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Original Article

CEACAM6 promotes tumor migration, invasion, and metastasis in gastric cancer

Yunqiang Zhang[1,2]†, Mingde Zang[1]†, Jianfang Li[1], Jun Ji[1], Jianian Zhang[1], Xiaolei Liu[1], Ying Qu[1], Liping Su[1], Chen Li[1], Yinyan Yu[1], Zhenggang Zhu[1], Bingya Liu[1]*, and Min Yan[1]*

[1]Shanghai Key Laboratory of Gastric Neoplasms, Department of Surgery, Shanghai Institute of Digestive Surgery, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China
[2]Shanghai First Maternity and Infant Hospital, Tongji University School of Medicine, Shanghai 200040, China
†These authors contributed equally to this work.
*Correspondence address. Tel: +86-21-64670644; Fax: +86-21-64373909; E-mail: byliu@sjtu.edu.cn (B.L.)/Tel: +86-21-64370045; Fax: +86-21-64373909; E-mail: ym10299@163.com (M.Y.)

Carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) shows increased expression in a wide variety of human cancers, and its over-expression is associated with enhanced migration, invasion, and *in vivo* metastasis. Here, we reported that CEACAM6 was up-regulated in gastric cancer (GC) cell lines and tumor tissues. Over-expression of CEACAM6 in MKN-45 and SGC-7901 GC cells promoted migration and invasion *in vitro* and metastasis in athymic mice, whereas migration and invasion of MKN-28 and SNU-16 GC cells were suppressed by knock-down of CEACAM6. We also observed that steroid receptor coactivator (C-SRC) phosphorylation was increased when CEACAM6 was over-expressed in SGC-7901 cells. Taken together, these results suggested that CEACAM6 functions as an oncoprotein in GC and may be an important metastatic biomarker and therapeutic target.

Keywords gastric cancer; CEACAM6; C-SRC; metastasis; invasion

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Introduction

Gastric cancer (GC) is the second most common cause of cancer-related death in the world [1]. Metastasis, the major obstacle to successful GC treatment, is a multi-step process that involves the dissemination of cancer cells to anatomically distant sites and their subsequent adaptation to local microenvironments [2]. The carcinoembryonic antigen (CEA) protein family belongs to an immunoglobulin protein superfamily, members of which play important roles in cell adhesion as well as cancer cell invasion and metastasis [2]. Abnormal expression of carcinoembryonic antigen-related cell adhesion molecule (CEACAM)1, CEACAM5, CEA CAM6, and CEACAM7, which are the best-characterized CEA family members, has been observed in human cancers. CEACAM1 expression is very dynamic in tumor tissues: the protein isoforms are considerably reduced in the early phases of many cancers including colon, prostate, liver, and breast cancers, indicating that it acts as a tumor suppressor protein. However, in other types of aggressive cancers such as melanoma, non-small-cell lung, gastric, thyroid, and bladder cancers, CEACAM1 is over-expressed and correlated with metastasis [3]. CEACAM7 is down-regulated or absent in a variety of epithelial-derived neoplasms and is considered as a tumor suppressor. In contrast, over-expression of CEACAM5 and CEACAM6 is detected in nearly 70% of solid tumors, including cancers of the gastrointestinal tract, breast, lung, and female reproductive system, and is associated with greater migration, invasion, and metastasis *in vitro* [4–9]. Expression of CEACAM6 is suggested to be an independent prognostic factor in colorectal cancer [8,9], and its level is associated with tumor stage, metastasis, and post-operative survival of patients with pancreatic cancer [10]. However, few studies have thus far been focused on CEA CAM6 expression in GC and the resulting data are still largely controversial. Oue *et al*. [11] found that CEACAM6 is over-expressed in GC but is not associated with any clinicopathological features, in keeping with Kinugasa *et al*. [12], whereas Zhao *et al*. [2] found that CEACAM6 expression in peripheral blood, detected by reverse transcription-polymerase chain reaction (RT-PCR), is associated with tumor stage. In the present study, we investigated CEACAM6 expression in GC tumors and its role in GC metastasis.

Materials and Methods

Tissues and cell lines

Primary tumor tissues and matched adjacent non-tumor tissues were obtained from 101 GC patients (male: $n = 74$, female: $n = 24$; age: 36–83 years, average 64.1 years; TNM stage: I,

II, $n = 38$; III, IV, $n = 60$) during radical gastrectomy at the Department of Surgery, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine from August 2010 to January 2012. Three patients' clinical parameters were unavailable. All samples were collected with patients' informed consent and confirmed by pathological examination. The pathological tumor staging was determined according to the International Union against Cancer staging system (2007). The GC cell lines, SGC-7901, and AGS, were purchased from the Culture Collection of the Chinese Academy of Science (Shanghai, China); MKN-45 and MKN-28 were obtained from the Japanese Cancer Research Resources Bank (Tsukuba, Japan); NCI-N87, BGC-823, SNU-1, SNU-16, and KATOIII were obtained from the American Type Culture Collection (Manassas, USA). GES-1, an immortalized gastric epithelial cell line, was a gift from Dr Feng Bi (Huaxi Hospital of Sichuan University, Chengdu, China). Cells were cultured at 37°C with 5% CO_2 in RPMI-1640 medium containing 10% fetal bovine serum (FBS). Exponentially growing cells were used for experiments.

Small interfering RNA expression vector construction and transfection

CEACAM6-specific small interfering RNA (siRNA) oligos with the following sequence were purchased from Biomics Biotechnologies Co., Ltd. (Nantong, China): CCGGACAG UUCCAUGUAUAdTdT (sense), UAUACAUGGAACUG UCCGGdTdT (antisense). *CEACAM6* cDNA was amplified from the SNU-16 cells by using RT-PCR with primer sequences of 5′-CCGGAATTCCCATGGGACCCCCCTCA GCCC-3¢ (forward) and 5′-TCCCCCGGGGCTATATCAG AGCCACCCTGG-3′ (reverse). The PCR products were purified and then ligated into a 19T-simple vector (Takara Biotechnology Co., Ltd., Dalian, China), followed by sequencing. The cDNA was then reconstructed into a pIRES2-EGFP vector at the *Eco*RI and *Sma*I restriction enzyme sites (Takara Biotechnology Co., Ltd.), coined herein as pIRES2-EGFP/pIRES2-CEACAM6. The siRNA (50 nM) was delivered to SNU-16 and MKN-28 cells by using Lipofectamine 2000 (Invitrogen, Carlsbad, USA). The pIRES2-EGFP and pIRES2-CEACAM6 plasmids were delivered to the SGC-7901 and MKN-45 cells by using Lipofectamine 2000 and the stable clones were selected by continuous treatment with G418 (1.2 mg/ml, Gibco, Newyork, USA). All transfections were carried out according to the manufacturer's protocol. Detection of CEACAM6 in cells transfected with siRNA was performed 48 h after transfection.

Detections of CEACAM6 expression

The 101 pairs of GC tumor and non-tumor tissue were embedded, by Shanghai Outdo Biotech Company, into two tissue arrays of paraffin blocks, one with 56 cases and the other with 45 cases. Immunohistochemical staining of the two array sections was performed according to DAKO's protocol, using mouse anti-CEACAM6 (1 : 100, ab78029 [9A6], Abcam, Cambridge, UK). The percentage (P) of positive cells was scored 0 for $<1\%$, 1 for 1%−10%, 2 for 11%−50%, 3 for 51%−75%, and 4 for $>75\%$ of the cells examined. The staining intensity (I) was graded as the following: 0 for no staining, 1 for light brown staining, 2 for brown staining, and 3 for dark brown staining. For statistical analysis, total scores ($P \times I$) from 0 to 3 were categorized as low CEACAM6 expression, whereas scores from 4 to 12 were categorized as high CEACAM6 expression. The scores were determined separately for each section by two independent experts under the same conditions. In rare cases, discordant scores were reevaluated and scored on the basis of consensus.

To detect the expression in cultured cells, a cell suspension containing $1-5 \times 10^5$ GC cells in 100 μl of phosphate-buffered saline (PBS) was incubated with anti-CEACAM6 (1 : 50) or PBS at room temperature for 2 h, and then incubated with a fluorescein isothiocyanate (FITC)-conjugated secondary antibody (goat anti-mouse; Santa Cruz Biotechnology, Santa Cruz, USA) at room temperature for 15 min in the dark. The FITC ratio was then used to detect CEACAM6-positive cells by flow cytometry (FCM). Moreover, cells were lysed by using M-PER reagents containing Halt Protease Inhibitor Cocktail kits (Pierce, Rockford, USA). Protein quantification was determined with a BCA Protein Assay kit (Pierce). Western blotting assay was carried out as described previously [13], with mouse anti-CEACAM6 (1 : 500, Abcam), rabbit anti-phospho-C-SRC (1 : 500, Cell Signaling, Boston, USA), and mouse anti-C-SRC (1 : 100, Santa Cruz Biotechnology) antibodies. Labeled bands were detected using the Odyssey Sa Infrared Imaging System (Gene Company Limited, Hongkang, China). Anti-GAPDH antibody (1 : 5000; Kangchen, Shanghai, China) was used for the loading control.

Cell migration and invasion assays

Cell migration and invasion were measured in Boyden chambers by using Transwell filters (Corning, New York, USA). Matrigel was used for the cell invasion assay but not for the cell migration assay. Cells (1×10^5) in 0.2 ml of serum-free medium were placed in the upper chamber, and the lower chamber was loaded with 0.6 ml of medium containing 10% FBS. Cells which migrated to the lower surface of the filters were stained with Crystal violet solution, and five fields of each well were counted after 24 or 48 h incubation at 37°C with 5% CO_2. Three wells were examined for each cell type and condition, and the experiments were conducted in triplicate. These assays were performed using SNU-16, MKN-28, SGC-7901, MKN-45, and their matched transfected cells.

Tumor metastasis assay in animals

Negative control (NC) cells, SGC-7901-CEACAM6, and SGC-7901, were injected into the tail veins of five male

nude mice (1.5×10^6 cells in 50 μl PBS per mouse) at age of 6 weeks from Shanghai experimental animal center of Chinese Academy of sciences (Shanghai, China), respectively. Mice were euthanized 2 months later and the main organs were dissected and examined for metastases. All animal experiments were performed with the approval of the Institutional Animal Use Care and Use Committee of Shanghai Jiao Tong University School of Medicine.

Statistical analysis

The relationships between CEACAM6 expression level and clinicopathological features were analyzed by using Pearson χ^2 test. Multivariate analysis was performed with logistic regression method. All statistical analyses were performed by using SPSS 13.0 software (SPSS Inc., Chicago, USA). $P < 0.05$ was considered statistically significant.

Results

The expression of CEACAM6 is up-regulated in GC

CEACAM6 expression level was significantly higher in the GC cell lines than in the GES-1 immortalized cells (**Fig. 1A**). The average expression level of CEACAM6 in the 101 GC samples (**Fig. 1B,D**) was significantly increased in tumor tissues, when compared with matched non-tumor tissues (**Fig. 1C**), with 78.2% (79 of 101) cases showing increased CEACAM6 expression in tumor tissues. Based on the immunohistochemical (IHC) staining scores, i.e. the difference between the tumor tissues (*T*) and matched non-tumor tissues (*N*), the 101 cases were divided into the high CEACAM6 expression ($T - N > 0$, $n = 80$) and the low CEACAM6 expression ($T - N < 0$, $n = 21$) groups. The high expression group was associated with lymph node metastasis ($P = 0.001$), but there was no correlation between CEACAM6 level and other clinicopathological parameters (i.e. age, gender, tumor site, tumor size, histological grade, tumor depth, and TNM stage). We further performed multivariate analysis for lymph nodes metastasis with logistic regression method, which rendered our data more convincing. It was revealed that CEACAM6 expression ($P = 0.006$) and differentiation ($P = 0.025$) were associated with lymph nodes metastasis, while age, gender, location, tumor size, and local invasion had no significance in this analysis model. The clinical features of the 101 GC patients were

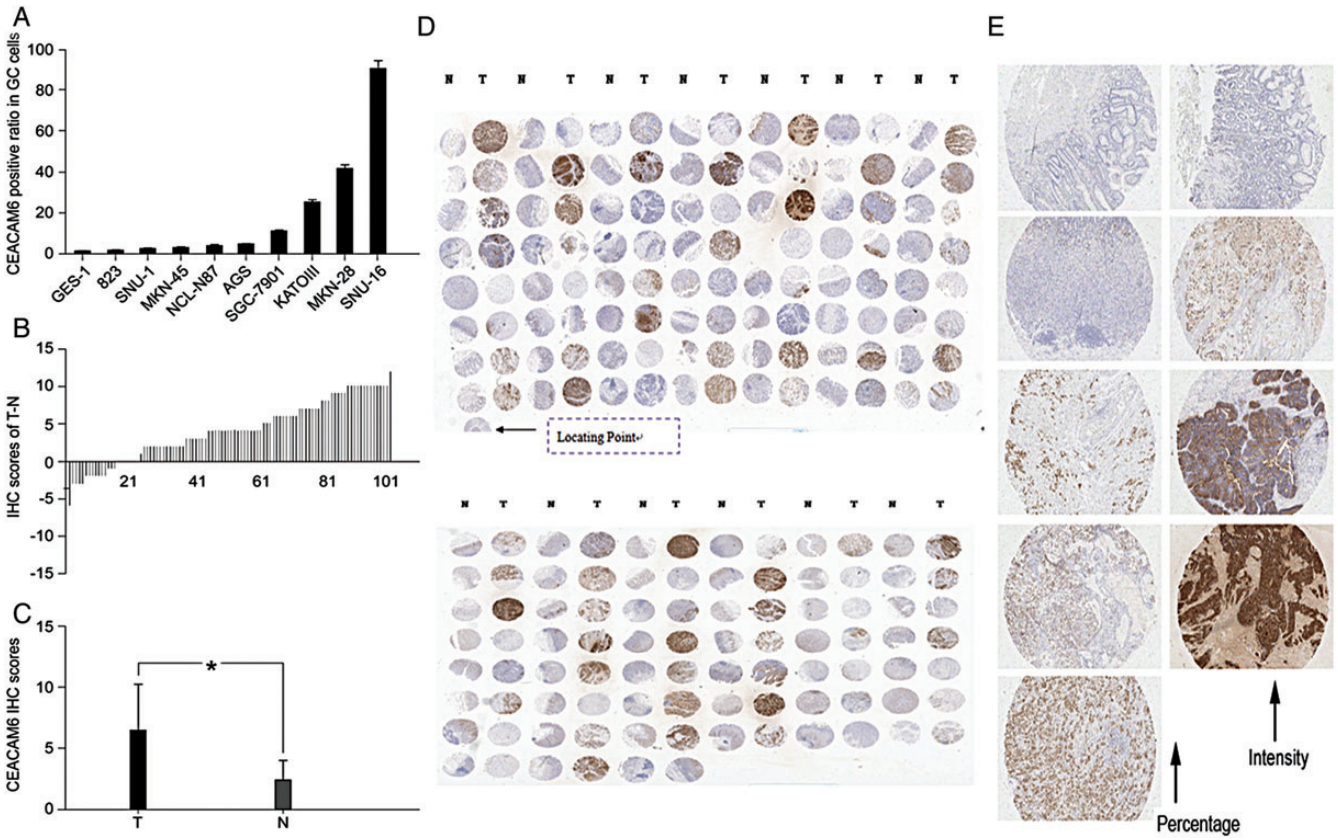

Figure 1. CEACAM6 is up-regulated in GC (A) CEACAM6 protein expression level was assessed by FCM in nine GC cell lines and the GES-1 immortalized gastric epithelial cell line. The CEACAM6-positive ratio is indicative of the CEACAM6 protein expression level. (B) CEACAM6 protein expression level was scored by IHC. Data are presented as the difference in the scores between the tumor tissues and the matched non-tumor tissues. (C) The mean and standard deviation of CEACAM6 protein level in the 101 GC tumors (black bar) and matched non-tumor tissues (gray bar) are shown. *$P < 0.01$. (D) Staining of CEACAM6 on IHC arrays. (E) Evaluating factors of IHC staining (*P*, *I*) as described in the 'Materials and Methods' section.

provided in Table 1. These results showed clearly that CEACAM6 was up-regulated in GC and the increase is associated with the lymph node metastasis.

CEACAM6 enhances GC cell migration

Because CEACAM6 level was much higher in the SNU-16 and MKN-28 than in the SGC-7901 and MKN-45 cells (**Fig. 1A**), we reduced the expression of CEACAM6 in the SNU-16 and MKN-28 cells with siRNA, or antagonized CEACAM6 in SGC-7901-CEACAM6 and MKN-45-CEACAM6 cells with a CEACAM6 antibody. Meanwhile, we over-expressed the CEACAM6 in SGC-7901 and MKN-45 cells by cDNA transfection, with the efficacy of these manipulations confirmed by western blotting assay (**Fig. 2A,B**). In the transwell assay, cell migration to the lower surface of the filter was decreased by 57.11% ($P < 0.01$) in siRNA-treated SNU-16 (SNU-16-SI) cells and by 52.08% ($P < 0.01$) in siRNA-treated MKN-28 (MKN-28-SI) cells (**Fig. 2C**). On the other hand, the migration showed increases by 5- or 2 fold in the SGC-7901-CEACAM6 or MKN-45-CEACAM6 cells which stably expressed CEACAM6, when compared with the corresponding NC ($P < 0.01$; **Fig. 2D**). Migration of the SGC-7901-CEACAM6 and MKN-45-CEACAM6 cells was inhibited almost to the NC levels when cells were treated with an anti-CEACAM6 monoclonal antibody. Taken together, these results suggested that CEACAM6 promotes GC cell migration *in vitro*.

Table 1. CEACAM6 total expression level and clinicopathological parameters in 98 GC

Clinicopathological parameters	CEACAM6 expression		*P*
	Low ($n = 21$)	High ($n = 77$)	
Age (year)			
<40	1	3	0.742
40–65	14	45	
≥65	6	29	
Gender			
Male	13	61	0.288
Female	8	16	
Location			
Distal 1/3	15	42	0.183
Middle and proximal 1/3	6	34	
Tumor size (cm)			
≤5	15	46	0.327
>5	6	31	
Differentiation			
Poorly, undifferentiated	18	61	0.505
Well, moderately	3	16	
Local invasion			
T1a, b, T2	5	17	0.866
T3, T4a, b	16	60	
Lymph node metastasis			
No	13	19	0.001*
Yes	8	58	
TNM stage			
I, II	11	27	0.149
III, IV	10	50	

*Statistically significant.

Three patients' clinical parameters were unavailable.

CEACAM6 enhances GC cells invasion

Judged by the amounts of cells capable of invading into the matrigel and moving to the lower surface of the filters, the invasion of SNU-16-SI and MKN-28-SI cells was decreased by 85.54% and 90.77% ($P < 0.01$; **Fig. 3A**), respectively. In contrast, the invasion of the SGC-7901-CEACAM6 and MKN-45-CEACAM6 cells, in which the CEACAM6 was ectopically expressed, showed 2.5- and 2.1-fold increases ($P < 0.01$; **Fig. 3B**), when compared with the corresponding controls. The invasion ability was decreased again when the SGC-7901-CEACAM6 and MKN-45-CEACAM6 cells were antagonized with an anti-CEACAM6 monoclonal antibody (**Fig. 3B**). These results showed that the CEACAM6 expression increased the invasiveness of GC cells. Furthermore, we normalized migration and invasion by proliferation assays to ensure the accuracy of our findings and observed that there was no significance in the different groups of the four cell lines ($P < 0.01$).

Over-expression of CEACAM6 increases P-C-SRC

Phosphorylation of C-SRC, an oncoprotein known to promote tumor cell migration and invasion [14], showed a positive correlation with the CEACAM6 level in SGC-7901 cells as detected by western blotting assay, but the total C-SRC protein levels remained unchanged (**Fig. 4**). These results suggested that the promotion of GC cell invasion and migration by CEACAM6 might be elicited in part by inducing C-SRC phosphorylation.

Over-expression of CEACAM6 increases metastasis *in vivo*

Two months after the CEACAM6-expressing GC cells or their NC controls were injected into a tail vein, four of five mice were found with metastases in the lung or liver, while no metastasis was discerned in any mouse receiving the NC cells (**Fig. 5A**). One mouse receiving CEACAM6-expressing cells also developed cachexia despite the same feeding conditions, whose body weight and size was far less than the others and its movement reduced obviously. It had widespread metastases throughout the pancreas and mesentery. The metastatic lesions were pathologically confirmed on hematoxylin-and-eosin stained sections (**Fig. 5B**).

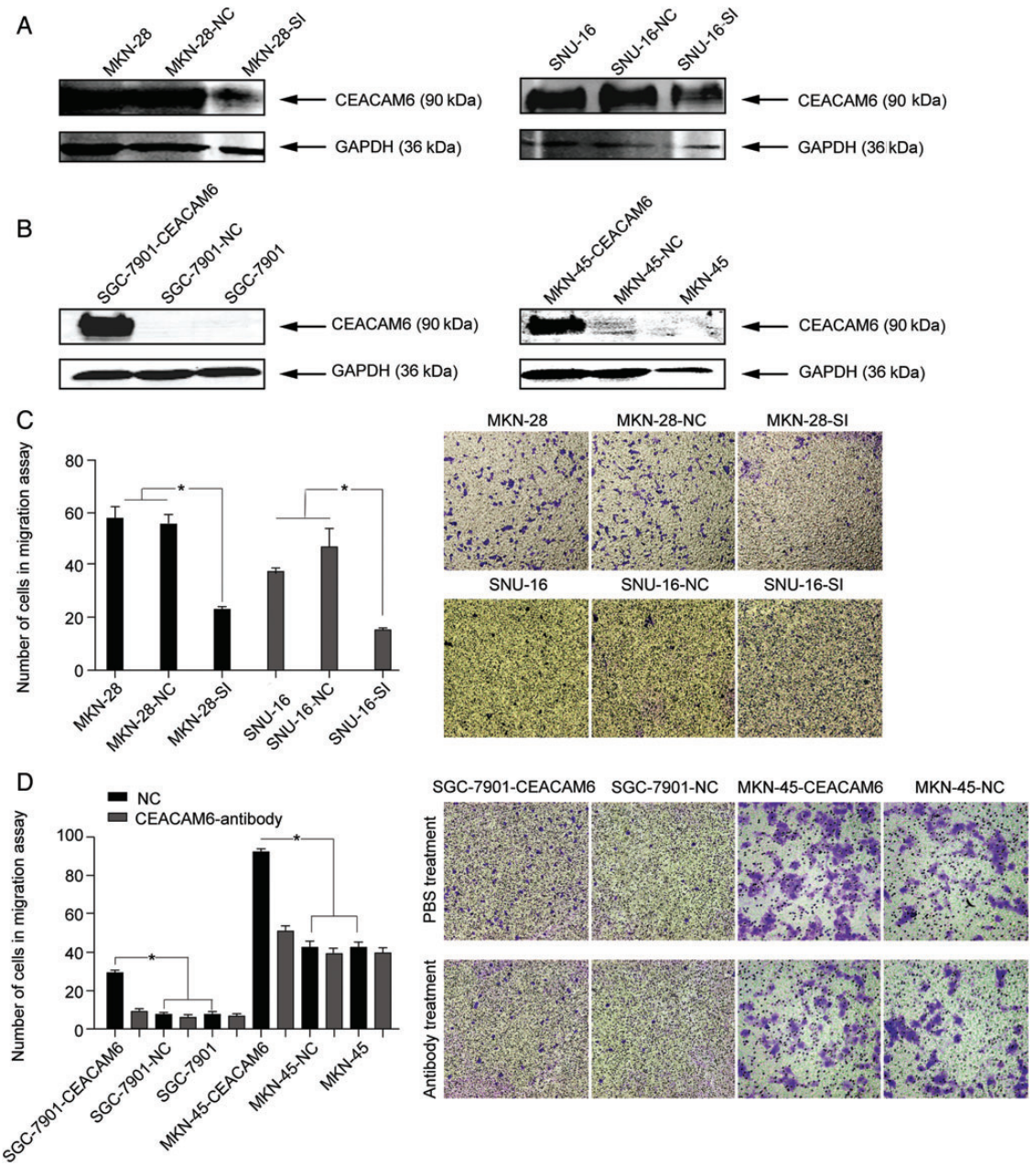

Figure 2. CEACAM6 promotes migration of GC cells (A) Confirmation of CEACAM6 knockdown in MKN-28 and SNU-16 cells by western blotting. (B) Confirmation of CEACAM6 over-expression in SGC-7901 and MKN-45 cells by western blotting assay. (C) CEACAM6 knockdown inhibited migration. Left, the number of cells migrating to the lower chambers in migration assays ($P < 0.01$); right, siRNA/NC/MOCK treatment groups ($\times 10$). *$P < 0.01$. (D) Cell migration was increased upon CEACAM6 over-expression but was decreased upon treatment with an anti-CEACAM6 antibody. Left, the number of cells migrating to the lower chambers in migration assays ($P < 0.01$); right, representative assay photos ($\times 4$). The bottom row was treated with an anti-CEACAM6 antibody and the upper row was treated with PBS. *$P < 0.01$.

Discussion

CEACAM6 is known to be oncogenic, as it inhibits cell differentiation and anoikis, causes the loss of cell polarity, and promotes cell adhesion, invasion, and metastasis [15–18]. The role of CEACAM6 in adhesion, invasion, and metastasis can be inhibited by the fragment of antigen binding (Fab′) of an anti-CEACAM6 antibody in breast, pancreatic, and colorectal cancers [19], and our *in vitro* results were in line with these reports. Another strategy to treat GC based on CEACAM6 is using antibody-drug conjugates, just as Govindan *et al*. reported [20]. Studies with pancreatic cancer have shown that the function of CEACAM6 is dependent on a C-SRC signaling pathway [14,21]. Our finding that CEACAM6 induced the C-SRC phosphorylation without affecting its total protein level in GC cells suggested that the activation of C-SRC via phosphorylation might indeed be a mechanism for CEACAM6 to promote tumor metastasis.

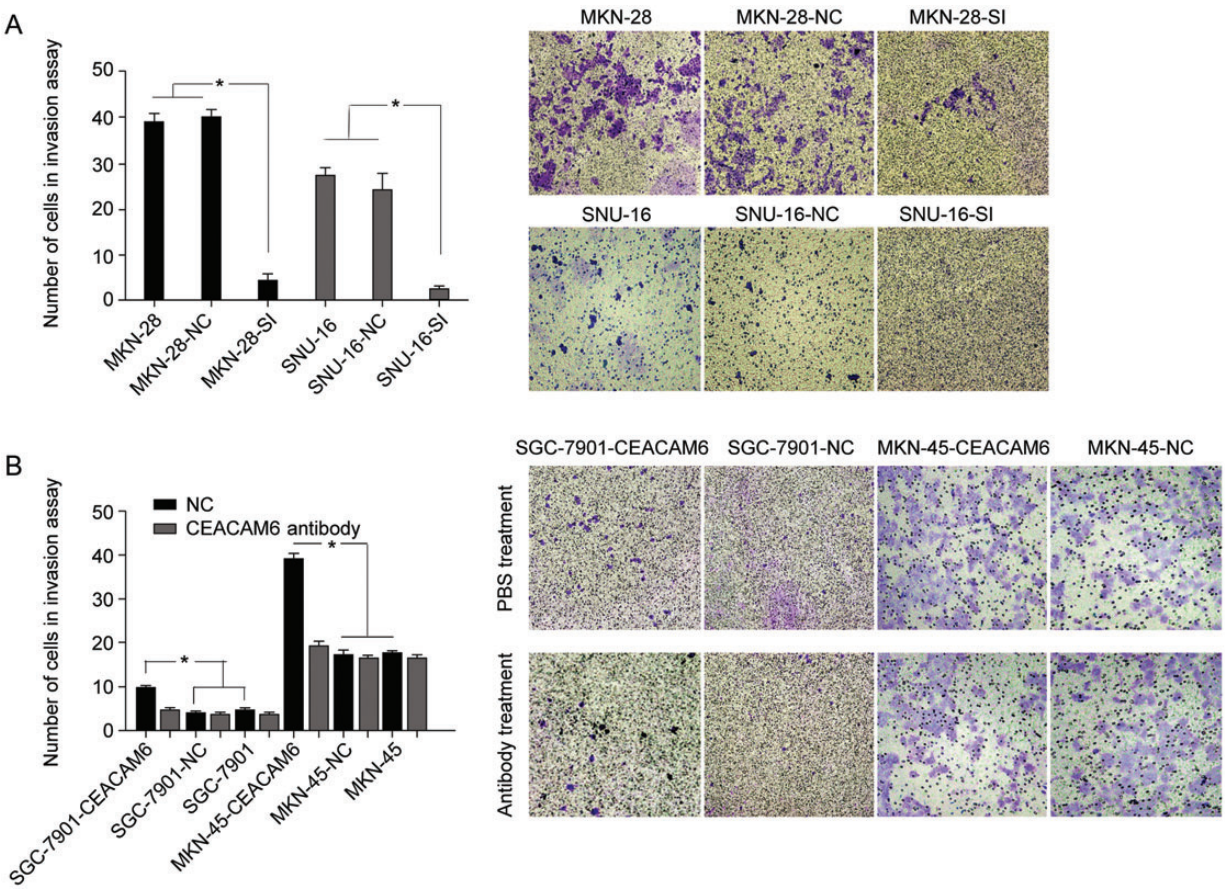

Figure 3. CEACAM6 promotes invasion of GC cells (A) CEACAM6 knockdown inhibited invasion. Left, the number of invading cells (mean ± SD, $P < 0.01$); right, siRNA/NC/MOCK treatment groups (×10). *$P < 0.01$. (B) Cell invasion was increased upon CEACAM6 over-expression but was decreased upon treatment with an anti-CEACAM6 antibody. Left, the number of invading cells (mean ± SD, $P < 0.01$); right, representative assay photos (×4). The bottom row was treated with an anti-CEACAM6 antibody and the upper row was treated with PBS. *$P < 0.01$.

Whether other molecules that have also been shown to mediate the oncogenic activities of CEACAM6, including fibronectin [22], integrin αvβ3 [14], SMAD3 and TGF-β [23], are also players in the CEACAM6 actions in GC invasion and metastasis remains to be further explored. CEACAM6 is also reported to play immunomodulatory roles in several diseases of human [24]. It can act as a receptor for adherent-invasive *Escherichia coli* and Neisseriae to invade into body [25,26]. *Helicobacter pylori* (HP) is a classic and crucial factor for GC [27]. Thus, an interesting question deserves to be researched: does CEACAM6 play oncogenic roles in GC through other pathways, such as acting as receptors for HP?

In summary, we showed in the present study that the CEACAM6 expression was increased in GC tumors and was positively associated with lymph node metastasis. Its over-expression could promote migration, invasion, and dissemination of GC cells *in vitro*, which might partly be elicited by inducing C-SRC phosphorylation. Meanwhile, there were some important points needed to be improved in our experiments: (i) survival of our study patients should be completed; (ii) C-Src phosphorylation should also be performed

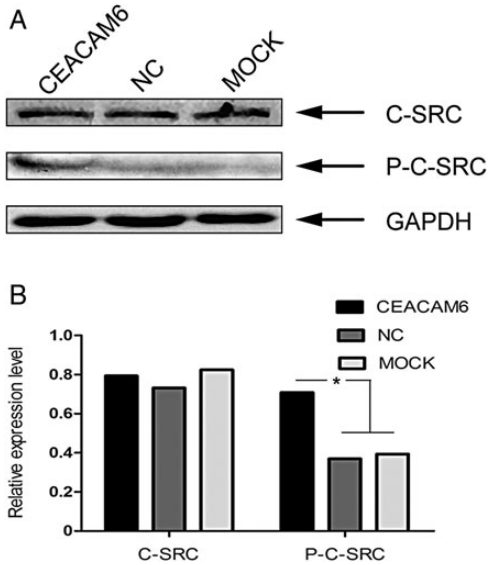

Figure 4. CEACAM6 induces C-SRC phosphorylation (A) Immunoblot showed that C-SRC phosphorylation was up-regulated in the SGC-7901-CEACAM6 cells compared with the NC and the mock, but the total C-SRC protein remained unchanged. (B) Quantification of C-SRC and P-C-SRC protein. Relative expression level of protein was calculated by ratio of gray scale: C-SRC/GAPDH and P-C-SRC/GAPDH. *$P < 0.01$.

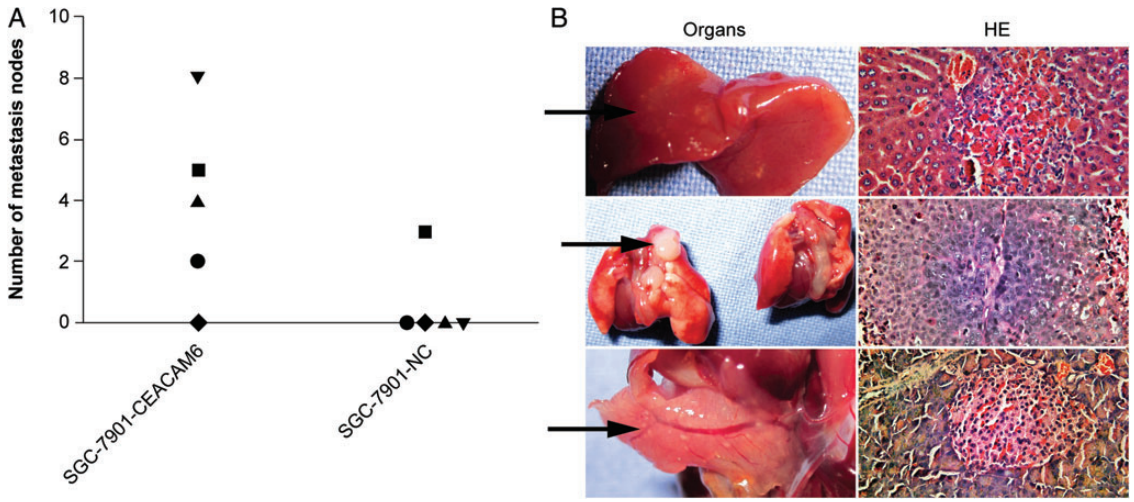

Figure 5. CEACAM6 promotes metastasis *in vivo* (A) Metastatic nodules in the organs of mice injected with the SGC-7901-CEACAM6 or SGC-7901-NC cells (mean ± SD, $P < 0.01$). Five different shapes stand for five different mice. (B) Left, metastatic nodules in the liver, lung, and pancreas (from top to bottom); right, matched hematoxylin-and-eosin staining (×20).

on cells those knocked down for CEACAM6, to ensure that this was not off-target effects; (iii) the *in vivo* metastatic data would be more convincing if mice were also injected with other GC cell lines or those targeted with an anti-CEA CAM6 shRNA for its knockdown; (iv) the expression of CEACAM6 in cells injected into mice 2 months later should be confirmed by IHC. Fortunately, all the points considered above are carried out in our ongoing follow-up study. Understanding the clinical significance of the CEACAM6 expression and its oncogenic mechanism may eventually lead to the discovery of a novel therapeutic target for GC treatment.

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