, a previous study has shown that the function of CEACAM6 is dependent on the c-SRC signaling pathway in pancreatic cancer (32).

In conclusion, the present study has demonstrated that CEACAM6 is significantly overexpressed in CCA tissues, which indicates that CEACAM6 could be utilized as a potential tumor EMT biomarker. Understanding the clinical significance of CEACAM6 expression and its oncogenic mechanism may eventually lead to the identification of a novel therapeutic target for human CCA treatment.

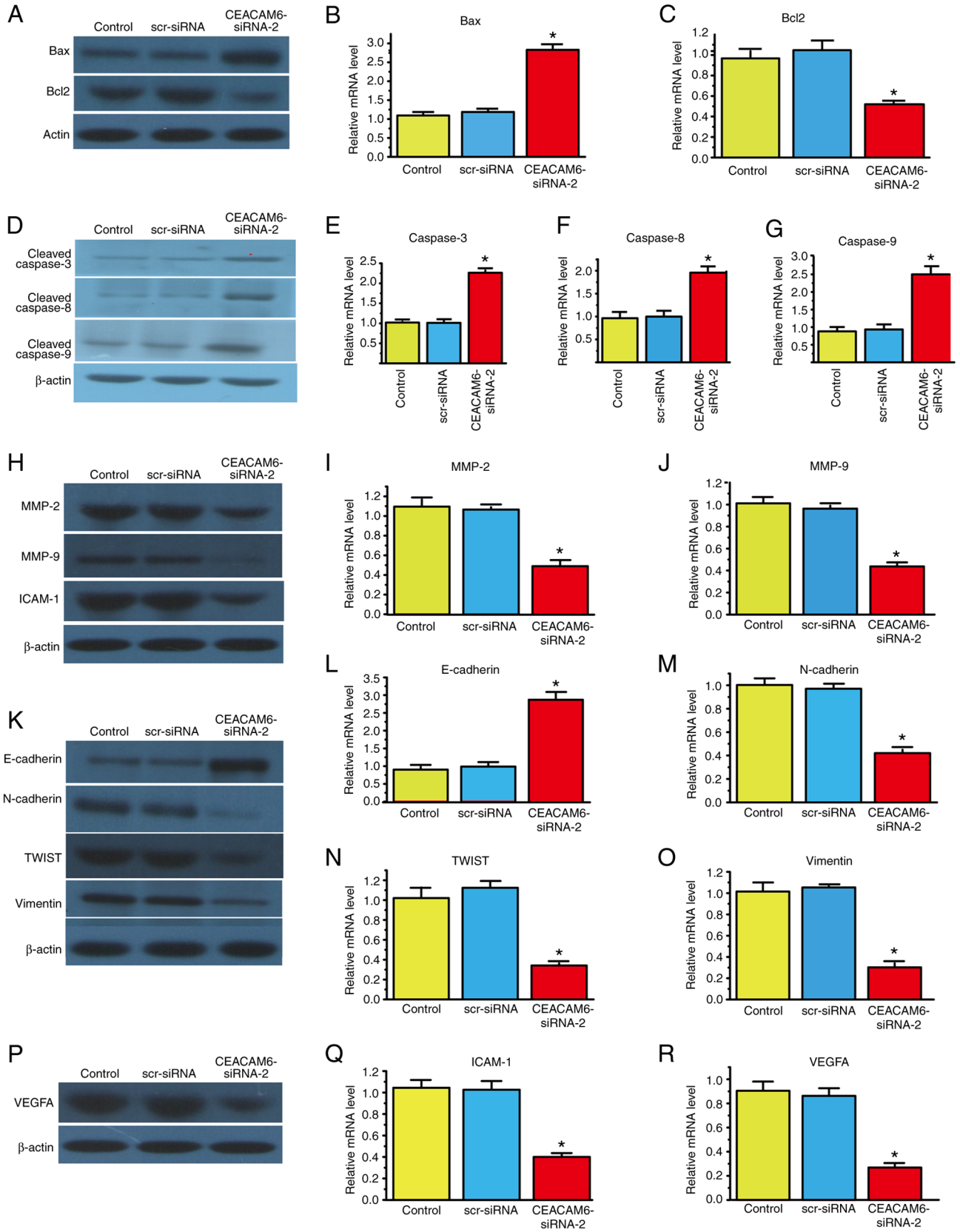

Figure 4. Effects of silencing CEACAM6 with CEACAM6-siRNA-2 after 48 h of transfection on the expression of apoptosis-related molecules and epithelial-mesenchymal transition markers. (A-G) Western blot and reverse transcription-quantitative PCR analyses were employed to detect the effects of silencing CEACAM6 on the expression of the pro-apoptotic protein Bax, cleaved caspases-3, -8 and -9, and the anti-apoptotic protein Bcl-2. (H-R) Western blot and reverse transcription-quantitative PCR analyses were used to detect the effects of silencing CEACAM6 on the expression of the extracellular matrix-related proteins MMP-2 and MMP-9, the epithelial cell marker E-cadherin, the interstitial cell marker N-cadherin, the intermediate filament protein vimentin, the transcription factor TWIST, the tumor nutrient vascular formation-related molecule VEGFA, and the tumorigenesis-promoting factor ICAM-1. Data are shown as the mean ± standard error of the mean (n=8), and were analyzed with one-way ANOVA followed by Student-Newman-Keuls post hoc test. *P<0.05 scr-siRNA vs. CEACAM6-siRNA. CEACAM6, carcinoembryonic antigen-related cell adhesion molecule 6; siRNA, small interfering RNA; scr, scramble; ICAM-1, intercellular cell adhesion molecule-1; TWIST, Twist-related protein.

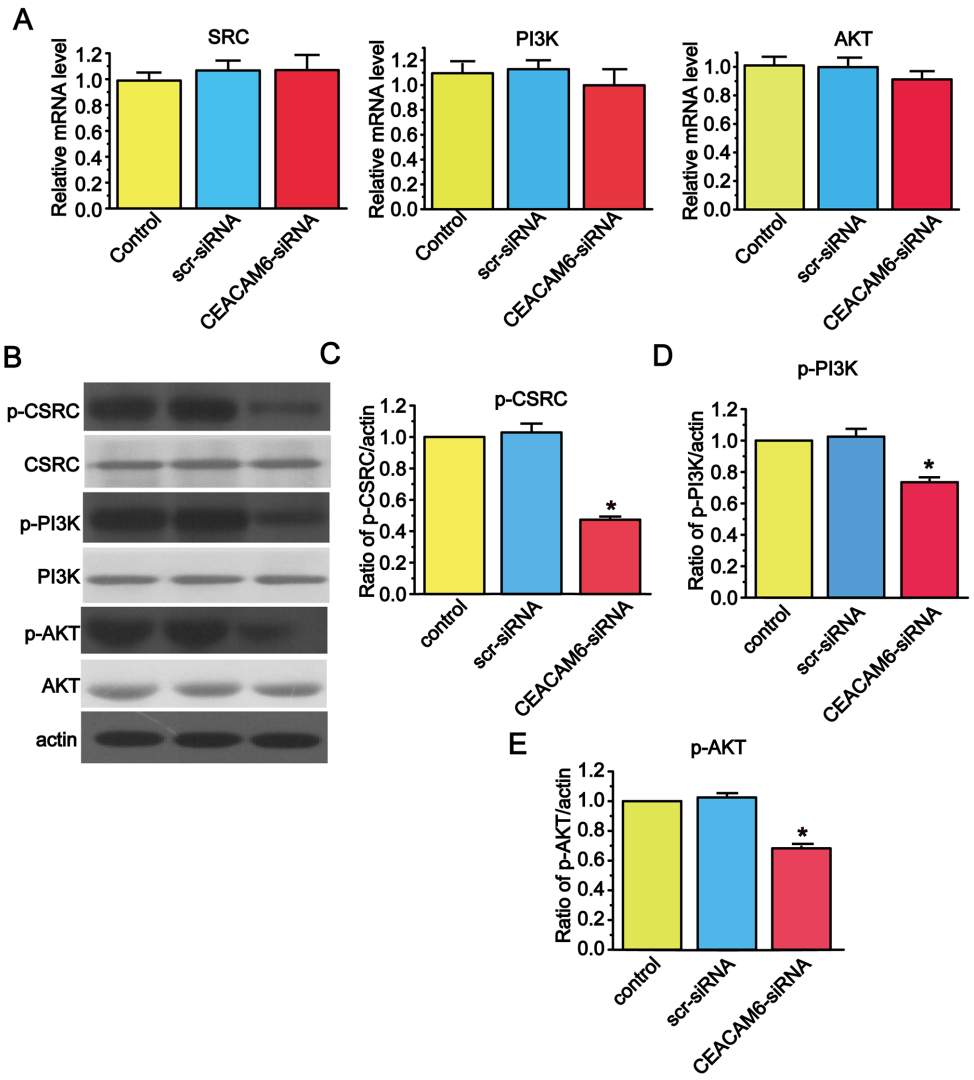

Figure 5. Effects of silencing CEACAM6 with CEACAM6-siRNA-2 after 48 h of transfection on the expression of members of the SRC/PI3K/AKT signal transduction pathway. (A) Reverse transcription-quantitative PCR analyses were performed to detect the effects of CEACAM6 silencing on the relative mRNA levels of SCR, PI3K and AKT. (B-E) Western blot analyses were conducted to detect the effects of CEACAM6 silencing on the levels of proteins associated with signal transduction (p-c-SRC, p-PI3K and p-AKT). Data are shown as the mean ± standard error of the mean (n=8) and were analyzed with one-way ANOVA followed by Student-Newman-Keuls post hoc test. *P<0.05 scr-siRNA vs. CEACAM6-siRNA. CEACAM6, carcinoembryonic antigen-related cell adhesion molecule 6; siRNA, small interfering RNA; c-SRC, cellular; p-, phosphorylated; scr, scramble.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

CL performed the experiments and acquired data. MW analyzed and processed data, and wrote and revised the manuscript. HL, BL, XY and WZ analyzed and interpretated data. HL, BL, XY and WZ confirm the authenticity of all the raw data. WW designed the experiments and gave final approval of the version to be published. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Ethics approval was from The Second Hospital of Hebei Medical University. Written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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