



# Carcinoembryonic antigen related cell adhesion molecule 6 promotes the proliferation and migration of renal cancer cells through the ERK/AKT signaling pathway

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**Background:** Carcinoembryonic antigen related cell adhesion molecule 6 (CEACAM6) is a versatile glycoprotein and a member of the CEACAM family. Studies suggested that it served as a diagnostic and prognostic biomarker in some malignancies. In addition, it is involved in tumorigenesis by stimulating proliferation, suppressing apoptosis, facilitating migration and invasion, promoting angiogenesis, and inducing drug resistance. In the present study, we demonstrated the oncogenic effects of CEACAM6 in clear cell renal cell carcinoma (ccRCC).

**Methods:** CEACAM6 expression was detected by quantitative real-time PCR (qRT-PCR), immunohistochemical staining and western blot in ccRCC tumor tissues and cell lines. Survival analysis was performed using the data of TCGA database. Cell proliferation and migration were detected by CCK-8 and transwell assays with the overexpression or silencing of CEACAM6. LY294002 was used to block the activation of PI3K/AKT pathway. Associated pathway proteins were detected by western blot.

**Results:** CEACAM6 was upregulated in ccRCC cell lines and tumor tissues. Longer overall survival was observed in patients with relatively low CEACAM6 levels. Furthermore, overexpression of CEACAM6 promoted the proliferation and migration of ccRCC cells. Conversely, shRNA-mediated CEACAM6 depletion modulated those changes. Further investigation demonstrated that the ERK/AKT signaling pathway activation played a pivotal role. In addition, PI3K/AKT pathway blockade abrogated the effects of CEACAM6 overexpression.

**Conclusions:** Aberrantly high expression of CEACAM6 is a stimulus for the formation and progression of ccRCC.

**Keywords:** Carcinoembryonic antigen related cell adhesion molecule 6 (CEACAM6); clear cell renal cell carcinoma (ccRCC); proliferation; migration

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## Introduction

Kidney cancers are common genitourinary cancers, more than 90% of which are renal cell carcinomas (RCCs) originating from the renal parenchyma (1). RCC is insensitive to conventional chemotherapy and radiotherapy (2). Therefore, surgical intervention, such as radical or partial resection of tumor-bearing kidneys, is the optimal treatment option for localized RCC (3). For metastatic RCC (mRCC), due to the expansion of therapeutic methods, resulting from the advent of targeted agents, systemic therapy using multi-kinase inhibitors, immunotherapies, surgery or a combination of them has been the mainstay of treatment (4–6). Although the management of RCC has been greatly improved in the past decade, many patients still suffer an unfavorable prognosis (7,8). A better understanding of the pathogenesis and further exploration of novel biomarkers or therapeutic targets is therefore urgently needed.

Carcinoembryonic antigen related cell adhesion molecules (CEACAMs) comprise a multitude of glycoproteins belonging to the immunoglobulin superfamily. They are anchored to the cell surface through a transmembrane domain or glycosylphosphatidylinositol (GPI) anchor (9), and they mediate intercellular adhesion by homotypic or heterotypic binding with other CEACAM family members (10). Studies have demonstrated that CEACAMs are implicated in numerous cellular processes, such as cell adhesion, cell proliferation and angiogenesis, and tumorigenesis (11,12).

As a versatile glycoprotein of the CEACAM family, extensive research suggests that CEACAM6 could serve as a biomarker for some human cancers. By detecting the CEACAM6 level, researchers found that high biliary CEACAM6 levels can predict the presence of an extrahepatic cholangiocarcinoma (13). In the current study on clinical samples, Upregulated levels of CEACAM6 indicate a poor prognosis for gastric carcinoma patients (14). In addition, CEACAM6 contributes to the development of some malignancies according to the clinical samples and cell culture experiments. For instance, CEACAM6 facilitates the proliferation of pancreatic carcinoma cells and promotes the metastasis of gastric cancers (15–17).

CEACAM family has not been extensively studied in RCC. Previous research indicates that CEACAM1 acts as a tumor suppressor gene in RCC (18). Ho *et al.* demonstrated the differential expression of CEACAM6 between patient-matched primary and mRCC (19). However, the role of CEACAM6 in RCC is still unclear. Hence, this study was

designed to investigate the function of CEACAM6 in RCC.

## Methods

### *Tissue samples and cell culture*

clear cell (ccRCC) tissues (n=15) and adjacent normal kidney tissues (n=15) were collected from ccRCC patients that underwent surgery in Shanghai Tenth People's Hospital after collecting informed consent and the approval of the Medical Ethics Committee. Human proximal tubule epithelial cell line HK-2 and ccRCC cell lines, containing 786-O, A498, OSRC-2 and SN12-PM6, were obtained from the American Type Culture Collection. All the cells grew in the media supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>.

### *RNA isolation and quantitative real-time PCR (qRT-PCR)*

Total RNA was extracted from frozen tissues using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. The concentration and purity of RNA was determined using an ND-2000 Spectrophotometer (Thermo Fisher Scientific, USA). Reverse transcription was performed using a PrimeScript RT reagent kit (TaKaRa, Japan), and qRT-PCR was performed with the KAPA SYBR FAST qPCR Kit (Kapa Biosystems, USA) using a 7900HT Fast Real-Time PCR System (Applied Biosystems, Japan). The CEACAM6 expression levels were normalized to  $\beta$ -actin. Data were analyzed using the  $2^{-\Delta\Delta Ct}$  method. The primer sequences were as follows: CEACAM6 forward primer: 5'-TCAATGGGACGTTCCAGCAAT-3'; CEACAM6 reverse primer: 5'-CACTCCAATCGTGATGCCGA-3'.  $\beta$ -actin forward primer: 5'-GGGACCTGACTGACTACCTC-3';  $\beta$ -actin reverse primer: 5'-TCATACTCCTGCTTGCTGAT-3'.

### *Western blot analysis*

Cell samples were treated with RIPA lysis buffer (Beyotime Biotechnology) containing protease inhibitors to extract protein. After quantification with a BCA protein assay kit (Thermo Fisher Scientific), equal amounts of lysates were loaded into each polyacrylamide gel well. Proteins of interest were separated by electrophoresis and transferred onto nitrocellulose membranes. Then, the protein bands were blocked with 5% nonfat milk for 2 h. Optimally

diluted primary antibodies and corresponding secondary antibodies were used for immunoreaction. Finally, protein bands were scanned with an Odyssey infrared imaging system (LI-COR Biosciences). All the antibodies, including CEACAM6, AKT, p-AKT, ERK, p-ERK, C-MYC, MMP-9, Survivin, and  $\beta$ -actin were purchased from Abcam.

### *Immunohistochemical staining*

Tissue samples were fixed with 10% formaldehyde and embedded in paraffin. Paraffin tissue blocks were sliced into sections. After deparaffinization with dimethylbenzene and rehydration with an ethanol gradient, endogenous peroxidase was inactivated by incubating with 3% H<sub>2</sub>O<sub>2</sub> for 10 min. Then the sections were immersed in 10 mM sodium citrate buffer solution (pH 6.0) and boiled with a pressure-cooker to retrieve the antigens. Bovine serum albumin was used for blocking non-specific binding sites. Afterward, the sections were incubated with a CEACAM6 antibody overnight at 4 °C and a corresponding secondary antibody for 40 min at 37 °C. Finally, the sections were stained with diaminobenzidine and counterstained with hematoxylin. Immunoreactivity was observed under a microscope.

### *Plasmid construction and cell transfection*

CEACAM6 overexpression and silencing vectors were obtained from Genechem (China). To elevate CEACAM6 expression, GV230 plasmids were linearized by restriction endonucleases. The CEACAM6 (NM\_002483) sequence was synthesized by PCR using the following primers. CEACAM6 (24388-1) Forward: TACCGGACTCAGATCTCGAGCGCCACCATGGGACCCCTCAGCCCC; CEACAM6 (24388-1) Reverse: GATCCCGGCCCCGCGGTACCGTTATCAGAGCCACCCTGGCCAGCAC. Then the gene segments were integrated into linearized GV230 plasmids. PCR was performed to distinguish the bacterial colonies. The primers used were as follows. CMV-F: CGCAAATGGGCGGTAGGCGTG; pEGFP-N-3: CGTCGCCGTCCAGCTCGACCAG. The sequence of identified transformants were verified by gene sequencing. Similarly, sequences targeting CEACAM6 (GTATTGGTTACAGCTGGTA) were used to interfere with CEACAM6 expression. The recombinant plasmids

or negative control vectors were transfected into RCC cell lines using Lipofectamine 2000 (Invitrogen).

### *Cell Counting Kit-8 (CCK-8) assay*

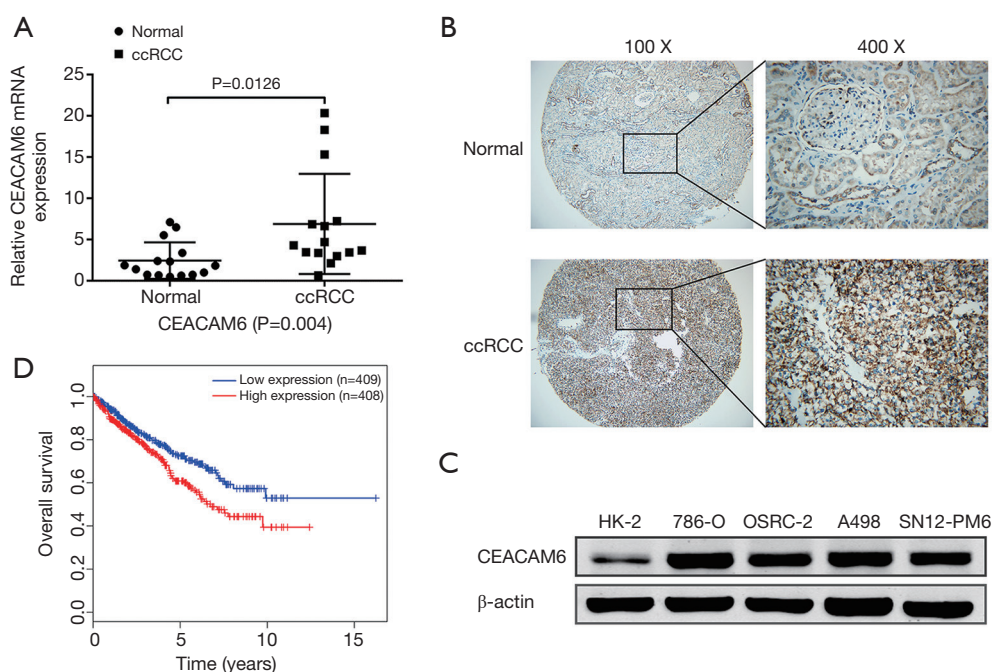
A CCK-8 assay was conducted to assess cell viability. Briefly, 786-O or A498 cells were seeded into 6-well plates and subsequently transfected with recombinant plasmids to alter CEACAM6 expression. Then the cells were harvested and a 100  $\mu$ L cell suspension was added into each well of the 96-well plates. Five duplicates were set for each group. For rescue assays, the cells were additionally treated with the PI3K/AKT pathway inhibitor LY294002 (Selleck, s1105) for 24 h. Finally, 10  $\mu$ L of CCK-8 was added to the culture medium after incubation. One hour later, the absorbance at 450 nm was measured using a microplate reader.

### *Transwell assay*

Cell migration capability was assessed by transwell assay using transwell chambers (Corning, 3422). Similarly, 786-O or A498 cells were seeded into 6-well plates and transfected with GV230-CEACAM6, sh-CEACAM6, or negative control vectors. Forty-eight hours later, the cells were digested and resuspended with serum-free medium. A 200  $\mu$ L cell suspension was added into upper chamber. Five-hundred  $\mu$ L complete medium containing 10% FBS were added into the lower chamber. For the rescue assay, the cells were additionally treated with LY294002 for 24 h. Finally, the chambers were washed with PBS. Cells migrating to the lower surface of the polycarbonate membrane were treated with 4% paraformaldehyde and 0.1% crystal violet. Migrated cells from multiple fields of vision were counted to assess cell migration.

### *Statistical analysis*

All the results were obtained from experiments conducted in triplicate. Data were expressed as mean  $\pm$  SD. Statistical analysis was performed with GraphPad Prism 6 (GraphPad Software, USA) using Student's *t*-test and one-way ANOVA. The correlation between CEACAM6 expression and overall survival of RCC patients was accessed by Kaplan-Meier analysis. Log-rank test was used to calculate *p* values.



**Figure 1** CEACAM6 was upregulated in both ccRCC tissues and cell lines and was associated with overall survival. ccRCC tumor tissues (n=15) and normal kidney tissues (n=15) were collected. (A) The relative mRNA levels of CEACAM6 were detected by qRT-PCR.  $P < 0.05$ ; (B) the increased expression of CEACAM6 was confirmed by immunohistochemical staining. The images were taken under a microscope with magnifications of  $\times 100$  and  $\times 400$ ; (C) the protein levels of CEACAM6 in several RCC cell lines and HK-2 cells were detected by Western blot; (D) Kaplan-Meier analysis of the correlation between CEACAM6 expression and overall survival for RCC patients was performed. P value was calculated by log-rank test.  $P < 0.01$ . CEACAM6, carcinoembryonic antigen related cell adhesion molecule 6; ccRCC, clear cell renal cell carcinoma; qRT-PCR, quantitative real-time PCR.

$P < 0.05$  denoted statistical significance.

## Results

### *CEACAM6 was upregulated in both ccRCC tissues and cell lines and was associated with overall survival*

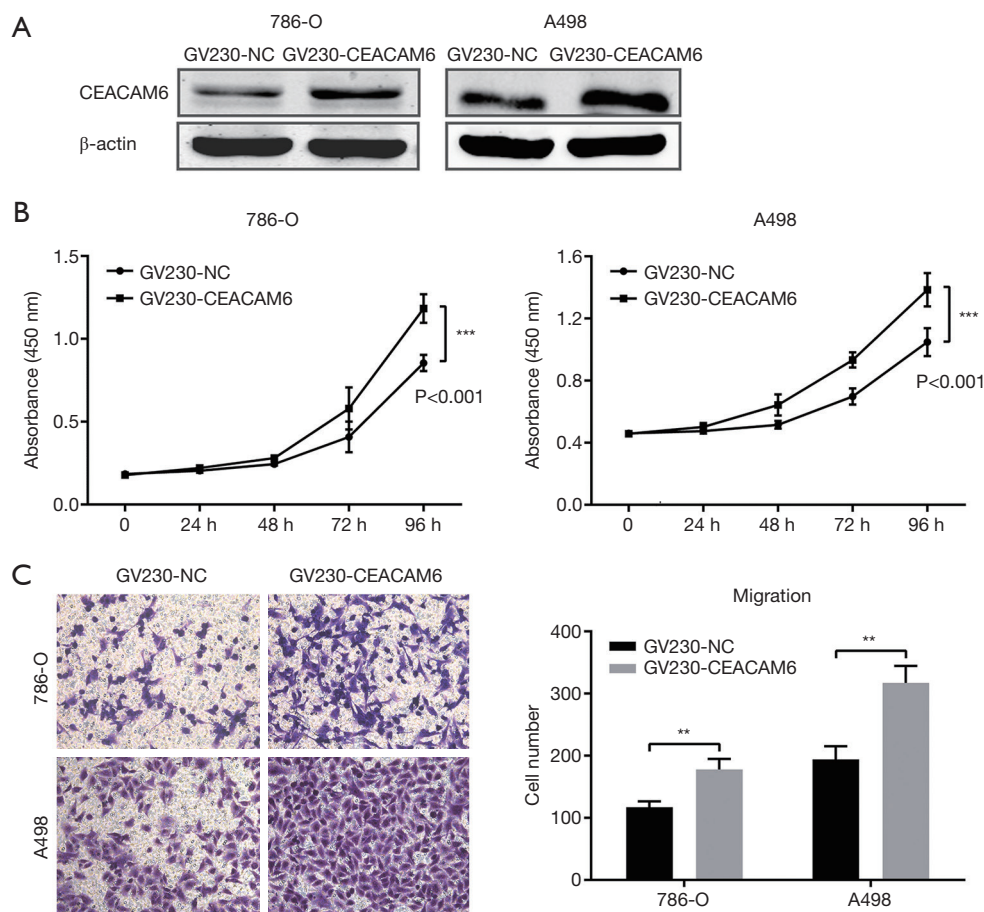
Several studies have shown that CEACAM6 deregulation arises in a multitude of malignancies and it is related to tumorigenesis and metastasis. These features indicate the potential of CEACAM6 in serving as a novel biomarker or therapeutic target. Herein, we detected the differential expression of CEACAM6 in ccRCC tissues (n=15) and normal kidney tissues (n=15). According to the results of qRT-PCR, CEACAM6 mRNA levels were significantly elevated in RCC tissues ( $P = 0.0126$ ) (Figure 1A). Moreover, immunohistochemical staining further confirmed CEACAM6 was overexpressed in ccRCC tissues compared with normal tissues (Figure 1B). Subsequently, we detected the expression of CEACAM6 in ccRCC cell lines. In line with

our other results, Western blotting revealed CEACAM6 protein levels were elevated in 786-O, OSRC-2, A498 and SN12-PM6 cells compared with HK-2 cells (Figure 1C). Accordingly, we can propose that the elevated level of CEACAM6 is a potential tumor promotor that is involved in the development of RCC.

In addition, the TCGA database was introduced to investigate the prognostic significance of CEACAM6 in RCC. The clinical data of 817 RCC patients was collected for Kaplan-Meier analysis. As shown in Figure 1D, patients with relatively high levels of CEACAM6 had shorter overall survival than those with low CEACAM6 expression, indicating a negative correlation between CEACAM6 expression and overall survival.

### *CEACAM6 deregulation modulated the proliferation and migration of ccRCC cells*

To investigate the pathophysiological roles of CEACAM6



**Figure 2** CEACAM6 overexpression facilitated the proliferation and migration of RCC cells. 786-O and A498 cells were transfected with GV230-CEACAM6 or GV230-NC (NC) to elevate CEACAM6 expression. (A) Transfection efficiency was confirmed by Western blot; (B) cell proliferation was detected by CCK-8 assay. The absorbances at 450 nm were examined after transfection for 0, 24, 48, 72, or 96 h; (C) cell migration was detected by transwell assay. Cells were stained by crystal violet,  $\times 200$ . Data were analyzed by Student's *t*-test. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . CEACAM6, carcinoembryonic antigen related cell adhesion molecule 6.

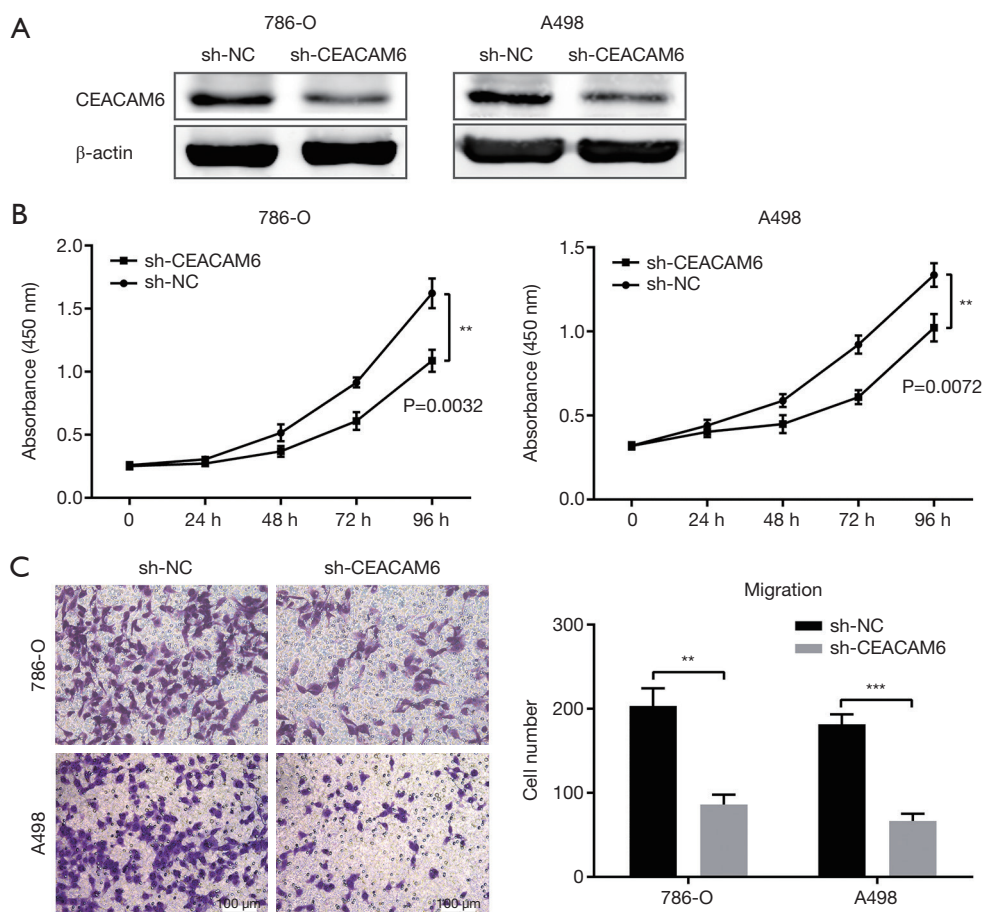
in ccRCC, recombinant plasmids containing CEACAM6 coding sequences were constructed to elevate the expression of CEACAM6 in 786-O and A498 cells. Transfection efficiency was confirmed by Western blot (Figure 2A). Then CCK-8 assays were performed to assess cell viability. Increased CCK-8 was observed in CEACAM6 overexpressing ccRCC cells (Figure 2B), implying that CEACAM6 facilitates cell proliferation. In addition, cell migration was detected by transwell assays. CEACAM6 overexpression stimulated the migration of ccRCC cells (Figure 2C). In contrast, specific gene silencing vectors were used to knock-down the endogenous expression of CEACAM6. As shown in Figure 3A, targeting CEACAM6 with shRNA successfully interfered with the expression of

CEACAM6. Subsequent tests revealed that cell proliferation and migration were significantly suppressed following CEACAM6 silencing (Figure 3B,C).

In summary, CEACAM6 contributed to the growth and migration of ccRCC cell lines, which provided additional evidence that CEACAM6 is involved in carcinogenesis. Moreover, CEACAM6 silencing was effective in suppressing these malignant phenotypes.

#### ***CEACAM6 exerted oncogenic effects in ccRCC through the ERK/AKT signaling pathway***

Subsequently, Western blotting was performed to clarify the underlying molecular mechanisms. It is well known



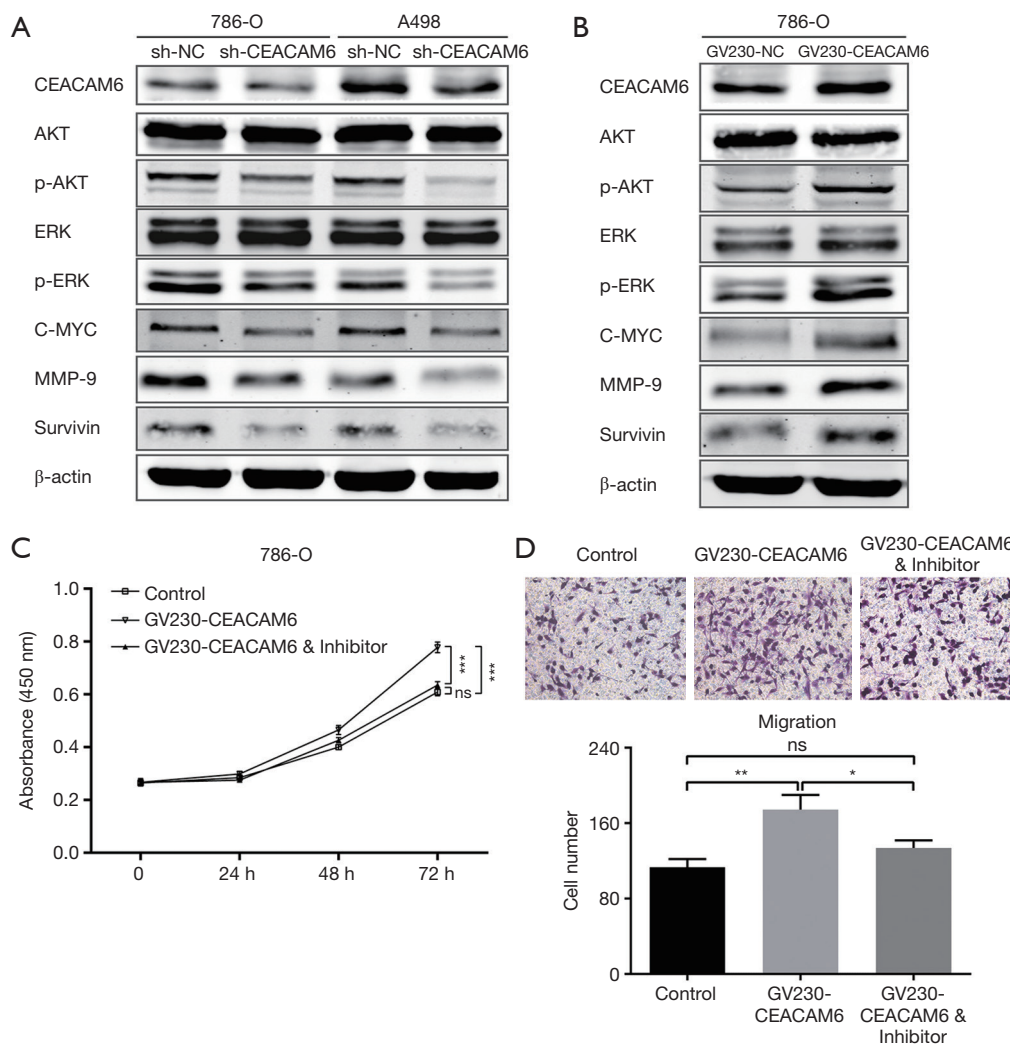
**Figure 3** CEACAM6 silencing suppressed the proliferation and migration of RCC cells. CEACAM6-specific shRNAs were used to induce gene silencing in 786-O and A498 cells. (A) CEACAM6 silencing was confirmed by Western blot; (B) after transfection for 0, 24, 48, 72, or 96 h, cell viability was assessed by CCK-8 assay; (C) cell migration was detected by transwell assay, crystal violet staining,  $\times 200$ . Data were analyzed by Student's *t*-test. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . CEACAM6, carcinoembryonic antigen related cell adhesion molecule 6.

that ERK and AKT are pivotal signals for the initiation and progression of cancers by modulating downstream signaling cascades (20-22). After reducing CEACAM6 expression with specific shRNA, we found that phosphorylated ERK and AKT levels decreased, indicative of inactivation of the ERK/AKT signaling pathway (Figure 4A). Furthermore, C-MYC and Survivin, two key oncogenes associated with cell growth and survival (23,24), and migration elements, such as MMP-9, were also downregulated (Figure 4A). On the other hand, to investigate the activation of these signals by overexpressing CEACAM6, 786-O cells were transfected with GV230-CEACAM6 plasmids. Figure 4B demonstrated the activation of the ERK/AKT signaling pathway with the increased protein levels of p-AKT, p-ERK, C-MYC, Survivin, and MMP-9. To further verify this mechanism,

CEACAM6 overexpressing 786-O cells were treated with a specific PI3K/AKT pathway inhibitor. The capabilities of cell proliferation and migration were assessed by CCK-8 and transwell assays. As shown in Figure 4C,D, increased cell proliferation and migration were abrogated by PI3K/AKT pathway blockage. The oncogenic effects of CEACAM6 were potentially ascribed to the activation of the ERK/AKT signaling pathway.

## Discussion

CEA, also known as CEACAM5, has been identified as a colon cancer-associated antigen (25). CEACAM6, another GPI-anchored glycoprotein, like CEA, has attracted recent attention as another potential cancer-associated antigen.



**Figure 4** CEACAM6 exerts oncogenic effects in RCC through the ERK/AKT signaling pathway. (A) The protein levels of several pivotal molecules were detected by Western blot following CEACAM6 silencing; (B) the expression of the above molecules following CEACAM6 overexpression. After transfection with GV230-CEACAM6 plasmids, 786-O cells were treated with LY294002 for 24 h. Cell proliferation and migration was detected by CCK-8 assay (C) and transwell assay (D) respectively. Cells were stained by crystal violet,  $\times 200$ . Data were analyzed by one-way ANOVA. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . CEACAM6, carcinoembryonic antigen related cell adhesion molecule 6; ns, no significant difference; RCC, renal cell carcinoma.

Overexpression of CEACAM6 has been observed in numerous malignancies, such as pancreatic cancer, gastric cancer, colorectal cancer, cholangiocarcinoma, breast cancer, head and neck cancer, mucinous ovarian neoplasms, leukemias, and RCCs (17,19,26-32). In the present study, we observed overexpression of CEACAM6 in ccRCC tissues by qRT-PCR and immunohistochemical staining, which was further validated in ccRCC cell lines. All of these indicate that overexpression of CEACAM6 may induce the

development of ccRCC.

CEACAM6 has been extensively studied as a diagnostic and prognostic marker in a diverse group of malignancies. For example, elevated CEACAM6 levels in atypical ductal hyperplastic lesions can predict the formation of invasive breast cancer (33). High levels of CEACAM6 means shorter overall survival for lung adenocarcinoma patients (34). Previous research revealed an association between CEACAM6 levels and RCC patient

prognosis (19). Similarly, we found that CEACAM6 expression was negatively correlated with the overall survival of RCC patients, according to survival analysis of the data from the TCGA database. Despite this, the diagnostic and prognostic significance of CEACAM6 in RCC requires further investigation.

By stimulating proliferation, suppressing apoptosis, facilitating migration and invasion, promoting angiogenesis, and inducing drug resistance, CEACAM6 serves as a determinant in the malignant transformation of cells and cancer progression (11,35). To explore the function of CEACAM6 in RCC, CCK-8 and transwell assays were performed in conjunction with the overexpression or silencing of CEACAM6. As expected, excessive CEACAM6 enhanced the capabilities of cell proliferation and migration, while CEACAM6 silencing brought about opposite results. To summarize, CEACAM6 is an oncogene for ccRCC.

Diverse molecular mechanisms are involved in the pathophysiological effects of CEACAM6. In pancreatic adenocarcinomas, overexpression of CEACAM6 facilitates cell invasion through c-Src-mediated increases in MMP-9 activity (27). Moreover, CEACAM6 overexpression induces the phosphorylation activation of AKT and caspase-mediated anoikis resistance, thereby promoting pancreatic cancer metastasis *in vivo* (36). In gastric cancers, CEACAM6 accelerates metastasis by stimulating the activation of the PI3K/AKT pathway and increasing epithelial-mesenchymal transition (16); CEACAM6 also facilitates angiogenesis through FAK signaling (37). In addition, post-translational modification also plays a critical role in regulating the biological functions of CEACAM6. N-acetylglucosaminyltransferase 5-mediated N-glycosylation of CEACAM6 promotes the aggressive phenotypes of oral squamous cell carcinoma through epidermal growth factor receptor activation triggered signaling cascades (38). According to our research, CEACAM6 silencing suppressed the phosphorylation activation of ERK and AKT and downregulated the expression of C-MYC, Survivin, and MMP-9, thereby restraining the malignant phenotypes of RCC cells. Moreover, PI3K/AKT pathway blockage abrogated CEACAM6 overexpression and increased cell proliferation and migration. More intricate regulatory mechanisms and other pathways beyond ERK/AKT signaling may also be involved and require further investigation.

Considering the excessive expression of CEACAM6 in diverse cancers, depletion of CEACAM6 may be a valid strategy for oncotherapy (39). According to

previous studies, miR-29a can suppress the formation and progression of lung adenocarcinomas by attenuating CEACAM6 expression (34). siRNA-mediated CEACAM6 silencing restrains metastasis of pancreatic adenocarcinomas and sensitizes cancer cells to gemcitabine (36,40). A designed humanized anti-CEACAM6 single chain variable fragment can also inhibit the formation of pancreatic cancer (41). Moreover, the integration of CEACAM6-shRNA and yCDglyTK in a recombinant plasmid is an effective gene therapy for pancreatic cancer (42). In addition, 8F5, a monoclonal antibody targeting CEACAM6, increases anoikis, represses tumor growth *in vivo*, and enhances the therapeutic effects of paclitaxel in lung adenocarcinomas (43). In this study, RNA interference induced loss of function of CEACAM6, which was also effective in suppressing the growth and migration of RCC cells. Collectively, it is promising that targeting CEACAM6 by some specific approaches may represent an effective treatment option for RCC.

In summary, our preliminary study revealed the carcinogenic and prognostic significance of CEACAM6 in ccRCC. It warrants further evaluation for development as a novel therapeutic target for RCC.

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## Footnote

*Conflicts of Interest:* The authors have no conflicts of interest to declare.

*Ethical Statement:* The authors are 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. This Study was approved by the Ethic Committee of The Affiliated Shanghai Tenth People's Hospital, Nanjing Medical University (2015-35).

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