EXPRESSION OF CELL ADHESION MOLECULE 4 IN GASTRIC CANCER TISSUE AND ITS EFFECT ON APOPTOSIS, AUTOPHAGIA, INVASION AND METASTASIS OF GASTRIC CANCER CELL LINE BGC-823

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ABSTRACT

Objective: To observe the effect of cell adhesion molecule 4 (CADM4) on apoptosis, autophagy, invasion and metastasis of gastric cancer BGC-823 cells.

Methods: Real-time quantitative polymerase chain reaction (Real-time PCR) was used to detect the expression of CADM4 in 60 cases of gastric cancer and paracancerous tissues, and the expression of CADM4 in 7 gastric cancer cell lines (HGC-27, BGC-823, MKN45, MGC-803, SGC-7901, NCL.N87, AGS) and normal gastric mucous membrane cells were detected. The overexpression of lentivirus carrier (LV-CADM4) by CADM4 was transiently transferred into BGC-823 cells, and the expression of CADM4 mRNA was detected by Real-time PCR. Apoptosis rate and mitochondrial membrane potential were detected by flow cytometry, apoptosis, autophagy and expression of invasion and metastasis related proteins were detected by Transwell invasion test and cell scratch test.; Western blot was used to detect apoptosis, autophagy and expression of invasion and metastasis related proteins.

Results: The expression level of CADM4 mRNA in gastric cancer was 0.32±0.05, which was significantly lower than that in paracancerous tissues (1.24±0.011) (P<0.001). The expression level of CADM4 in gastric cancer cells was significantly lower than that in HGC-27, MKN45, MGC-803, SGC-7901, NCL.N87, AGS and GES.1 cells (P<0.001). Compared with the blank control group and negative virus group, LV-CADM4 could significantly increase the apoptosis rate of BGC-223 cells (all P<0.001), at the same time, the mitochondrial membrane potential and the ability of invasion and metastasis of BGC-823 cells were significantly decreased (P<0.001). The expression of B cell lymphoma/leukemia-2 (bcl-2), p62, matrix metalloprotease (MMP)-9 protein, bcl-2-associated X protein (bax), cysteinyl aspartate specific protease (Caspase)-9 and microtubule related protein 1 light chain 3 were significantly down-regulated (all P<0.001).

Conclusion: The expression of CADM4 is down-regulated in gastric cancer tissues and cell lines. Up-regulation of CADM4 can induce apoptosis and autophagy of gastric cancer BGC-823 cells through mitochondrial pathway, and decrease the ability of invasion and metastasis of gastric cancer cells.

Keywords: Gastric cancer, phagocytosis, autophagy, invasion, metastasis.

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Introduction

The cell adhesion molecule (CADM) gene encodes an immunoglobulin superfamily molecule, which mainly includes four subfamilies, CADM1, CADM2, CADM3 and CADM4. Cell adhesion factor prevents malignant transformation and metastasis by maintaining adhesion between epithelial cells. It has been confirmed that CADM1 is located on chromosome 11q23.2 and plays a role as tumor inhibitor gene in non-small cell lung cancer. Some studies have also shown that the expression of CADM1 is often inactivated in various cancers. The deletion of CADM1 and protein 4.1B expression leads to the development and progress of breast cancer, especially in invasion and metastasis. The low or abnormal expression of CADM1 in tumors is significantly correlated with lymphatic invasion and advanced tumor stage, suggesting that CADM1 expression may be used as a prognostic biomarker to
assist in effective individualized therapy\(^7,8\). Therefore, CADM1 plays a role as a tumor inhibitor in non-small cell lung cancer and breast cancer. Cell adhesion molecule 4 (CADM4) is located on chromosome 19q13.31. CADM4 has significant homology with tumor inhibitor gene in lung cancer\(^9,10\). CADM4 encodes immunoglobulin (Ig) superfamily cell adhesion molecule, also known as NEC-4, IG superfamily molecule 4C, or tumor inhibitor in lung cancer 1-like gene 2 (TSLL2)\(^11-13\). Previous studies have shown that CADM4 may play a role as a tumor inhibitor in clear cell carcinoma of the kidney and inactivate in breast and colon cancer\(^14-16\). CADM4 is involved in cell-cell adhesion and is expressed in many tissues, including brain, prostate, heart, kidney, pancreas, small intestine and large intestine. Studies have shown that the expression of CADM4 is lost or significantly decreased in primary prostate cancer, and the loss of CADM4 expression may be related to malignant progress\(^17\). However, the role of CADM4 in the development of gastric cancer is rarely reported. The purpose of this study was to observe the expression of CADM4 in gastric cancer and to investigate the effect of CADM4 on apoptosis, autophagy, invasion and metastasis of gastric cancer BGC-823 cells.

### Materials and methods

#### General data
Paraffin specimens of 80 cases of gastric cancer tissues surgically removed in our hospital from January 2018 to August 2019 were selected, all of which were confirmed by pathology. Meanwhile, normal adjacent tissues more than 5 cm away from the cancer foci were selected as the control. There were 48 males and 32 females with the age of (56.17±8.40) years. None of the patients had received radiotherapy or chemotherapy before surgery, and the clinicopathological data were complete. Human gastric cancer cell lines (HGC-27, BGC-823, MKN-45, MGC-803, SGC-7901, NCL-N87, AGS) and normal gastric mucosa cells GES-1 were purchased from Shanghai cell resource center, Chinese Academy of Sciences. RPMI 1640 medium is produced by Invitrogen. Real-time reverse transcription kit is produced by TaKaRa company of Japan. Trizol and real-time fluorescence quantitative kit were purchased from Shanghai cell resource center, Chinese Academy of Sciences. RPMI 1640 medium is produced by Invitrogen. Real-time reverse transcription kit is produced by TaKaRa company of Japan. Trizol was used to extract total RNA from the cells, and samples were added according to the kit instructions for reverse transcription reaction and amplification. Ct values of each sample and each gene amplification were obtained with the analysis software provided by the instrument, with p-actin was the reference gene, and the relative value of target gene expression was RQ=Ct

#### LV-CADM4 construction and transfection
Single cell suspension was prepared from logarithmic growth stage cells and inoculated in a 6-well plate culture. When the cells reached 30% fusion, appropriate amount of virus was added according to the complex number of cell infection (MOI value was 20). The cells were divided into three groups: blank control group (without transfection), negative virus control group (with green fluorescent protein (GFP) only), and LV-CADM4 overexpression group (with both GFP and CADM4 genes). After 72 h of infection, the infection efficiency was observed by GFP fluorescence intensity, and the fluorescence rate >80% was collected for subsequent experiments.

#### Real-time PCR was used to detect the mRNA expression of the target gene
Trizol was used to extract total RNA from the cells, and samples were added according to the kit instructions for reverse transcription reaction and amplification. Ct values of each sample and each gene amplification were obtained with the analysis software provided by the instrument, with p-actin was the reference gene, and the relative value of target gene expression was RQ=Ct

#### Membrane associated protein V-fluorescein isothiocyanate (Annexin V-FITC)/c ingot iodide (PI) double staining to detect apoptosis
Select logarithmic long-term cell vaccination in 6 orifice, pancreatic enzyme digestion cells, phosphate buffer solution (PBS) washing cells 3 times, add 5 μl FITC Annexin V 1 and 5μl PI dyeing, blending, avoid light reaction at room temperature for 15 min, flow cytometry instrument to detect cell apoptosis rate.
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Mitochondrial membrane potential was used to detect the autophagy function of cells

Cells in the paired growth stage were selected and inoculated in the 6-well plate. The cell density was adjusted to 1x10^6 cells/well, and 0.5 ml JC-1 dye was added to each well incubated in dark for 20 min, the cells were washed with PBS for 3 times, and the mitochondrial membrane potential was detected by flow cytometry.

Cell migration ability was tested by cell scratch experiment

Cells were inoculated on the 6-well plate, and a horizontal line was drawn on the back of the plate with Marker pen.48 h after transfection, the cells were cleaned with PBS, and then cultured with serum-free RPMI 1640 medium. The migration rate of cells was determined according to the cell coverage.

Transwell invasion assay tested the invasion ability of cells

Cells were collected and added to serum-free RPMI 1640 medium for re-suspension to prepare single-cell suspension, and the cell density was adjusted to 2x10^5/ml. The upper chamber of Transwell was added with 400 micron cell suspension, and the lower chamber was added with 600 micron RPMI 1640 medium containing 10% bovine blood and egg white (BSA), and cultured at 37°C and 5% CO2 for 24 h. Cotton swabs wiped away the cells that did not pass through, the membrane was fixed with methanold, and the crystal violet stain was performed. Microscopic counts of invading cells.

Western blot detection of target protein expression

Total protein was extracted from the sample, and transferred to PVDF membrane by electrophoresis. 10% skim milk powder was sealed for 2 hours. Each antibody was added in turn, and the color was developed and fixed by chemiluminescence. The ratio of the target protein to the internal reference absorbance indicated the target protein phase pair expression level.

Statistical method

SPSS 13.0 was applied software statistical analysis, measurement data were expressed as mean ± standard deviation (x ± s). One-way anova was used for comparison among the three groups, and SNK method was used for pairwise comparison with P<0.05 means the difference is statistically significant.

Results

Expression of CADM4 in gastric cancer tissues

Real-time PCR results showed that the expression level of CADM4 in gastric cancer tissues and adjacent tissues was 0.32±0.05, 1.24±0.11, respectively, the difference was statistically significant (t=-68.10, P<0.001).

Relationship between CADM4 expression and clinicopathological parameters of gastric cancer

CADM4 expression level was 0.31±0.06 in males, 0.33±0.05 in females; It was 0.32±0.06 in ≤60 years old and 0.3±0.05 in >60 years old, 0.33±0.07 in tumor ≤5 cm, 0.31±0.05 in >5 cm, 0.42±0.08 in stage I, 0.21±0.06 in stage II-IV, 0.40±0.05 in well differentiated cells and 0.19±0.04 in poorly differentiated cells, 0.42±0.07 in T1-T2 and 0.22±0.06 in T3-T4.

The rate of no lymph node metastasis and lymph node metastasis were 0.45±0.05 and 0.19±0.04, respectively.

CADM4 in patients without lymph node metastasis was significantly correlated with TNM stage, degree of cell differentiation, depth of tumor invasion and lymph node transformation (t=13.28, P<0.001; t=20.74, P<0.001; t=13.72, P<0.001; t=25.68, P<0.001), but there was no significant correlation with sex, age and tumor size (t=1.56, P=0.123; t=0.78, P=0.438; t=1.47, P=0.123).

Expression of CADM4 in gastric cancer cell lines

Real-time PCR showed the expression levels of CADM4 in gastric cancer cell HGC-27, BGC-823, MKN-45, MGC-803, SGC-7901, NCL-N87 and AGS were lower than that in GS-1, while the expression levels of BGC-823 were the lowest (F=18.04, P<0.001). Therefore, BGC-823 cells were selected for subsequent experiments.

Effect of LV-CADM4 transfection on CADM4 expression in BGC-823 cells

Real-Time PCR results showed that BGC.823 cells were transfected with LV.After CADM4, CADM4 mRNA expression was significantly higher than that of the blank control group and the negative virus control group, and the difference was statistically significant (t=10.46, P<0.001). T=8.15, P<0.001); There was no statistically significant difference between the blank control group and the negative virus control group ( =1.03, P=0.637).
The effect of transfection of LV. CADM4 on the apoptosis of BGC-823 cells

Flow cytometry results showed that the apoptosis rate of BGC-823 cells after transfection with LV-CADM4 was significantly higher than that of the blank control group and the negative virus control group.

There was no significant difference between the blank control group and the negative control group (Table 1).

The effect of transfection of LV. CADM4 on the mitochondrial membrane potential of BGC-823 cells

Flow cytometry results showed that BGC was transfected with LV-CADM4 compared with the control group with negative virus. The mitochondrial membrane potential decreased significantly in BGC-823 cells (P<0.001).

The difference between the blank control group and the negative control group was not statistically significant (P=0.706), and the fluorescence intensity was in LV-CADM4 overexpression group, blank control group and negative virus control group were 4.43±0.64, 12.47±1.57, 12.25±1.44, F=127.77, P<0.001, respectively.

The effect of transfection of LV. CADM4 on invasion and metastasis of BGC-823 cells

Transwell invasion test and cell scratch test results showed that the number of membrane penetrating cells and scratch healing rate of BGC-823 cells transfected with LV-CADM4 were lower than those of the negative virus control group.

There was no statistically significant difference between the blank control group and the negative control group (Table 2).

Discussion

Overexpression of CADM4 and inactivation of tumor inhibitor genes are important reasons for the development of gastric cancer (18-20). The role of CADM4 in the occurrence and development of malignant tumors has been concerned. The results showed that CADM4 expression was down-regulated in gastric cancer tissues and cells, and its expression was related to TNM stage, degree of cell differentiation, depth of tumor invasion and lymph
node metastasis, suggesting that CADM4 may play an important role in the development of gastric cancer. The results showed that LV-CADM4 could up-regulate the expression of CADM4 mRNA. After overexpression of lentivirus infection with CADM4, the apoptosis rate and autophagy of BGC-823 cells in LV-CADM4 overexpression group were significantly increased, while the mitochondrial membrane potential and invasion and metastasis ability were significantly decreased. At the same time, the expression of apoptosis inhibitor bcl-2, autophagy marker p62 protein was down-regulated, and the expression of apoptosis-promoting protein bax, autophagy marker LC3, Beclin1 protein was up-regulated in LV-CADM4 overexpression group, which led to the increase of mitochondrial permeability and reoptosis protease activating factor (Apaf)-1, and then activated Caspase-9 to initiate apoptosis. In addition, the expression of cell adhesion molecule 4 in gastric cancer tissue and its effect on apoptosis, autophagia, invasion and metastasis of BGC-823 cells.

References


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