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Overexpression and clinical significance of carcinoembryonic antigen-related cell adhesion molecule 6 in colorectal cancer

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ABSTRACT

Background: Carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) inhibits anoikis and affects the malignant phenotype of cancer cells. In this study, we analyzed CEACAM6 as a gene that is highly upregulated in colon cancer tissues, and examined the assertion that CEACAM6 might be a suitable candidate tumor marker for the diagnosis of colon cancer.
Methods: CEACAM6 gene expression in human colon tissues was performed by tissue microarray and analyzed using RT-PCR (each of normal and tumor tissue, $n=40$) and immunohistochemical and clinicopathological (colon cancer patients, $n=143$) analyses.
Results: CEACAM6 transcriptional and translational levels were significantly upregulated in human tumor tissues compared to non-tumor regions, and clinicopathological analysis revealed a significant correlation between CEACAM6 protein expression and Dukes' stage ($p<0.001$). High expression levels of CEACAM6 were significantly associated with lower overall survival ($p<0.001$) and shorter recurrence-free survival ($p<0.001$). We demonstrated that knockdown of CEACAM6 with *CEACAM6*-specific small interfering RNA in colorectal cancer cells attenuated invasivity (35%); conversely, the overexpression of CEACAM6 increased invasiveness.
Conclusions: CEACAM6 is significantly upregulated in colon cancer tissues and is closely associated with poor prognosis, indicating that CEACAM6 might be used as a tumor biomarker and a potential therapeutic target for colon cancer.

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1. Introduction

Colorectal cancer originates from uncontrolled cell growth in the colon, rectum, or appendix of the gastrointestinal tract. More than 1 million individuals develop colorectal cancer every year worldwide [1], and the mortality rate of colorectal cancer is nearly 33% in developed countries [2]. The 5-year survival rate in colorectal cancer patients is less than 60% in Europe, and 1/3 of individuals who develop colorectal cancer in developed countries ultimately succumb to the disease [2]. Survival is directly related to early stage detection of cancer, but overall is poor for symptomatic cancer. Typically, the disease is not detected until the cancer is quite advanced. Survival rates for early stage detection are approximately 5 times greater than for late stage cancers.

The carcinoembryonic antigen (CEA) family of proteins has long been thought to have an important role as cell-adhesion molecules in cancer, and CEA is one of the most widely used tumor markers in serum immunoassay determinations. However, the inter- and intracellular physiological functions of CEA and its potential involvement in tumorigenesis remain elusive. The CEA-related cell adhesion molecule (CEACAM) protein family has been implicated in various intercellular adhesion and intracellular signaling processes that mediate cellular growth and differentiation. The CEACAM family may also influence the immune responses associated with infection, inflammation, and cancer [3], having important roles in vascularization, tumorigenesis, and apoptosis [4,5]. For example, CEACAM5 was first described as a gastrointestinal oncofetal antigen [6]; CEACAM6 was shown to be overexpressed in

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pancreatic cancer, breast cancer and leukemia [7–10]; and CEACAM8 was highly expressed in acute leukemia [10]. However, CEACAM1 was shown to be downregulated in various tumor types, including prostate, breast, colorectal and endometrial tumors [11].

CEACAM6 is a single-chain glycosylphosphoinositol (GPI) anchored immunoglobulin (Ig)-like glycoprotein and is a member of the human CEA family [7]. CEACAM6 inhibits anoikis (apoptosis induced by inadequate adhesion to extracellular matrix) and modulates the malignant phenotype of cancer cells [8,9,12–16]. The anoikis-resistance of CEACAM6 results in cell survival under anchorage-independent conditions and is a characteristic associated with tumorigenesis and metastasis [9,17]. Knock-out of the CEACAM6 gene enhances cell anoikis, increases caspase activation in response to anchorage-independent conditions, and downregulates the cell survival pathway of Akt [16]. The expression of CEACAM6 on cell membranes also increases cell invasiveness through a c-src-dependent mechanism. Furthermore, it has been reported that CEACAM6 is overexpressed in a wide variety of carcinomas, including pancreas and colorectal cancers [7–9], and CEACAM6 overexpression has been shown to be associated with poor clinical outcome [13]. Functionally, CEACAM6 has been implicated in cellular adhesiveness, invasiveness, and metastatic behavior of tumor cells. CEACAM6 overexpression also disturbs the ordered tissue architecture that is observed in 3-D cultures of several colon carcinoma cell lines [18].

2. Materials and methods

2.1. Patient samples

Colon cancer samples were obtained from patients who underwent routine surgery for colon cancer at the Department of Surgery, Eulji University Hospital (Daejeon, South Korea), between January 2000 and June 2005. For immunohistochemical study, 143 colon cancer tissues and paired normal mucosal tissues taken from a site distant from the tumorous lesion were fixed in a 10% neutralized buffered formalin solution for 24 h, and for microarray and RT-PCR, frozen tissues were used. Each patient's clinical status was classified according to the pathological grade of the tumor, tumor size, lymph node involvement, and Dukes' staging system for colon cancer. All patients were advised of the procedures and were provided with written informed consent approved by the Institution's Ethics Committee.

2.2. Microarray analysis of colon cancer tissues

Total RNA was extracted from colon cancer tissues of 66 patients with stages II and III cancer using the Qiagen kit according to the manufacturer's manual (Qiagen, Hilden, Germany). The quantified RNA was used for tissue microarray analysis on 48-K Illumina chips (Illumina Inc., CA). Total RNA samples were labeled using the Illumina TotalPrep RNA Amplification Kit (Ambion Inc., CA, USA), and 1.5 μg of biotin-labeled combinational RNA (cRNA) was hybridized at 58 °C for 16 h with the Illumina Sentrix Human-6 Expression BeadChip (version 2). The hybridized biotinylated cRNA was detected with streptavidin–indocarbocyanin and was quantified using the Illumina BeadArray Reader Scanner according to the manufacturer's instructions. Array data were processed and analyzed using Illumina BeadStudio software (version 3.0). Java Treeview (http://jtreeview.sourceforge.net/, accessed on March 2010) was used to observe the pattern of gene expression in tumors compared with normal tissues.

2.3. Cell culture, transfection, luciferase reporter assay

Human colon cancer cell lines (SW620, SW480, Colo205, HT29, HCT116, SNUC1, and LoVo) were obtained from the Korean Cell Line Bank (Seoul, South Korea). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco-BRL, Grand Island, NY) or RPMI-1640 medium supplemented with heat-inactivated 10% fetal bovine serum (Gibco-BRL) and antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin) and maintained at 37 °C in an incubator containing a humidified atmosphere of 5% CO_2. Cells at 60% confluence were plated 1 day before transfection and then transfected with siRNA specific for CEACAM6 (10–50 nmol/l; sense, 5′-CUG ACA UUA GCA GCA UCU U (dTdT)-3′; antisense, 5′-AAG AUG CUG CUA AUG UCA G (dTdT)-3′) and CEACAM6 overexpression plasmid (1 μg) using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. LoVo cells were transfected with siRNAs, pGL2CDH1-luciferase reporter, and pCMV-β-gal plasmids for 3 days. The cells were lysed and *CDH1* promoter activity was assayed using a luminometer. The cells were lysed with lysing buffer (Promega, Madison, WI), the cleared lysates were transferred to 96-well plates, and luciferase assay reagent (Promega) was added. The light intensity of the reaction was determined using a plate-reading luminometer (Turner Designs, Sunnyvale, CA).

2.4. RNA extraction and RT-PCR analysis

Total RNA was extracted from the normal/tumor tissue pairs from patients with colorectal cancer and colon cancer cell lines using TRI reagent (Molecular Research Center, OH, USA) according to the manufacturer's instructions. After quantification of RNA, the proSTAR first strand RT-PCR kit (Stratagene, La Jolla, CA) was used for cDNA synthesis. Primers used in this study are as follows: CEACAM6 (sense, 5′-CGC ATA CAG TGG TCG AGA GA-3′; antisense, 5′-GTC ATG TTG CCA TTG GAC AG-3′); and *ACTB* (sense, 5′-GAT CAT TGC TCC TCC TGA GC-3′; antisense, 5′-ACT CCT GCT TGC GAT CCA C-3′). For real-time RT-PCR analysis, SYBR, primers, and cDNAs were mixed and the reaction was performed for 40 cycles in a Thermal Cycler Dice (Takara, Japan). Data were analyzed using TP800 software (Takara).

2.5. Immunohistochemistry (IHC)

Tissue specimens obtained through therapeutic procedures were fixed in neutral buffered formalin (10% v/v formalin in water, pH 7.4) and embedded in paraffin wax. Serial sections 4 μm thick were cut and mounted on charged glass slides (Superfrost Plus; Fisher Scientific, Waltham, MA). In brief, the tissue sections were deparaffinized, and antigen retrieval was carried out in citrate buffer (pH 6.0) for 10 min. The sections were then treated with 3% H_2O_2 in methanol to quench the endogenous tissue peroxidase activity, followed by incubation with 1% BSA to block nonspecific binding. Tissue sections were incubated with monoclonal anti-CEACAM6 antibody (1:100 dilutions) and were stained with a standard EnVision-HRP kit (Dako, Carpinteria, CA).

2.6. Assessment of immunostaining

Each slide was evaluated for CEACAM6 immunoreactivity using a semiquantitative scoring system for both the intensity of the stain and the percentage of positive neoplastic cells. The percentage of cells displaying a stronger staining intensity than the adjacent mucosal epithelium was scored as 1 (0–24% tumor cells stained), 2 (25–49%), 3 (50–74%), or 4 (75–100%). For the purpose of statistical analyses, the median of this series (25% of malignant cells showing a stronger intensity than adjacent colon mucosal epithelium) was used as a cutoff value to distinguish tumors with low (<25%) or high (>25%) levels of CEACAM6 expression. The relationship between the results of the immunohistochemical study and the clinicopathologic parameters was determined using SAS® software (v9.01; SAS Institute, Cary, NC). The cutoff date for vital status analysis was August 2009.

2.7. Cell invasion assay

Invasion assays were performed using a cell invasion assay kit (Chemicon, Temecula, CA) according to the manufacturer's instructions.

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