Down-regulation of CEACAM1 in breast cancer

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Abstract

Carcinoembryonic antigen-related adhesion molecule 1 (CEACAM1) is a type 1 transmembrane glycoprotein belonging to the CEA family, which has been found to exist as either soluble forms in body fluids or membrane-bound forms on the cell surface. Aberrant CEACAM1 expression is associated with tumor progression and has been found in a variety of human malignancies. Increasing interest has been devoted to the expression of CEACAM1 in breast cancer, but most of these findings are contradictory. The aim of this study was to investigate CEACAM1 expression in breast cancer in greater detail. Using immunohistochemical staining, we found that CEACAM1 expression was reduced or lost in breast cancer tissues compared with noncancerous breast tissues. In addition, soluble CEACAM1 levels in the culture medium of breast cancer cell lines were significantly lower than those in a nontumorigenic breast epithelial cell line. Confocal laser scanning microscopy consistently showed that breast cancer cell lines have relatively low expression of membrane-bound CEACAM1. Furthermore, CEACAM1 mRNA and protein expression levels were down-regulated in breast cancer cell lines as measured using real-time reverse transcriptase-polymerase chain reaction and western blot analysis, respectively. Taken together, our results demonstrate a systematic down-regulation of CEACAM1 in breast cancer and suggest that a strategy to restore CEACAM1 expression may be helpful for the treatment of breast cancer.

Key words: CEACAM1, breast cancer, down-regulation

Introduction

The human carcinoembryonic antigen-related adhesion molecule 1 (CEACAM1), previously known as biliary glycoprotein or CD66a and a type 1 single-pass transmembrane glycoprotein, is a member of the CEA family, which belongs to the immunoglobulin superfamily [1]. CEACAM1 has a broad distribution due to its expression in a variety of proliferating and quiescent epithelia, endothelia, hematopoietic cells, monocytes, granulocytes, T cells, B cells, and natural killer cells [2,3]. CEACAM1 is a multifunctional protein that functions as a homophilic or heterophilic adhesion molecule, a modulator of immunity and inflammation, and a stimulator of angiogenesis, and can regulate cell polarization [3]. CEACAM1 also plays a role in insulin metabolism, acts as a promoter of cholesterol crystallization, and serves as a binding receptor for certain bacterial strains [3]. There are 12 known CEACAM1 isoforms, resulting from differential splicing and proteolytic processing [4]. Nine of the 12 known CEACAM1 isoforms are anchored to the plasma membrane via the transmembrane domain, whereas three isoforms lacking the transmembrane domain appear to exist in soluble form [4–6]. Thus, CEACAM1 isoforms are categorized into two separate groups: soluble CEACAM1 forms and membrane-bound CEACAM1 forms.
Recently, accumulating evidence has indicated that CEACAM1 expression is altered during oncogenesis, and it mediates various key signal transduction pathways in tumor progression. CEACAM1 expression has been shown to be down-regulated in many types of malignancies, such as colorectal cancer [7], hepatoma [8], renal cell carcinoma [9], prostate carcinoma [10], and bladder carcinoma [11]. These findings together with the inhibitory effect of CEACAM1 on tumor growth, which is observed in some malignancies, lead to the postulation that CEACAM1 acts as a tumor suppressor [9,12]. However, recent studies have shown that CEACAM1 is over-expressed in several malignancies, including pancreatic adenocarcinoma [13], gastric carcinoma [14], malignant melanoma [15], thyroid cancer [16], and lung cancer [17]. Although the change of CEACAM1 expression in different types of malignancies is contradictory, most studies suggest that aberrant CEACAM1 expression may play a pivotal role in tumorigenesis.

Aberrant CEACAM1 expression has also been observed in breast cancer tissues in several studies. However, no consensus has been reached. Although some reports [18,19] have indicated that there is a partial reduction or loss of CEACAM1 expression in breast cancer compared with normal tissue, others [20] have demonstrated that CEACAM1 is expressed to the same extent in both normal and malignant breast tissues, showing no down-regulation in breast cancer. Thus, it is of interest to investigate CEACAM1 expression in breast epithelia in more detail. One intrinsic limitation in analyzing primary tumor tissues is the contamination of normal cells, which might lead to underestimation or overestimation of the degree of abnormality in these tissues. Although gene expression profiles in cancer cell lines may not always reflect gene expression patterns in vivo, cancer cell lines are not contaminated with other cells. Therefore, in addition to immunohistochemical analysis of tissue samples, we also examined the expression of CEACAM1 in a panel of six human breast cancer cell lines. First, we compared the soluble CEACAM1 levels in the conditioned media of breast cancer cell lines and in a normal breast epithelial cell line, MCF 10A. Subsequently, the expression of membrane-bound CEACAM1 was evaluated by immunofluorescence (IF) staining. In addition, the CEACAM1 expression levels in breast cancer cell lines were verified at the mRNA and protein level using quantitative real-time reverse transcriptase polymerase chain reaction and western blot analysis, respectively.

**Materials and Methods**

**Patients and specimens**

Human benign (n = 40) and malignant (n = 41) breast tissue samples were collected from patients who underwent surgical resection at Shanghai Sixth People’s Hospital, and were subsequently diagnosed with breast cancer or benign disease based on histopathological evaluation. Moreover, the matched nontumor adjacent tissues were also collected from 28 cases of the 41 malignant specimens mentioned above. The clinical characteristics of the patients are shown in Table 1. None of the patients had received chemotherapy or radiotherapy before surgery. The samples were immediately snap-frozen in liquid nitrogen after resection and stored at −80°C. All tissue samples were routinely cut at 5 µm thickness for serial sections. One section of each sample was stained with hematoxylin–eosin (H&E) for histopathological evaluation. This study was approved by the Ethics Committee of Shanghai Jiao Tong University in accordance with the Declaration of Helsinki, 1975 (as revised in 1983). All individuals provided written informed consent before participation in the study.

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**Cell culture**

Human breast cancer cell lines (BT-549, Hs 578T, T-47D, MCF7, MDA-MB-231, and MDA-MB-468) were obtained from the Institute of Biochemistry and Cell Biology, the Chinese Academy of Sciences (Shanghai, China). BT-549 and T-47D were cultured in RPMI1640 medium (Gibco, Grand Island, USA), Hs 578T and MCF7 were cultured in Dulbecco’s modified Eagle’s medium (Gibco), and MDA-MB-231 and MDA-MB-468 were cultured in Leibovitz’s L-15 medium (Gibco). MCF 10A, a basal-like nontumorigenic, immortalized mammary epithelial cell line, was included in this study as the normal control, which was cultured in mammary epithelial growth media (Lonza, Walkersville, USA). All cell lines were cultured at 37°C in a humidified atmosphere, and media were supplemented with 10% fetal bovine serum.

**Immunohistochemistry**

To determine the expression and location of CEACAM1, specimens were stained using the immunohistochemistry method, which was performed on breast tissue specimens with the mouse monoclonal anti-CEACAM1 antibody (Abcam, Cambridge, UK; 1:75 dilution). The sections were fixed in 4% acetone at 4°C for 10 min, and endogenous peroxidase was blocked by immersing the sections in a 3% solution of hydrogen peroxide in methanol for 10 min. After being washed three times in phosphate-buffered saline (PBS; pH 7.4) for 5 min, the sections were blocked with 5% bovine serum albumin (BSA) in PBS at room temperature for 1 h. The excess serum albumin was rinsed off with PBS, and the sections were incubated with primary antibody in a humidity chamber overnight at 4°C. After that, sections were rinsed with PBS before incubation with the biotinylated secondary antibody (DAKO, Glostrup, Denmark) in a humidity chamber for 40 min at 37°C. After rinsing with PBS, the streptavidin–peroxidase complex reagent (StrepABComplex/HRP Duet, DAKO) was added. Diaminobenzidine and hydrogen peroxide were used for visualization. For negative controls, the primary antibody was replaced by PBS. Histological and immunohistochemical evaluation were performed.
Enzyme-linked immunosorbent assay
The soluble CEACAM1 levels in the conditioned media of breast cancer cells were measured using an enzyme-linked immunosorbent assay (ELISA) kit (RayBiotech, Atlanta, USA) according to the manufacturer’s instructions. Briefly, a 96-well microplate was precoated with anti-human CEACAM1 antibody (RayBiotech), which can recognize the extracellular domain. Before use, all of the reagents and samples were allowed to warm to room temperature (18–25°C). The standard dilution series ranged from 20.58 to 15,000 pg/ml. First, 100 µl of each standard or serum sample was added to the appropriate wells and incubated for 2.5 h at 24°C with gentle shaking. After discarding the solution and washing four times, 100 µl of prepared biotinylated anti-human CEACAM1 antibody (RayBiotech) was added to each well and incubated for 1 h. After washing away the unbound biotinylated antibody, 100 µl of horseradish peroxidase (HRP)-conjugated streptavidin (RayBiotech) was added to the wells and incubated for 45 min, and 100 µl of 3,3′,5,5′-tetramethylbenzidine one-substrate reagent was added after five washes. Subsequently, 50 µl of stop solution was added to each well, and the plate was immediately read at 450 nm.

Immunofluorescence staining
To investigate the expression of membranous CEACAM1, Immunofluorescence (IF) staining was performed. For indirect IF, cells were grown on glass coverslips (24-well plates). The cells were fixed with cold methanol (−20°C) for 10 min at 4°C and then washed with PBS. The coverslips were blocked with 1% BSA/PBS for 1 h at room temperature and incubated overnight with anti-CEACAM1 antibody (Abcam) at a 1:40 dilution. Then, extensive washing was performed and cells were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody (Jackson ImmunoResearch, Philadelphia, USA) at 1:400 dilutions for 1 h at room temperature. Finally, the coverslips were washed three times with PBS again, and the fluorescence was visualized using an inverted fluorescence microscope (Olympus, Tokyo, Japan). Negative controls were processed similarly, but the primary antibody incubation step was eliminated. Three replicate wells were prepared for each cell type. Fluorescence images were captured and quantified using the ImageJ software (NIH, Bethesda, USA). Camera exposure and light settings were kept constant during each experiment and relative fluorescence intensities on cells were assessed as described by Mu et al. [21].

Quantitative real-time polymerase chain reaction
Total RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer’s instructions. The purity and concentration of RNA were determined by a spectrophotometer at 260 nm. Complementary DNA (cDNA) was generated by the PrimeScript RT reagent kit (Takara Biotechnology, Otsu, Japan). GAPDH was used as an internal control for each sample. Quantitative real-time polymerase chain reaction (qPCR) was performed using specific primers for CEACAM1 (forward, 5′-CAGTACCCCTGAATGTCACCCTAGT-3′; reverse, 5′-GTTCCATTTGATAAGCCAGGAGTAC-3′). Briefly, 1 µg of total RNA was denatured for 5 min at 70°C and cooled for 5 min on ice. Reverse transcriptase was added to a total volume of 20 µl, and reverse transcription was performed for 15 min at 37°C followed by 5 s at 85°C, according to the protocol recommended by the manufacturer. The cDNA was either used immediately for PCR amplification or stored at −20°C for further analysis. qPCR was performed in a total reaction volume of 20 µl on a thermal cycler ABI7500 (Applied Biosystems, Foster City, USA) for 40 cycles (denaturation: 30 s at 95°C, annealing: 20 s at 60°C, and extension: 15 s at 72°C). After PCR amplification, melting curve analysis was performed to verify the specificity of the test. For quantification of CEACAM1 mRNA, the 2−ΔΔCt (ΔCt = Ct,GAPDH − Ct,CEACAM1) method was employed.

Western blot analysis
Cells were lysed in radioimmunoprecipitation assay buffer containing 5% phenylmethanesulfonyl fluoride, a protease inhibitor, for 1 h on ice. After the mixture was centrifuged at 12,000 g for 10 min at 4°C, insoluble materials were removed. The total protein concentration in the cell lysates was determined using a bicinchoninic acid Protein assay kit (Pierce, Rockford, USA). Identical amounts (30 µg of protein) of cell lysates were boiled for 5 min, and then were electrophoretically separated on 10% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, USA). The PVDF membranes were blocked with Tris-buffered saline (TBS) containing 5% nonfat milk powder at room temperature for 1 h, and immunoblot analysis was performed with anti-CEACAM1 antibody (1:10000) at 4°C overnight. GAPDH was used as an internal control. The membranes were washed with TBS/Tween-20 buffer three times (5 min each) and then incubated with HRP-conjugated polyclonal secondary antibody (Abcam) for 1 h at room temperature. The immunoblots were visualized using the enhanced plus chemiluminescence assay (Pierce, Rockford, USA) according to the manufacturer’s instructions. Quantification of the blots was conducted using Image-Pro Plus software (version 6.0; Media Cybernetics, Bethesda, USA).

Statistical analysis
The data are presented as the mean ± standard deviation (SD) and represent at least three experiments. The statistical significance of the differences between two different groups was analyzed with Student’s t-test, using SPSS19.0 statistics software (SPSS, San Diego, USA), and P values <0.05 were considered statistically significant.

Results
Reduction or loss of CEACAM1 expression in human breast cancer tissues
To determine the expression and location of CEACAM1, breast tissue samples were immunohistochemically stained using a monoclonal antibody. Previous studies have shown that CEACAM1 expression is chiefly confined to the apical surface of ductal and lobular epithelial cells in normal breast tissue [18–20]. Here, we consistently observed predominant expression of CEACAM1 on the luminal surface of breast epithelium as shown in Fig. 1. Using the semi-quantitative scale, CEACAM1 expression was evaluated according to the methods described by Kang et al. [22]. Briefly, the staining intensity was classified as follows: 0, no expression; 1, weak expression; 2, moderate expression; and 3, strong expression. Among 41 malignant samples, 11 cases were negative for CEACAM1 expression and others showed weak or moderate expression. In contrast, CEACAM1 expression was positive in all 40 benign samples and 28 nontumor adjacent tissues. Of the benign cases, nine cases showed strong expression of CEACAM1, others showed moderate or weak CEACAM1 staining. In addition, two cases of the nontumor adjacent tissues exhibited a strong staining for CEACAM1. Furthermore, quantitative integrated optical density analysis of the expression of CEACAM1 was performed and is presented in Fig. 1, showing a clear reduction or loss of CEACAM1 in malignant breast tissues.
Low level of the soluble CEACAM1 in conditioned media of breast cancer cell lines

Soluble CEACAM1 was quantified in the conditioned media of breast cell lines as described by Markel et al. [23]. Briefly, breast cells were seeded in a constant number of $2 \times 10^5$ cells per well in six-well plates and cultured for 72 h. Owing to different proliferation rates, the concentrations of soluble CEACAM1 were normalized according to the number of cells at the time of harvesting. Cultures were still <80% confluence at the time of harvest, and trypan blue staining revealed that >95% of cells were visible. The conditioned medium was collected and centrifuged followed by quantitation of CEACAM1 using ELISA. In breast cancer cell lines, variable normalized CEACAM1 levels were observed, ranging from low level (8.04 ± 1.06 pg/10^5 cells) to high level (13.95 ± 3.10 pg/10^5 cells). For the normal control cell line MCF 10A, the normalized soluble CEACAM1 concentration was 24.82 ± 4.63 pg/10^5 cells. Compared with MCF 10A, breast cancer cell lines had lower soluble CEACAM1 levels, and statistical analysis revealed that the differences were significant (Fig. 2).

Decreased expression of membrane-bound CEACAM1 in breast cancer cells

As CEACAM1 includes soluble isoforms and membrane-bound isoforms, fluorescence microscopy was used to measure the fluorescence intensity of membranous CEACAM1 staining in breast cancer cells. IF staining results indicated that CEACAM1 was expressed at the cell surface of all of these cell lines. Quantitative analysis showed that the relative fluorescence intensities in the breast cancer cell lines were significantly lower than those in MCF 10A, the control cell line. As shown in Fig. 3, the relative fluorescence intensity for MCF
10A was 32.46 ± 5.10, whereas the intensities of breast cancer cell lines ranged from 5.44 ± 1.97 to 13.84 ± 3.66.

Down-regulation of CEACAM1 at mRNA and protein levels in breast cancer cell lines

To provide further evidence for the expression of CEACAM1, we also measured CEACAM1 mRNA and protein levels in breast cancer cell lines. After normalization to GAPDH, it was obvious that the CEACAM1 mRNA expression was down-regulated in breast cancer cell lines. The levels of CEACAM1 mRNA expression in most breast cancer cell lines (Hs 578T, MCF7, T-47D, BT-549, and MDA-MB-231) decreased to 10%–20% compared with those in the control MCF 10A cell line (Fig. 4A). To confirm that the decreases in CEACAM1 transcripts were consistent with the decreased protein levels, we performed western blot analysis. Similarly, the quantitative analysis revealed that the relative protein expression levels of CEACAM1 in six cancer cell lines range from 0.11 ± 0.06 to 0.32 ± 0.12, which were considerably lower than those in the control MCF 10A cells (1.0 ± 0.10) (Fig. 4B).

Discussion

In recent years, an increasing number of studies have shown that aberrant expression of CEACAM1 contributes to the initiation and progression of cancers [24]. Moreover, the physiological and pathological roles of CEACAM1 have been demonstrated in most malignant tumor types, and CEACAM1 seems to be of great importance in the diagnosis and treatment of cancers [4]. Therefore, the dysregulated expression of CEACAM1 and its clinical significance has become a focus of cancer studies.
To date, analysis of the expression levels of CEACAM1 in breast cancer is still controversial. To provide further evidence, in this study, we investigated CEACAM1 expression in both breast cancer tissues and breast cancer cell lines. Our data demonstrated a systematic down-regulation of CEACAM1 in breast cancer, including: (i) loss or reduced immunohistochemical staining in cancer tissues; (ii) lower soluble CEACAM1 levels in the conditioned media of breast cancer cell lines than that in the control cell line; (iii) lower amount of membrane-bound CEACAM1 in breast cancer cell lines compared with the control cell line; (iv) down-regulation of CEACAM1 mRNA expression in breast cancer cell lines; and (v) decreased expression of CEACAM1 protein in breast cancer cell lines. Although the aberration of CEACAM1 expression in breast cancer in previous studies was contradictory, their views were consistent regarding the location of CEACAM1 in breast epithelial cells. In normal breast tissues, CEACAM1 expression was mostly confined to the apical surface of ductal and lobular epithelial cells.[19–20]. In this study, using the immunohistochemistry method, we also found that CEACAM1 was expressed in normal breast epithelium with an apical localization.

In a previous study, using the immunohistochemistry method, Rethdorf et al. [19] analyzed the expression of CEACAM1 in a series of breast tissue samples, including 5 normal breast tissues, 5 fibrocystic lesions, 12 breast hyperplasia, and 115 breast cancers (obtained from Department of Gynecological Histopathology, Clinic of Gynecology and Obstetrics, University of Hamburg, Hamburg, Germany). The results of their study indicated a partial reduction or loss of CEACAM1 expression in carcinomas compared with normal tissue and benign proliferative breast lesions. In addition, Wang et al. [18] reported that among 60 pairs of breast cancer tissues and corresponding nontumor adjacent tissues (obtained from Department of Pathology, Qilu Hospital, Shandong University, Jinan, China), 65% of the cancer tissues had lower CEACAM1 expression than that of adjacent tissues. Furthermore, 20% of the cancer tissues exhibited no CEACAM1 expression, which is consistent with our observation. However, Huang et al. [20] investigated CEACAM1 expression in breast lesion samples (obtained from Division of Anatomic Pathology, City of Hope National Medical Center, Duarte, USA) using immunohistochemistry and mRNA analysis methods. Their results suggested that CEACAM1 was expressed to the same extent in normal and malignant breast tissues, demonstrating no down-regulation of CEACAM1 expression in breast cancer.

To avoid contamination with normal cells, we analyzed CEACAM1 expression in six breast cancer cell lines. These experimental results at the cellular level were consistent with our immunohistochemical analysis of tissue samples, both demonstrating that CEACAM1 expression was significantly down-regulated in breast cancer. However, our study has an important limitation. Only six breast cancer cell lines were analyzed in the present study, and the small
sample size may increase the bias of the results. Additional studies on a greater number of samples are needed to confirm these results.

In summary, our data indicate that CEACAM1 expression is systematically down-regulated in breast cancer. In terms of CEACAM1’s tumor suppressive role, restoration of CEACAM1 expression in breast cancer could be a promising anti-cancer strategy. Further studies are required to better understand the molecular mechanisms of CEACAM1-mediated biological functions before it can be explored for clinical applications.

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References