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Effect of CEACAM-1 knockdown in human colorectal cancer cells

ZHONG-MIN HAN, HE-MEI HUANG and YONG-WU SUN

Department of Medical Technology, Zhengzhou Railway Vocational and Technical College, Zhengzhou, Henan 450052, P.R. China

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Abstract. Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM-1) is the major antigen of the CD66 cluster of granulocyte differentiation antigens. The present study aimed to assess the biological function of CEACAM-1 on the growth of human colorectal cancer (CRC) cells *in vitro*. Treatment of cultured CRC HCT-8 cells with CEACAM-1-specific siRNA successfully downregulated CEACAM-1 expression by 61% compared with control cells. The effects of CEACAM-1 downregulation on HCT-8 cell proliferation and apoptosis were then assessed via Cell Counting kit-8 assay and flow cytometry, respectively. The results demonstrated that siRNA-induced CEACAM-1 downregulation significantly inhibited proliferation and increased apoptosis, but had no significant effect on cell cycle progression in HCT-8 cells. Together, these results suggest that CEACAM-1 activity is critical to CRC growth, and thus, CEACAM-1 may be a promising therapeutic target for the treatment of CRC.

Introduction

Malignant tumor growth is a common and serious disease condition that may affect any number of different organs and tissues throughout the body, and incur varied and complex symptoms that are often life threatening. Colorectal cancer (CRC) is one of the most common gastrointestinal malignancies, and localizes to the rectum or the junction of the rectum and the sigmoid colon. In fact, CRC is the fourth most common type of cancer internationally, following gastric, esophageal, and lung cancer (1,2).

Malignant tumors are often characterized by both tissue architecture disruption, and differentiation derangement. Cell adhesion dysregulation contributes to tumor invasion and metastasis (3), such that the abnormal expression of various cell adhesion molecules induces a loss of cell-cell binding, thereby promoting tumor differentiation and malignant invasion (4). Carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) are members of the glycosylphosphatidylinositol (GPI)-linked immunoglobulin (Ig) superfamily (5). CEACAM-1 is a CEACAM subtype that is also known as biliary glycoprotein I or CD66a (6,7). Previous studies have demonstrated that CEACAM-1 expression is reduced in several tumor types, such as melanoma, lung, colon and ovarian cancer, compared with the corresponding normal tissues (8-14). This suggests that CEACAM-1 may function to inhibit carcinogenesis. In addition, CEACAM-1 has been reported to promote the apoptosis of various cells, including pulmonary and mammary epithelial cells, oral keratinocytes, cancer cells, Jurkat T cells, and cardiomyocytes (13,15,16).

To date, the expression of CEACAM-1 in CRC has not been investigated. In the present study, CEACAM-1 expression was silenced in a CRC cell line, and its effects on cell growth and apoptosis were examined. The findings provide the first evidence that decreased CEACAM-1 expression promotes CRC progression.

Materials and methods

Cell culture. HCT-8 cells (Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China) were maintained (37˚C, 5% CO_2) in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (all from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 μg/ml penicillin, and 100 μg/ml streptomycin. The cells were passaged every 2-3 days, using 0.02% EDTA and 0.1% trypsin.

Small-interfering RNA (siRNA) design and cell transfection. Vectors carrying either siRNA specific to CEACAM-1 or a negative control siRNA were generated by GeneChem Co., Ltd. (Shanghai, China). Three CEACAM-1-specific siRNAs were designed, as follows: siRNA1 sense, 5'-CAGCCACAGAAAUAAUUUATT-3' and antisense, 5'-UAAAUUAUUUCUGUGGCUGTT-3'; siRNA2 sense, 5'-CCGUCAAAUUGUAGGAUAUTT-3' and antisense, 5'-AUAUCCUACAAUUUGACGGTT-3'; and siRNA3 sense, 5'-GAGCUCUUUAUCCCUAACATT-3' and antisense, 5'-UGUUAGGGAUAAAGAGCUCTT-3'. A siRNA encoding a nonsense sequence (sense, 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense, 5'-ACGUGACAC

Correspondence to: Dr Zhong-Min Han, Department of Medical Technology, Zhengzhou Railway Vocational and Technical College, 9 Qiancheng Road, Zhengzhou, Henan 450052, P.R. China
E-mail: hanzhongmin1@163.com

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GUUCGGAGAATT-3'), was designed and used as a negative control (GenePharma Co., Ltd., Shanghai, China). HCT-8 cells were cultured until they reached 50% confluence, washed with PBS, and transfected with 50 nM CEACAM-1-specific or control siRNA, according to the manufacturer's instructions. Cells were collected 48 h post-transfection, and maintained in fresh medium for 24 h prior to further experimentation and/or analysis.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). CEACAM-1 expression was analyzed via RT-qPCR, as previously described (17). Briefly, total RNA was extracted from CRC cells, using an RNeasy Mini kit (Invitrogen; Thermo Fisher Scientific, Inc.). Genomic DNA was removed from the extracted total RNA via DNase I digestion, and then the total RNA was reverse transcribed to generate cDNA, according to the manufacturer's instructions (PrimeScript™ 1st strand cDNA Synthesis kit; Takara Biotechnology Co., Ltd., Dalian, China). qPCR was performed in triplicate in a 10 μl reaction mix consisting of 4 μl template DNA (0.05 μg/μl), 5 μl SYBR-Green (Takara Biotechnology Co., Ltd.), 0.2 μl each forward and reverse oligonucleotide (10 μM each) and 0.6 μl deionized water. The thermocycling conditions were as follows: 95˚C for 10 min, followed by 40 cycles of 95˚C for 10 sec, 60˚C for 30 sec, and 72˚C for 30 sec. The primers used for qPCR were: CEACAM-1 forward, 5'-CAGGGGCTTCTG CTCACAGC-3' and reverse, 5'-AGTTGCTTCTTCACA AGAT-3'; β-actin forward, 5'-GGCTGTGGAGACAAA AATGACCTC-3' and reverse, 5'-AGGCTTGGGCTTGAA TGGAGTC-3'. The expression level was estimated with the $2^{-\Delta\Delta Cq}$ method (18).

Western blot analysis. CEACAM-1 expression levels were determined via western blot analysis. CRC cells were washed twice with PBS, and collected via centrifugation. Proteins were then extracted with cell lysis buffer (CST Biological Reagents Company, Ltd., Shanghai, China) containing 1 mM phenylmethylsulfonyl fluoride, and the protein concentration of each sample was determined using the BCA protein assay reagent kit (Beyotime Institute of Biotechnology, Lianyungang, China), and bovine serum albumin was used as a standard. The samples were denatured, and 50-80 μg were separated via polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were incubated first (4˚C, overnight) with anti-CEACAM-1 (cat. no. AF1857; Novus Biologicals, Ltd., Cambridge, UK; dilution, 1:200) and anti-β-actin (cat. no. ab8227; Abcam, Cambridge, UK; dilution, 1:4,000) antibodies. The membranes were then incubated with horseradish peroxidase-conjugated rabbit anti-human secondary antibodies (cat. no. ab6759; Abcam; dilution, 1:3,000) for 12 h at 4˚C, and finally with enhanced chemiluminescence substrate (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The resulting blots were analyzed using ImageJ software (NIH, Bethesda, MA, USA).

Cell proliferation. Cell proliferation was assessed by a CCK-8 assay (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions. Typically, cells were plated at a density of 1.5-2x10^3 cells/well in 96-well plates. After 48 h (to allow cell adherence to occur), cells were incubated with colorimetric substrates. Colorimetric changes were then measured in a multi-well

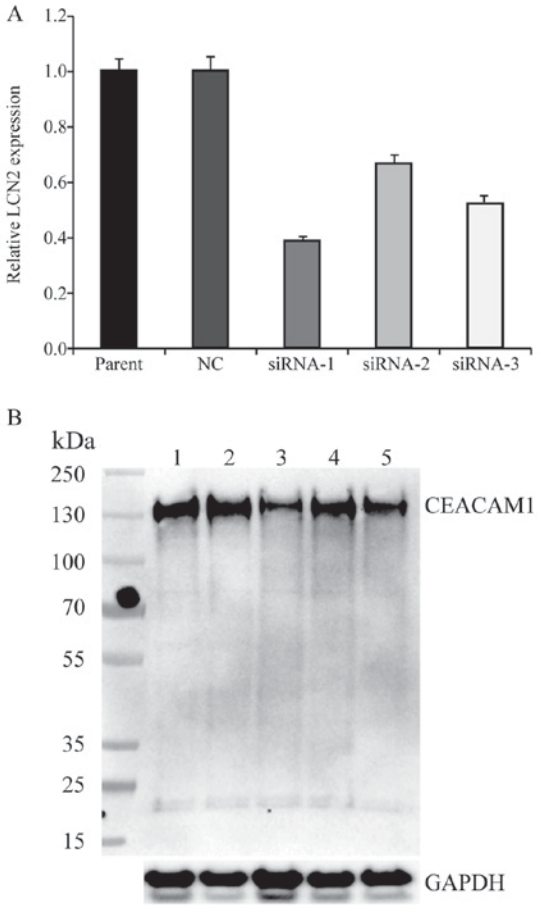

Figure 1. Knockdown of CEACAM-1 expression in HCT-8 cells. (A) CEACAM-1 mRNA expression was suppressed using three different siRNA constructs in HCT-8 cells. Parental untreated cells and cells transfected with negative control (NC) siRNA were used as controls. The experiment was performed in triplicate. (B) Representative immunoblot of CEACAM-1 protein expression in HCT-8 cells, where GAPDH was used as a loading control. Lane 1, untreated group; lane 2, NC siRNA; lane 3, siRNA-1; lane 4, siRNA-2; lane 5, siRNA-3. CEACAM-1, carcinoembryonic antigen-related cell adhesion molecule 1; si, small interfering; NC, negative control.

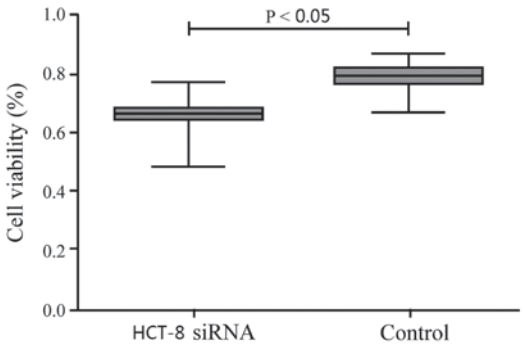

Figure 2. Downregulation of CEACAM-1 inhibits colorectal cancer cell proliferation. HCT-8 cells transfected with either CEACAM-1-siRNA or a negative control siRNA were assessed for cell proliferation using the CCK-8 assay. Results are presented relative to the control group. CEACAM-1, carcinoembryonic antigen-related cell adhesion molecule 1; si, small interfering.

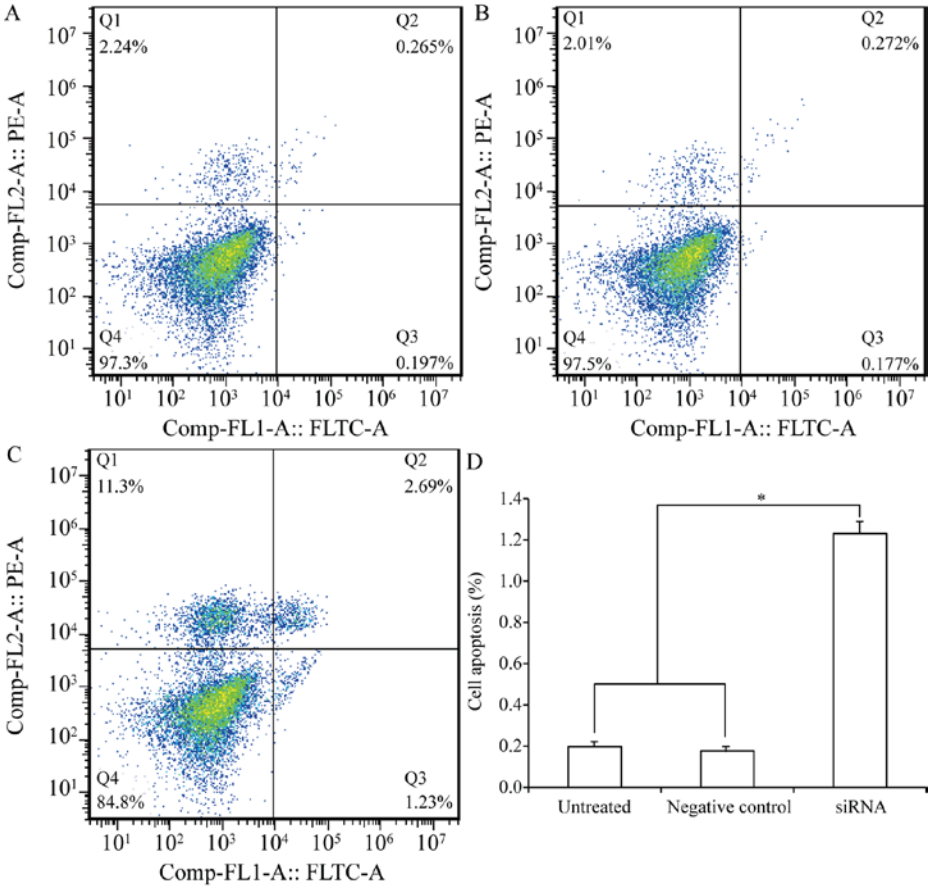

Figure 3. Downregulation of CEACAM-1 promotes colorectal cancer cell apoptosis. Representative plots are shown from flow cytometry analysis following Annexin V staining. (A) Untreated group. (B) Negative control siRNA group. (C) CEACAM-1-specific siRNA group. (D) Quantification of the results. *P<0.01. CEACAM-1, carcinoembryonic antigen-related cell adhesion molecule 1; si, small interfering.

spectrophotometer (MR5000 Multiplate Reader; Dynatech, Denkendorf, Germany), and cell survival following treatment was expressed as a % of viable cells relative to control cell values. All experiments were independently conducted three times, and the results of the three experiments were then averaged.

Cell cycle assay. Cells were fixed, washed with PBS, treated with RNaseA, and stained (37˚C, 30 min) with 25 μg/ml propidium iodide (PI). The samples were then analyzed via flow cytometry, and the cell cycle phase distribution was quantified using Modfit Software (BD Biosciences, Franklin Lakes, NJ, USA). The proliferative index was calculated to represent the % of cells identified to occur in the S/G_2/M phase.

Apoptosis assay. Cells were collected, stained with Annexin V-fluorescein isothiocyanate (FITC) and 7-Aminoactinomycin D using an Annexin V-FITC Apoptosis Detection kit (KeyGen Biotech Co., Ltd., Nanjing, China) according to the manufacturer's instructions, and analyzed via flow cytometry (BD Biosciences).

Statistical analysis. All results are presented as the mean ± standard error of the mean. Differences between two groups were evaluated by the unpaired Student's t-test. One-way analysis of variance with post-hoc analysis by Bonferroni's test was performed to evaluate the data generated by the cellular viability and apoptosis assays. Additional statistical analyses were performed by Student's t-tests. P<0.05 was considered to indicate a statistically significant difference.

Results

Knockdown of CEACAM-1 in HCT-8 cells. To investigate the function of CEACAM-1 expression in CRC cells, three separate siRNA sequences, comprising CEACAM-1-siRNA1, CEACAM-1-siRNA2, and CEACAM-1-siRNA3, were designed. When testing their efficacy, the three sequences induced a 61.3, 32.4 and 47.3% decrease in CEACAM-1 mRNA expression, respectively, in the CEACAM-1-knockdown cells compared with the negative control HCT-8 cells (Fig. 1A). The results from western blot analysis revealed a concordant reduction in CEACAM-1 protein expression levels compared with the control group (Fig. 1B). The CEACAM-1-siRNA1 sequence was selected for use in subsequent experiments, since it induced the greatest reduction in *CEACAM-1* expression.

CEACAM-1 downregulation inhibits cell proliferation. CEACAM-1 knockdown significantly decreased the numbers of viable HCT-8 cells compared with the control group (Fig. 2),

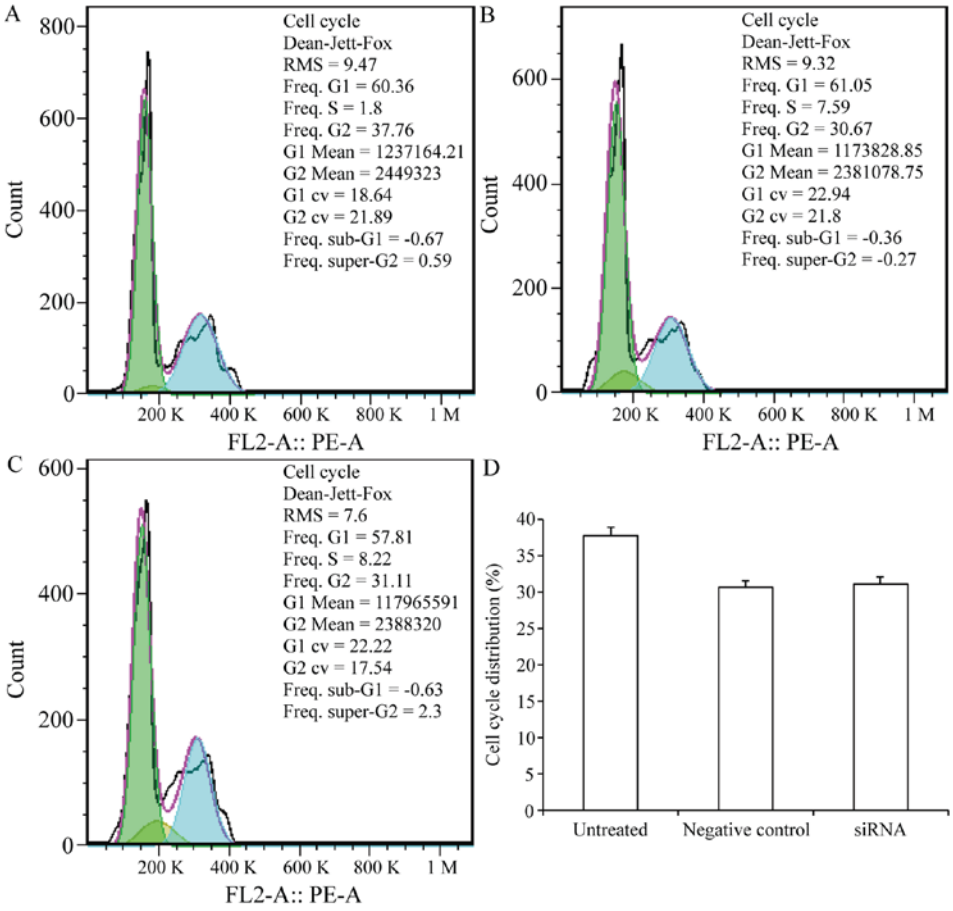

Figure 4. Effect of CEACAM-1 downregulation on the cell cycle distribution of colorectal cancer cells. Representative plots are shown from flow cytometry analysis following propidium iodide staining. (A) Untreated group. (B) Negative control siRNA group. (C) CEACAM-1-specific siRNA group. (D) Quantification of the results. CEACAM-1, carcinoembryonic antigen-related cell adhesion molecule 1; si, small interfering.

suggesting that CEACAM-1 silencing inhibited CRC cell proliferation.

CEACAM-1 downregulation increases cell apoptosis. Next, the effect of CEACAM-1 silencing on apoptosis was determined in CRC cells. The results from Annexin V cytometric analysis revealed that CEACAM-1 downregulation resulted in a significant increase in the apoptotic rate of HCT-8 cells (1.23% in the CEACAM-1-knockdown group compared with 0.197% in the control group; $P<0.01$; Fig. 3). The experiment was performed in triplicate.

CEACAM-1 might function to inhibit carcinogenesis, which is often associated with both cell cycle arrest and activation of the cell death pathway. Therefore, the cell cycle phase distribution was examined in the CEACAM-1-knockdown cells to determine whether CEACAM-1 knockdown has an effect in cell cycle arrest. The results revealed no significant difference in the % of G_2/M-phase cells that occurred in the CEACAM-1-knockdown compared with the control group ($P>0.05$; Fig. 4). The experiment was performed in triplicate.

Discussion

To date, the function of CEACAM-1 expression in malignant tumors remains unclear. Previous studies have investigated CEACAM expression via either immunohistochemical or serum expression analyses (19,20). To elucidate its functional role in CRC, the present study assessed the effect of silencing CEACAM-1 expression on the viability and proliferation of a CRC cell line CEACAM-1 belongs to a diverse family of GPI-linked Igs that combine the structural features of the Ig superfamily with the functional properties of cadherins (21).

In the present study, CEACAM-1 expression was inhibited in the HCT-8 cell line via three custom-designed siRNA sequences, achieving a maximal reduction in CEACAM-1 mRNA expression by 61.3% compared with the control cells, and a similar reduction in CEACAM-1 protein production. Subsequently, it was determined that CEACAM-1 downregulation significantly inhibited cell proliferation and promoted cell apoptosis, suggesting that CEACAM-1 may have a potential clinical use in the treatment of CRC. Consistent with these results, a previous study has demonstrated that CEACAM-1 knockdown results in the decreased proliferation and migration of human pancreatic adenocarcinoma Pac 5061 cells (17). Thus, it can be concluded that CEACAM-1 is likely an important modulator of CRC.

Collectively, the results of the present study demonstrated that CEACAM-1 downregulation in CRC cells significantly inhibited cell proliferation and promoted apoptosis. Thus, CEACAM-1 may be a critical mediator of CRC cell growth and progression, and as a result, a promising potential target for novel CRC treatment strategies.

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Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

Z-MH designed, analyzed the experiments and wrote the manuscript. H-MH and Y-WS performed the experiments and co-wrote the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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