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Expression profiling of CEACAM6 associated with the tumorigenesis and progression in gastric adenocarcinoma

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ABSTRACT. Carcinoembryonic antigen-related cellular adhesion molecule 6 (CEACAM6) is a member of the immunoglobulin superfamily and has been recently reported to affect the neoplastic, metastatic, and invasive ability of malignant cells by regulating intracellular signaling pathways during tumorigenesis and progression. We investigated the expression and amplification of CEACAM6 in relation to the clinicopathological and biological significance of gastric adenocarcinoma. Expression of CEACAM6 mRNA in 75 primary gastric adenocarcinom and 20 adjacent tissues compared to normal gastric mucosas were explored using real-time quantitative-polymerase chain reaction. Immunohistochemical assays were conducted to evaluate the expression and tissue distribution of CEACAM6 protein. Overexpression of CEACAM6 mRNA in both gastric adenocarcinoma (2.513 ± 0.869) and adjacent tissues (1.171 ± 0.428) was significantly higher than the relative expressions in non-neoplastic specimens (0.594 ± 0.513) ($P < 0.01$). CEACAM6 protein was present in 52 (69.33%) gastric adenocarcinomas, but

not in normal gastric tissues. Adenocarcinomas with elevated CEACAM6 expression were significantly associated with lymph node metastases and advanced stages. There were no relationships between CEACAM6 expression and tumor size, histological differentiation, or different subtypes, respectively. Moreover, higher expression of CEACAM6 was found to be correlated with short postoperative survival time of patients with gastric cancer. Amplification and upregulation of CEACAM6 expression was observed in human gastric adenocarcinomas, which may be correlated with the generation or transformation of malignant cells, tumor aggressive progression, and clinical outcome. CEACAM6 may be a valuable biomarker screening for gastric tumor and novel predictor for patients in advanced stages of gastric cancer.

Key words: CEACAM6; Tumorigenesis; Gastric adenocarcinoma; RQ-PCR; Molecular marker

INTRODUCTION

Targeted strategies and individual therapies have played a major role in the management of cancer. Predictive markers used for the identification of tumor stages represent powerful tumor monitoring targets with predictive and prognostic significance, particularly in gastric adenocarcinoma. Gastric carcinoma is the most common malignant tumor in China and is often accompanied by metastasis and invasion during advanced stages, resulting in a high mortality rate. Regarding neogenetic colonies and lymph node metastasis, several molecular alterations in intercellular or extracellular matrix interactions and angiogenesis are thought to play a critical role in tumor dissemination and processes, some of which have contributed to the development of rationally designed molecular targeted therapies (Chen et al., 2003). Carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) is a glycosyl phosphatidyl inositol (GPI)-linked immunoglobulin super family member, which is thought to have important functions of disrupting tissue architecture, affecting cell differentiation, anti-anoikis activity, contributing to tumor invasion and metastasis (Hammarstrom et al., 1980; Ordonez et al., 2000), and even chemoresistance in human tumors, including colorectal, pancreas, cholangiocarcinoma, lung, prostate, breast, and female reproductive system tumors (Gaglia et al., 1988; Chevinsky, 1991; Hasegawa et al., 1993; Genega et al., 2000; Duxbury et al., 2005; Ieta et al., 2006; Wang et al., 2009). Several recent studies have demonstrated that this novel oncoprotein influences intracellular signaling events and may be involved in early molecular events leading to colorectal cancer. Silencing of the CEACAM6 gene was shown to impair metastasis and suppresses tumor growth (Jass et al., 2002).

However, studies examining the expressions, biological functions, and potential clinical implications of CEACAM6 are limited, particularly in gastric tumors. In this study, we quantitatively evaluated CEACAM6 mRNA transcripts using real-time quantitative polymerase chain reaction (PCR) and detected the expression of the corresponding protein using immunohistochemistry in a total of 75 gastric adenocarcinomas and 20 adjacent tumor tissues;

these results were compared with those of 20 normal gastric mucosas. Furthermore, the correlations between CEACAM6 expression and tumor clinicopathological features, development, and malignant progresses, as well as the significance of clinical outcome in gastric tumors were examined.

MATERIAL AND METHODS

Patients and specimens

Gastric adenocarcinoma tumor tissues and 20 corresponding non-neoplastic gastric mucosas were obtained from 75 patients who had undergone surgical gastrectomy from 2008-2011 in ShengJing Hospital affiliated China Medical University after obtaining informed consent from all patients. The patients included 52 men and 23 women with a mean age of 64.0 ± 7.31 years (range 33-75 years); none of the cases received preoperative chemotherapy and/or radiation therapy. Tumor tissues and normal gastric mucosas were immediately snap-frozen in cryovials containing 1 mL TRIzol® reagent (Invitrogen; Carlsbad, CA, USA) and stored at -80°C until analyses. Surgical specimens were also processed for routine histopathological analysis. Pathological parameters were evaluated according to the World Health Organization (WHO) criteria for grade and tumor-node-metastasis (TNM) system for stage classification proposed by the International Union against Cancer (Fenoglio-Preiser et al., 2000; American Joint Committee on Cancer, 2002). In the current study, 19 patients were in TNM stage I, 23 in stage II, 20 in stage III, and 13 in stage IV. Histology showed well-differentiated adenocarcinoma in 19 patients, moderately differentiated adenocarcinoma in 20 patients, and poorly differentiated adenocarcinoma in 36 patients. Forty-eight patients had lymph node metastasis, whereas 27 patients had no regional lymph node metastasis.

Total RNA isolation and cDNA synthesis

Total RNA was isolated from gastric adenocarcinoma and non-neoplastic gastric tissues using TRIzol® reagent according to manufacturer instructions and was evaluated for purity and concentration as previously described using a nucleic-acid quantitative analysis apparatus (DU800; Beckman; Brea, CA, USA). Two micrograms total RNA was reverse-transcribed for cDNA synthesis in 25 μL mixture, containing 2 μL 0.5 μg/μL random primer, 4 μL 10 mM dNTPs (Promega; Madison, WI, USA), 1 μL 1 U/μL RNase inhibitor, and 1.5 μL 200 U/μL MMLV murine MLV-reverse transcriptase (Invitrogen) (Ogawa et al., 2005). The reverse transcription reaction was performed in an ABI 9700 PCR system (Applied Biosystems; Foster City, CA, USA) at 42°C for 60 min, followed by heating at 95°C for 10 min. The cDNA products were stored at -80°C.

Real-time quantitative RT-PCR amplification of CEACAM6

RQ-PCR assays were performed with 4 μL cDNA (2.5 ng/μL related to RNA concentration), 1 μL 2 μL each primer, and 5 μL Power SYBR Mastermix (Applied Biosystems) in 10 μL total reaction volume per reaction, on a 96-well plate. Primers for CEACAM6 (NM_002483)

(Zhao et al., 2011) and β-actin, which used as an endogenous control, were as follows: CEACAM6 forward 5'-CGCATACAGTGGTCGAGAGA-3'; reverse 5'-GTCATGTTGCCATTGGACAG-3'; β-actin forward 5'-GAGACCTTCAACACCCCAGCC-3'; reverse 5'-GGAGTACAGGT CTTTGCGGATG-3'. RQ-PCR amplifications were conducted on an ABI PRISM 7500 Sequence Detection System with the following protocol: 2 min at 95°C, followed by 45 cycles for 15 s at 95°C and 1 min at 60.5°C, followed by 10 s at 72°C. Negative controls were used to ensure that no amplification occurred in the absence of target cDNA and all assays were run in triplicate.

To confirm the purity and specificity of all products amplified, a melting curve of the final RQ-PCR products was generated and analyzed using the ABI Prism Dissociation Curve Software (Applied Biosystems). Single and multiplex standard curves were produced with 5-fold serial dilutions of target and endogenous genes to examine the reaction efficiency and reproducibility of the assay. Relative expression of CEACAM6 was calculated and normalized to β-actin values according to the formula: Δ(Rt-Et)/Δ(Rn-En), where *Rt* is the threshold cycle number for the reference gene observed in the tumor, *Et* is the experimental gene in the tumor, *Rn* is the reference gene in the normal sample, and *En* is the experimental gene in the normal sample. Rn and En values were averaged from the 20 normal mucosa samples.

Immunohistochemistry

Serial 4-μm-thick sections from formalin-fixed paraffin-embedded tissue samples were deparaffinated, rehydrated using a descending concentration series of alcohol, and washed with Tris-buffered saline (50 mM Tris, 150 mM NaCl, pH 7.4) as previously described. Antigen retrieval was performed by microwave-heating the sections in 10 mM sodium citrate buffer, pH 6.0, for 10 min. After quenching the endogenous peroxidase activity with 3% hydrogen peroxide in methanol for 15 min and blocking non-specific binding, primary polyclonal rabbit antibody against human CEACAM6 protein (Sigma; St. Louis, MO, USA) was added at a 1:200 dilution, after which slides were incubated at 4°C overnight. Secondary goat anti-rabbit antibody (DAKO; Carpinteria, CA, USA) was incubated with the samples at room temperature for 30 min after further washing with Tris-buffered saline. Sections were incubated with Strep ABC complex/horseradish peroxidase (1:100; DAKO) for 30 min at 37°C. Chromogenic immunolocalization was performed by exposure to 0.05% diaminobenzidine tetra hydrochloride as described by the manufacturer. Finally, the slides were counterstained with hematoxylin, mounted with glycerine/gelatin, and analyzed using light microscopy.

Stains were evaluated independently by 2 researchers who were blinded to the clinicopathological features of the specimen. Negative control staining was conducted either by omission of the primary antibody, by the use of non-immune serum, or using irrelevant antibodies, to ensure the sensitivity of the reaction in all cases. Immunoreactive tissue of an invasive gastric adenocarcinoma with known overexpression of CEACAM6 was used as a positive control. Samples showing no evidence of nuclear or cytosolic staining or those with evidence of only rare scattered positive cells (<10%) were considered to be negative. CEACAM6 was first scored according to the specific location in the membrane and cytoplasmic staining intensity as follows: 0 (negative), 1 (weak), 2 (moderate), and 3 (strong).

Next, frequency was graded from 0-4 based on the percentage of positive cells, as follows: grade 0 (<5%); grade 1 (5-25%); grade 2 (25-50%); grade 3 (50-75%), and grade 4 (>75%). Based on the product of the intensity and frequency grades, IHC results were classified using a 4-point scale as follows: index score 0, negative (-); index score 1-4, weakly positive (+); index score 5-8, moderately positive (++), and index score 9-12, and strongly positive (+++).

Statistical analysis

The association between the CEACAM6 expression and individual clinicopathological variables was analyzed using the Student *t*-test and Fisher exact test. To analyze survival curves, patients who had died from causes other than gastric cancer were excluded from the analyses. The differences in survival between subgroups of patients were compared using the log-rank test and survival curves were plotted using the Kaplan-Meier method. Statistical data are reported as the means ± standard deviation, and a P value < 0.05 was considered to be statistically significant.

RESULTS

Amplification of CEACAM6 mRNA by RQ-PCR

To assess CEACAM6 expression, we used real-time fluorescent quantitative PCR to quantitatively evaluate mRNA as a biomarker surrogate of gene expression in tissue samples.

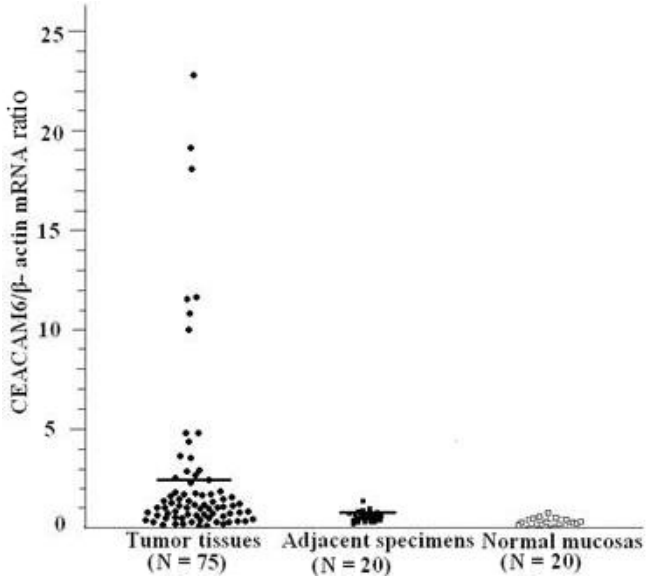

Figure 1. Expressions of CEACAM6 mRNA in gastric adenocarcinoma tissues, adjacent tumor specimens, and non-neoplastic mucosas. Differential expressions of CEACAM6 mRNA in gastric adenocarcinoma tissues, corresponding 20 adjacent tumor mucosas and 20 non-neoplastic tissues obtained from 75 patients were determined by real time quantitative PCR. The horizontal line represents the mean for the CEACAM6/β-actin ratio, and the relative expression levels of CEACAM6 mRNA in each group is 2.513 ± 0.869, 1.171 ± 0.428, and 0.594 ± 0.513, respectively.

Detection of CEACAM6 and β-actin mRNA was sensitive and precise (Ct < 30). Melting curves showed localized unimodality for each sample, indicating non-specific fluorescence derived from byproducts such as primer-dimers. The transcripts of CEACAM6 mRNA detected on RQ-PCR showed an increasing trend from normal to malignant and adjacent tissues. Expression of CEACAM6 mRNA in both gastric adenocarcinomas (2.513 ± 0.869) and adjacent tissues, which were located within 5 mm of the study specimens (1.171 ± 0.428), were significantly up-regulated compared to tumor-free samples (0.594 ± 0.513) ($P < 0.01$) (Figure 1).

Overexpression of CEACAM6 protein

The immunohistochemical assay showed that the expression pattern of CEACAM6 protein was homogenous throughout gastric adenocarcinoma cells, and staining intensity was moderate to strong mainly in the cytoplasm and/or plasma membranes, compared with no expression in normal gastric tissues or focal weak nuclear and/or cytosolic staining of some scattered stroma. Epithelial cells adjacent to the tumor tissues were all negative. Seven (9.3%) of the 75 investigated adenocarcinoma samples were negative, while 16 (21.3%) were weakly (1+), 30 (40%) were moderately (2+), and 22 (29.3%) were strongly (3+) immunoreactive. Therefore, positive immunostaining (2+ and 3+) for CEACAM6 protein was detected in 52 (69.33%) of the 75 gastric adenocarcinomas, which correlated with the upregulated levels of CEACAM6 transcripts observed using RQ-PCR. (Figure 2A-D, Table 1).

Correlation of CEACAM6 expressions with tumor variables

To analyze the relationship between the presence and absence of protein overexpression levels of CEACAM6 and tumor clinicopathological characteristics, tumor samples were considered low expression if IHC was classified as 0 or 1+ and overexpression as 2+ and 3+. A statistically significant association between adenocarcinoma staining for CEACAM6 and lymph node metastasis was observed (51.85 *vs* 79.17%, $P = 0.003$) (Table 1). Similarly, statistical results revealed clear differences in CEACAM6 expression between the early stage I + II and advanced stage III + IV (52.38 *vs* 90.91%, $P = 0.001$). However, tumors showing overexpression were generally well differentiated, but the correlation between these parameters was not statistically significant (68.42 *vs* 69.6%, $P = 0.941$). In addition, the remaining tumor variables, including tumor size and histological types, showed no significant association with positive staining for CEACAM6 in this group of patients, and there was no obvious age or other demographic difference between patients with CEACAM6-negative and CEACAM6-positive adenocarcinomas.

Effect of CEACAM6 expression on prognosis

According to survival analysis using the Kaplan-Meier method, patients with lower or absent CEACAM6 expression showed significantly longer overall postoperative survival (median 43 months) compared with those with higher expression of CEACAM6 (median 17 months). Higher expression of CEACAM6 was found to be associated with poor survival of gastric cancer patients ($P = 0.046$, log-rank) (Figure 3).

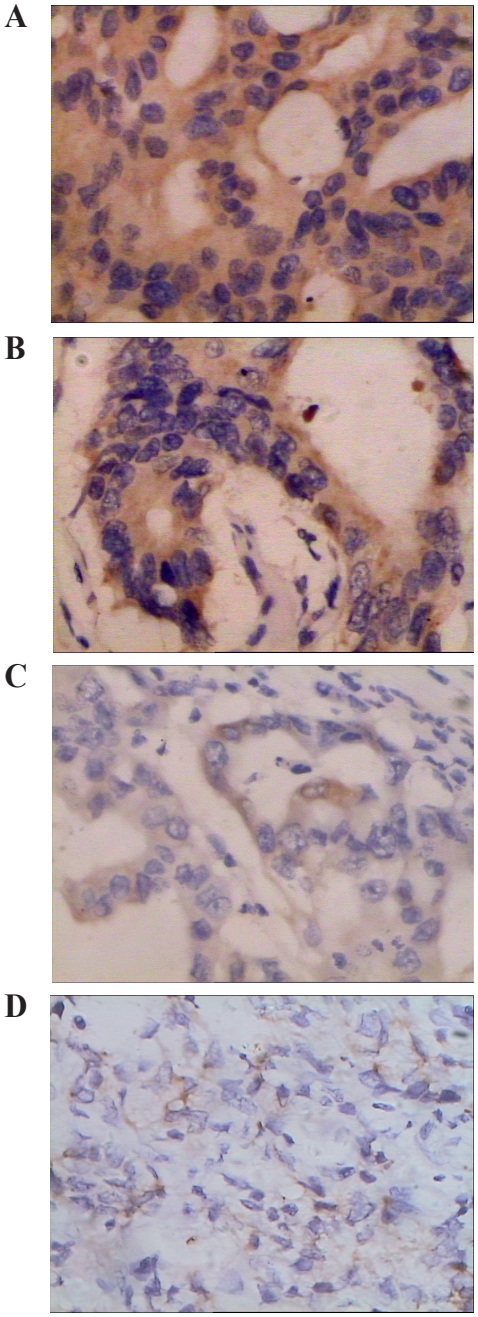

Figure 2. Representative examples of CEACAM6 immunohistochemistry. Immunohistochemical staining of gastric adenocarcinoma specimens, adjacent tumor tissues and normal gastric mucosas. **A.** Adenocarcinoma cells revealed distinctly labeled with strong (3+) membrane cytoplasm and nucleus staining pattern (Magnification 200X). **B. C.** A majority of stain was observed in adjacent tumor cells exhibiting moderate (2+) to weak (1+) intensity. (200X). **D.** The tumor shows no staining at all, except a few faint cytoplasmic staining. (200X).

Table 1. Associations between CEACAM6 expression and clinicopathological characteristics.

Variables	Total	CEACAM6 expression		P value
		Overexpression	Low expression	
Age (years)				
≥60	38	30	8	0.067
<60	37	22	15	
Gender				
Male	52	33	19	0.097
Female	23	19	4	
Tumor size (cm)				
<5	21	14	7	0.755
≥5	54	38	16	
Histology differentiation				
Well	19	13	6	0.941
Moderate or poor	56	39	17	
Lymph node metastases				
Negative	27	14	13	0.003
Positive	48	38	10	
Lauren's classification				
Intestinal type	38	26	12	0.697
Diffuse type	26	19	7	
Other unclassified	11	7	4	
TNM Stage				
I + II	42	22	20	0.001
III + IV	33	30	3	

TNM = tumor-node-metastasis. *P values which represent the comparison of patients who had CEACAM6-positive tumors with patients had CEACAM6-negtive tumors, were calculated by the Fisher exact test.

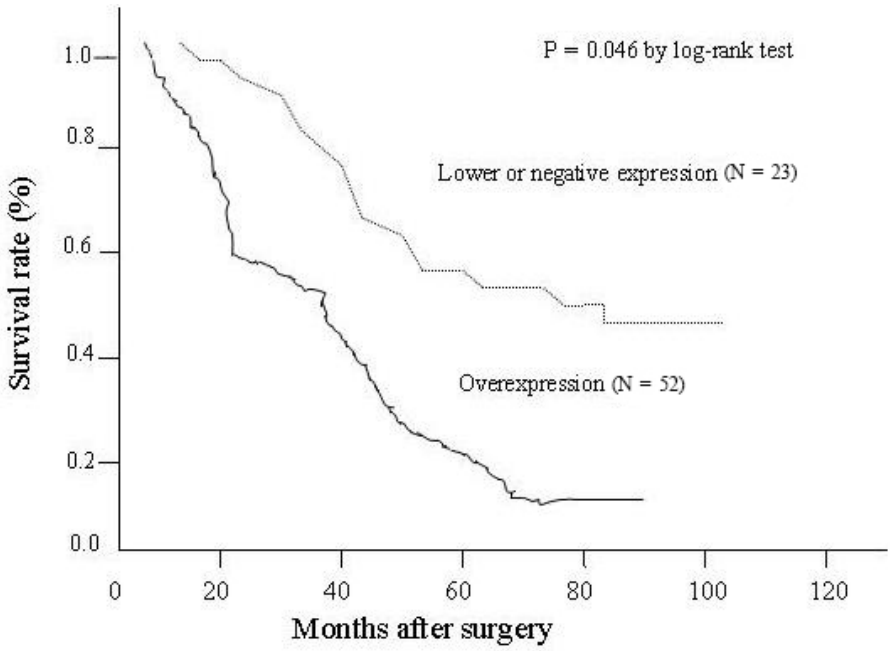

Figure 3. Kaplan-Meier disease-free survival curves in patients with gastric adenocarcinoma according to the level of CEACAM6 expression. The survival rate for patients in the high expression group was significantly higher than that for patients in the low expression group (P = 0.046, log-rank test).

DISCUSSION

Carcinogenesis and metastasis of gastric cancer is a complex multistep malignant process that involves continuous signaling cascades related to cell migration, proliferation, differentiation, apoptosis, and survival. Over the past decades, molecular therapies based on understanding the molecular mechanisms underlying gastric tumorigenesis and progression have promoted new targeted therapies directed at tumor-specific molecules, inducing cellular apoptosis, blocking expressions of oncogene related to metastasis and invasiveness, correcting genetic abnormalities, enhancing tumor sensitivity to chemotherapy, or modulating the immune response against tumors (Kim et al., 2008; Moss et al., 2008). The human CEACAM family consists of membrane-linked and secretory glycoproteins, members of which are highly glycosylated and belong to the immunoglobulin gene super-family. This protein is in the same family as human carcinoembryonic antigen CEA [CEA (since re-designated CEACAM5)], which was initially thought to be a tumor-specific antigen and a tumor marker, which was widely used for patient management. CEACAM6 was observed principally on neutrophils and some epithelia (Arrieta et al., 2009) and was thought to be a novel molecular target in relation to tumorigenesis. In this study, we quantitatively examined CEACAM6 mRNA transcriptional levels and the expression of its corresponding protein to determine the relationship between clinicopathological variables and prognostic significance of gastric cancer.

An RQ-PCR approach was applied to detect CEACAM6 gene expression on the molecular level. CEACAM6 levels in samples obtained from 75 gastric adenocarcinoma and 20 adjacent tumor tissues were clearly increased, with relative expression levels of 2.513 ± 0.869 and 1.171 ± 0.428, respectively. By contrast, PCR results showed lower expression of 0.594 ± 0.513 in 20 non-neoplastic specimens. Statistical significance was observed between adenocarcinoma and non-neoplastic tissues, as well as between adjacent-tumor tissues and non-neoplastic tissues ($P < 0.01$). Expression of CEACAM6 protein as assessed by IHC displayed an increasingly strong trend in the sequence of non-neoplastic adjacent malignant gastric tissue samples, which coincided with the amplification of CEACAM6 mRNA in RQ-PCR. According to these data, CEACAM6 may play a role in human gastric carcinogenesis and participate in the malignant transformation of neoplastic cells in gastric adenocarcinomas. This result is consistent with the finding that CEACAM6 expression is an early event in human colorectal cancers and plays a role in both early carcinogenesis and subsequent tumor progression (Yasui et al., 2004; Jass, 2005). Previous studies using animal models support that CEACAM6 is functionally important in tumorigenesis, cellular adhesion, growth, invasion, and metastasis, and inhibition of CEACAM6 expression by RNA interference or antibody targeting improved survival of mice with metastases (Ilantzis et al., 2002; Blumenthal et al., 2005). Several experiments *in vitro* and *in vivo* demonstrated that CEACAM6 may impede myogenic, adipogenic, neurogenic, and colonic differentiation programs (Duxbury et al., 2004a,b), inhibit anoikis and apoptosis in colon and pancreatic cancer cells, disrupt cell polarization and tissue architecture (Soeth et al., 2001; Duxbury et al., 2004c), enhance liver metastasis (Capurso et al., 2006), increase chemoresistance (Duxbury et al., 2004d), and increase higher incidence of spontaneous colon tumor and lung tumor in a transgenic mouse model (Chan et al., 2006). In the present study, CEACAM6 in 52 (63.33%) gastric adenocarcinoma specimens was observed to be associated with lymph node metastasis ($P = 0.003$) and advanced stage ($P = 0.001$). These observations indicate that CEACAM6 is a specific determinant of malignant cellular behavior and progression in gastric adenocarcinoma. This is supported by studies in pancreatic and

cholangiocarcinoma cancer showing that overexpression of CEACAM6 correlates with aggressive growth (Duxbury et al., 2004e,f; Olnes and Erlich, 2004) and that adhesion molecules play a role in the steps leading to malignant cell metastasis. However, there was a tendency of higher rate from well differentiation to moderate and poor differentiation, and no statistical significance was obtained (P = 0.0941). Similarly, male gender, tumor size, and pathologic subtypes were not found to be associated with higher expression rates of CEACAM6. Chan et al. (2006) reported that cellular differentiation and extreme colonocyte hyperproliferation in transgenic colons were completely blocked when CEACAM6 was highly expressed, which is a tumor-like property. However, there are various differences, including tissue specific properties and approaches, between the two studies.

Moreover, we observed that median postoperative survival time of patients with higher expression of CEACAM6 was significantly shorter than that in patients with lower expression of CEACAM6 (17 *vs* 43 months, P = 0.046 by log-rank test), indicating the overexpression of CEACAM6 is associated with poor clinical outcome. Expression of CEACAM6 has also been reported to be an independent prognostic significance factor in colorectal cancer patients and of improved survival in pancreatic cancer (Thompson et al., 1991; Jantscheff et al., 2003; Logsdon et al., 2003). However, whether a correlation exists between the expression of CEACAM6 and clinically reduced survival, particularly in gastric tumors, as well as the evaluation of CEACAM6 as a useful prognostic factor for gastric tumors, should be further investigated.

Several adhesion molecules have been found to be up- or downregulated in gastric cancer and may be new targets for molecular therapy, such as the cancer-specific monoclonal antibodies against the E-cadherin mutations. These molecules may be used as very specific agents to treat gastric cancer related to toxins, drugs, or radiolabelling. CEACAM6 functions as an intercellular adhesion molecule because of parallel and antiparallel self-binding of their extracellular domains (Ordonez et al., 2007; Akinc et al., 2008). Small CEACAM6-containing lipid rafts can cluster together to form larger rafts and co-cluster their associated signaling elements and may underlie the activation observed of downstream signaling cascades such as the integrin signaling pathway. Neutralizing antibodies directed against integrin inhibit gastric cancer peritoneal dissemination in nude mice, as well adhesion polypeptides, which block the binding of integrin to the extracellular matrix reduced peritoneal implantation of gastric cells (Matsuoka et al., 1998; Kawamura et al., 2001). However, the present study highlighted CEACAM6 as a useful molecular target for diagnosis and therapy.

In conclusion, we quantitatively analyzed amplification of the CEACAM6 gene and the overexpression of CEACAM6 protein in patients with gastric adenocarcinoma, which was significantly correlated with lymph node metastasis, advanced stage, and postoperative survival time. These observations indicate that CEACAM6 contributes to molecular pathogenesis, adverse pathologic progression or aggravation, as well as the clinical prognosis of gastric cancer. Taken together, this study highlights that CEACAM6 expression may be a valuable biomarker for screening of gastric tumors and an aggressive phenotype of malignant stages. Our results will benefit patients with gastric cancer and should facilitate the use of individual therapies during early disease stages.

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