Cell motility and spreading promoted by CEACAM6 through cyclin D1/CDK4 in human pancreatic carcinoma

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Abbreviations: CEACAM6, carcinoembryonic antigen-related cell adhesion molecule 6; CEA, carcinoembryonic antigen

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Abstract. Carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) belongs to the human carcinoembryonic antigen (CEA) family. Numerous lines of studies have indicated that altered expression of CEACAM6 may have a role in carcinogenesis and development. However, few studies have defined functional roles and mechanisms of action. In the present study, the relationship between clinical and pathological parameters was also analyzed. The relative CEACAM6 protein expression of pancreatic carcinoma was significantly higher than that in non-cancerous tissue. Different clinical stages and lymph node metastasis between groups were significantly different (P<0.05). We used siRNA and forced-expression in multiple cell lines to define the role of CEACAM6 in the regulation of proliferation of pancreatic carcinoma in vitro and in vivo. Knockdown of endogenous CEACAM6 decreased proliferation of BxPC-3 and SW1990 cells. These changes significantly reduced cyclin D1 and CDK4 protein levels. Conversely, overexpression of CEACAM6 in MIA PaCa-2 cells stimulated proliferation and increased cyclin D1 and CDK4 protein levels. Our results confirm that CEACAM6 promoted cell proliferation, and these changes were mediated by cyclin D1/CDK4. These observations contribute to our understanding of the important roles of CEACAM6 in pancreatic carcinoma development and progression and could be a promising molecular target for the development of new diagnostic and therapeutic strategies of pancreatic carcinoma.

Introduction

Pancreatic carcinoma is one of the most frequently occurring gastrointestinal malignancies and the incidence rate is showing an upward trend worldwide (1). The prognosis for patients with advanced pancreatic carcinoma remains poor with a 5-year survival rate of <5% (2). Among the most significant determinants of the poor prognosis associated with this malignancy are the highly aggressive loco-regional invasion and early metastasis that characterize this malignancy, such that the majority of patients present with advanced, surgically unresectable disease (3). Gemcitabine and erlotinib are the only agents that are approved for the treatment of pancreatic carcinoma. However, both drugs induce a poor response in patients and their use can result in patients developing multiple drug resistance (4,5). Although in recent years, great progress has been observed with regard to investigations on the molecular pathogenesis of pancreatic carcinoma, the clinical treatment of pancreatic carcinoma remains a challenge. Therefore, novel therapeutic approaches to this malignancy are needed.

Carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6), also known as CD66c or NCA-90, as well as another 6 members of the CEACAM subgroup, belong to the human carcinoembryonic antigen (CEA) family (6). Numerous lines of studies have indicated that altered expression of CEACAM6 may have a role in carcinogenesis and development. Increased CEACAM6 gene expression has been found in lung, breast, colorectal, hepatocellular and pancreatic carcinomas (7-12). Yet, the relationship between the differentially expression of CEACAM6 in tumor compared to normal tissue and its biological function needs further investigation. In the present study, we used immunohistochemistry to explore CEACAM6 protein in pancreatic carcinomas. The relationship between clinical and pathological parameters was also analyzed. Furthermore, we examined the role of CEACAM6 gene expression in human pancreatic carcinoma. The aim of the present study was to increase the knowledge on the treatment of pancreatic carcinoma.
Materials and methods

Samples. A total of 42 cases of pancreatic carcinoma and 12 cases of non-cancerous specimens were selected between February 2005 and January 2010 from the Xiangya Hospital of Central South University. The resected tissue samples were immediately cut into small pieces and snap-frozen in liquid nitrogen until use. All procedures were conducted in accordance with the Helsinki declaration, and with approval from the Ethics Committee of Xiangya Hospital. Written informed consent was obtained from all the participants.

Immunohistochemistry. Routine paraffin sectioning, dewaxing and hydration using 3% hydrogen peroxide were performed to remove endogenous peroxidase. Microwave antigen was retrieved and blocked with fetal calf serum (FCS) for 2 h. Approximately 50 µl (1:25) of goat polyclonal anti-human CEACAM6 was added, and the mixture was incubated at 4°C overnight. Approximately 50 µl of biotinylated goat anti-rabbit IgG secondary antibody working solution was added, and the mixture was incubated 37°C for 30 min, followed by diaminobenzidine coloration. The sample was dyed with hematoxylin, separated using ethanol and hydrochloric acid, saturated with lithium carbonate until the color returned to blue, and then dehydrated with gradient alcohol as well as xylene. Mounting with neutral resin followed. The negative control used was phosphate-buffered saline (PBS) in place of the primary antibody. The stained cells were divided into five grades based on cell counting, namely, 0 (no staining), 1 (<25% stained cells), 2 (25-50% stained cells), 3 (50-75% stained cells), and 4 (>75% stained cells). Based on both of the above scoring methods, the immunohistochemistry results were classified into four, namely, negative (0), weakly positive (1-2 score), positive (3-4 score), and strongly positive (5-7 score) (13).

Cell lines. Pancreatic carcinoma cell lines, BxPC-3, SW1990 and MIA PaCa-2, were maintained by our laboratory. They were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco). Cells were maintained at 37°C atmosphere of humidified air with 5% CO₂.

Semi-quantitative polymerase chain reaction. RNA isolated from cells was reverse-transcribed and amplified using the One-Step reverse transcription polymerase chain reaction (RT-PCR) system (Fermentas). The sets of primers for CEACAM6 receptor subunit are: sense, 5’TACAAAGCCGA AAGATGGATG-3’ and antisense, 5’GTATTGGTTACAGCTGGTA-3’. As a negative control, we used shRNA vector without hairpin oligonucleotides (NC).

To transfection of the plasmid expression vector encoding human CEACAM6, the DNA sequencing containing the full-length CEACAM6 (1034 bp) open reading frame flanked by XhoI (sense) and BamHI (antisense) restriction sites was PCR amplified from BxPC-3 cells. Primer sequences used were sense 5’-CCGCCTGAGACCTGGGAGTCTCGGGA GGGAC-3’ and antisense 5’-CGCGGATCCCTATATCAGA GCCACCTTGCCAGC-3’. The resulting fragment was inserted into pcDNA3.1(+) to generate pcDNA3.1-CEACAM6. The desired sequence was confirmed by direct DNA sequencing.

Cell transfection. For transfection, the human pancreatic carcinoma cell lines were plated onto 24-well plates, and transfected with these vectors using FuGENE6 (Roche, Indianapolis, IN, USA) according to the instructions of the manufacturer, followed by 200 µg/ml of neomycin selection. The cells were harvested 15 days later to analyze the knockdown effect on CEACAM6 by RT-PCR using the primers shown above and by western blot analysis using rabbit anti-human polyclonal antibody against CEACAM6 (Abcam).

Impact of CEACAM6-siRNA on the growth of pancreatic carcinoma cells

CCK-8 analysis. For the CCK-8 analysis, cells were seeded with serum-free medium at a density of 10³ cells/well in 96-well plates (n=6), grown overnight, washed in PBS, and incubated with 10% FBS DMEM at 37°C, 5% CO₂, for varying periods and exposed to fresh media every other day. During the last 4 h of each day's culture, the cells were treated with the Cell Counting kit-8 (CCK-8, (4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium (Vazyme Biotech Co., Ltd., Lakewood, NJ, USA). The OD at 450 nm were measured for detecting the cell viability.

Colony formation analysis. For the colony formation analysis, cells at 1,000 cells/well in 6-cm plates were incubated with serum-free medium for 24 h, and then cultured in DMEM with 10% FBS at 37°C, 5% CO₂ for 2 weeks. The cell colonies

Western blot analysis. The cells were washed with cold PBS and lysed in Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol and 0.01% bromophenol blue) for 5 min at 95°C. Cell lysates were analyzed by SDS-PAGE and transferred electrophoretically to polyvinylidene difluoride membrane. The blots were probed with specific antibodies by a secondary detection step. The immunoreactive proteins were revealed by an ECL kit. Western blot analysis was carried out using the following antibodies: rabbit anti-CEACAM6 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-cyclin D1 antibody (Cell Signaling Biotechnology, Danvers, CA, USA), rabbit anti-cyclin E1 antibody (Abcam Biotechnology), rabbit anti-CDK4 antibody and rabbit anti-CDK2 antibody (both from Santa Cruz Biotechnology).

Knockdown and overexpression of CEACAM6 vector construction. To knock down CEACAM6 expression, we used GV102 vector encoding a small hairpin RNA directed against the target gene in BxPC-3 and SW1990. The target sequences for CEACAM6 were 5’-GTATTGGTTACAGCTGGTA-3’. G419
were washed twice with PBS, fixed by 4% paraformaldehyde for 15 min and stained with Giemsa for 25 min. Individual clones with >50 cells were counted. Clone forming efficiency for individual type of cells was calculated, according to the number of colonies/number of inoculated cells x 100%.

Flow cytometric analysis. For the flow cytometric analysis, cells were incubated with serum-free medium for 24 h, and then cultured in DMEM with 10% FBS at 37°C 5% CO₂, then harvested at 70-80% confluence and resuspended in fixation fluid at a density of 10⁶/ml. A total of 1,500 µl propidium iodide (PI) solution was added, and the cell cycle was detected by FACSCalibur (Becton-Dickinson).

Tumor formation in nude mice. The influence of CEACAM6 silencing on the tumor development of pancreatic carcinoma in vivo was examined. Briefly, BxPC-3/si-CEACAM6, BxPC-3/NC, SW1990/si-CEACAM6 and SW1990/NC cells (1x10⁷) were suspended in 0.2 ml of extracellular matrix gel and injected subcutaneously in the left back flank of the animals. The 6-week-old BALB/c nude (nu/nu) mice (Slac Laboratory Animal Center, Changsha, China) were divided into four groups: i) the mice were injected with BxPC-3/NC cells (n=3); ii) the mice were injected with BxPC-3/si-CEACAM6 cells (n=3); iii) the mice were injected with SW1990/NC cells (n=3); and iv) the mice were injected with SW1990/si-CEACAM6 cells (n=3). Tumor variables were measured every 5 days by an electronic caliper, and tumor volume was calculated using a standard formula (14): Tumor volume = width² x length x 0.5. At the end of the experiment, all the mice were sacrificed and individual tumor weights were measured.

Statistical analysis. The values are presented as the means with the SPSS software (version 13.0; SPSS, Inc., Chicago, IL, USA) for significance. The analysis of variance and t-test were applied in comparing the intergroup difference of measurement data. P<0.05 was considered to indicate a statistically significant difference. The diagrams were drawn by GraphPad Prism 5.

Results

The expression of CEACAM6 and clinicopathological parameter. To explore the role of CEACAM6 in pancreatic carcinoma, we first examined the CEACAM6 protein expression level in 42 cases of pancreatic carcinoma and 12 cases of non-cancerous specimens by immunohistochemistry. The expression level of CEACAM6 protein in pancreatic carcinoma tissues was increased compared with the non-cancerous ones. Under high magnification, CEACAM6 was expressed as light yellow or brown in the cytoplasm and cell membrane (Fig. 1).

The relative CEACAM6 protein expression of pancreatic carcinoma was significantly higher than that in non-cancerous tissue. Different clinical stages and lymph node metastasis between groups were significantly different (P<0.05). However, no statistically significant difference was found between pancreatic carcinoma and non-cancerous specimens, which were from different gender, as well as from different age. There was also no statistically significant difference among the different tumor size of pancreatic carcinoma (P>0.05). All of the analysed parameters indicated its potential role in the progression of pancreatic carcinoma (Table I).

We documented CEACAM6 expression in BxPC-3, SW1990 and Mia PaCa-2 cell lines. The results of semi-quantitative RT-PCR and western blot analysis showed that CEACAM6 was detected in BxPC-3 and SW1990 cells, but not in Mia PaCa-2 cells (Fig. 2).
The effect of RNAi. To investigate the biological significance of CEACAM6 overexpression in pancreatic carcinoma cell lines BxPC-3 and SW1990, we constructed siRNA expression vectors (si-CEACAM6) specific to CEACAM6 transcripts and
transfected them into BxPC-3, and SW1990 cells that endogenously expressed high levels of CEACAM6, as shown in Fig. 3A and B. A knockdown effect was observed by RT-PCR and western blot analysis when we transfected si-CEACAM6, but not in negative control NC (Fig. 3A and B).

**Impact of CEACAM6-siRNA on the growth of pancreatic carcinoma cells.** Subsequently, we examined the effect of decreased CEACAM6 on BxPC-3/si-CEACAM6, BxPC-3/NC, SW1990/si-CEACAM6 and SW1990/NC cell growth by CCK-8 assays. Following a 6-day period, the growth of BxPC-3/NC and SW1990/NC cells was much more rapid than BxPC-3/si-CEACAM6 and SW1990/si-CEACAM6 cells, and significantly high number of BxPC-3/NC and SW1990/NC cells was observed from day 3 (Fig. 3C and D).

To investigate the possible function of CEACAM6 on the growth of BxPC-3 and SW1990 cells, the dynamics of BxPC-3/si-CEACAM6, BxPC-3/NC, SW1990/si-CEACAM6 and SW1990/NC cell growth were determined by colony formation assay. As a result, the average colony number of BxPC-3/si-CEACAM6 and SW1990/si-CEACAM6 cells decreased compared with BxPC-3/NC and SW1990/NC cells. Therefore, the low number of cell colonies from BxPC-3/si-CEACAM6 and SW1990/si-CEACAM6 cells demonstrated that the down-regulation of CEACAM6 expression inhibits the growth of BxPC-3 and SW1990 cells (Fig. 3E-H).

To further explore the cause for the decrease in cell viability, we examined the effects of CEACAM6 RNAi on the cell cycle. As shown in Fig. 4, BxPC-3 and SW1990 cells with CEACAM6 siRNA showed blocking of the cell cycle in G1 phase. These data indicated that the down-regulation of CEACAM6 expression arrests BxPC-3/si-CEACAM6,
SW1990/si-CEACAM6 cell cycling at G0/G1 phase, which may inhibit the growth of BxPC-3 and SW1990 cells. **Overexpression of CEACAM6 promotes cells of MIA PaCa-2 proliferation.** Given that downregulation of CEACAM6 inhibi-
ited BxPC-3/NC and SW1990 cells proliferation in vitro, we considered that CEACAM6 could promote MIA PaCa-2 cell development. To test this possibility, MIA PaCa-2 cells were transfected with plasmid pCDNA(+3.1-CEACAM6 encoding CEACAM6. Comparing to the control (MIA PaCa-2-C), cells transfected with plasmid encoding CEACAM6 (MIA PaCa-2-CEACAM6) had increased levels of CEACAM6 mRNA and protein (Fig. 5A). CCK-8 analysis showed that the proliferation of MIA PaCa-2-CEACAM6 cells was much higher than MIA PaCa-2-C cells (Fig. 5B). Colony formation analysis showed a larger amount of cell colonies from MIA PaCa-2-CEACAM6 cells. The results demonstrated that upregulation of CEACAM6 expression promoted cell proliferation in vitro (Fig. 5C and D).

Cell proliferation was also detected by flow cytometry, results showed MIA PaCa-2-CEACAM6 decreased the cell cycle in G1 phase and increased the cell cycle in S phase, which may promote the proliferation of MIA PaCa-2 cells (Fig. 5E and F). This result is in line with the above analysis.

Tumor formation in nude mice. Given that downregulation of CEACAM6 inhibited pancreatic carcinoma cell proliferation and upregulation of CEACAM6 promoted pancreatic carcinoma cell proliferation in vitro, we hypothesized that CEACAM6 could promote pancreatic carcinoma cell development in vivo. To further determine the role of CEACAM6 in tumorigenicity and development of pancreatic carcinoma cells, BxPC-3/si-CEACAM6 and SW1990/si-CEACAM6 or BxPC-3/NC and SW1990/NC cells were injected subcutaneously into nude mice. The development of the tumors was monitored for 40 days. As shown in Fig. 6A and B, CEACAM6 knockdown tumors emerged later and grew slowly compared to control tumors. At the end of the experimental period, the final weights of CEACAM6 knockdown tumors (0.392±0.065 and 0.491±0.057 g) were found to be markedly lighter than controls (0.658±0.098 and 0.739±0.072 g) (Fig. 6C and D). RT-PCR and western blot analysis of CEACAM6 in xenograft tumors indicated that increased CEACAM6 expression had been maintained throughout the experimental time course (Fig. 6E and F). Collectively, these data evidenced that CEACAM6 promoted xenograft tumor development in vivo.

Discussion

CEACAM6 is a single-chain GPI-anchored immunoglobulin (Ig)-like glycoprotein and is a member of the human CEA family (15). Jantscheff et al (16) showed that CEACAM6 overexpression was associated with poor clinical outcome in colorectal cancer. In the present study, we show for the first time a significant role for CEACAM6 in the regulation of pancreatic carcinoma cell proliferation and cyclin D1/CDK4 expression. These findings indicate a new mechanism for CEACAM6-mediated cell proliferation that may act through the regulation of cyclin D1/CDK4 expression.

We first demonstrated that the two kinds of pancreatic carcinoma cells express CEACAM6. The expression of CEACAM6 in pancreatic carcinoma cells indicated that CEACAM6 exerted a potential role in the cell regulation. In the present study, BxPC-3 and SW1990 cells were used as a pancreatic carcinoma tumor cell model. It has been reported that CEACAM6 overexpression independently predicted poor overall survival and disease-free survival, whereas CEACAM1 or CEACAM5 was not significantly associated with these outcomes. CEACAM6 overexpression leads to morphological changes that are similar to epithelial-mesenchymal transformation (17), increased invasiveness (17), increased chemoresistance (18) and resistance to anoikis (19-21), whereas CEACAM6 appears to exert its pro-invasive effect in a c-Src-dependent manner, at least in part through the upregulation of MMP-9 activity (22).
The overexpression of CEACAM6 genes has been widely associated with a variety of carcinomas (23,24). Therefore, our results are consistent with previous observations that CEACAM6 genes are highly expressed in a variety of carcinomas. CEACAM6, in particular, plays a role in pancreatic carcinoma cell growth, motility and cellular proliferation. In the present study, we performed siRNA-mediated knockdown of CEACAM6 in the high CEACAM6-expressing pancreatic carcinoma cell line. Conversely, we overexpressed CEACAM6 in the low CEACAM6-expressing MiaPaCa-2 cell line to determine how CEACAM6 regulates pancreatic carcinoma growth. The results show that CEACAM6 exerted a specific role in pancreatic carcinoma cell proliferation, knockdown of CEACAM6 induced a decrease in pancreatic carcinoma proliferation, while overexpression of CEACAM6 in MiaPaCa-2 cells significantly promoted cell growth. These results support the hypothesis that CEACAM6 modulates the growth and oncogenesis of human pancreatic carcinoma cells.

Since cell proliferation is controlled by progression through the cell cycle, which is regulated by many proliferative signaling cascades, we carried out flow cytometric analysis to identify cell cycle distribution. Normal cell cycle follows the ordinary steps, but cancer cells grow without regulation. The rate of progress in cell cycle is decided by cyclins and cyclin-dependent kinases (CDKs). Entering of each phase is controlled by specific cyclin-CDK complex. CDK is a member of serine-threonine kinase family because a cyclin binds to a CDK and starts the phosphorylation of its serine and threonine site (25,26). It has been reported that the G1-S check-point is mainly regulated by a series of cyclins and cyclin-dependent kinase (CDK), which were predominantly cyclin D/CDK4, CDK6, cyclin E/CDK2 (27). Cyclin D is a key regulatory enzyme in cell proliferation and plays an important role in the progression from the G1 to S phase in the cell cycle (28). In early G1 phase, cyclin D binds to CDK4 to form the cyclin-CDK complex and subsequently activates the Rb protein by phosphorylation. Phospho-Rb (p-Rb) is the major negative regulator of cell division and exerts most of its effect in the first two thirds of the G1 phase of the cell cycle (29). In the present study, we examined the effect of cyclin D1/CDK4 on pancreatic carcinoma cell growth. As observed by others in multiple cell types (30), cyclin D1/CDK4 activation promoted cell proliferation and stimulated the replication of cells. The present study further identified cyclin D1/CDK4 as a novel downstream target of CEACAM6.

In summary, our present data support a novel mechanistic role for the CEACAM6 modulation of pancreatic carcinoma cell proliferation via the expression of cyclin D1/CDK4. This finding contributes to the pro-proliferation effect of CEACAM6 in pancreatic carcinoma cell growth, motility and cellular proliferation. This finding will yield new potential targets for the treatment of pancreatic carcinoma tumors and other pancreatic carcinoma cell proliferation dysfunction that involve the aberrant expression of CEACAM6 gene.

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