

CD44V6 regulates gastric carcinoma occurrence and development through up-regulating VEGF expression

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Abstract. – OBJECTIVE: Gastric carcinoma (GC) is one of the most common malignant tumors around the world. It is featured as high morbidity, poor prognosis, and short survival, thus seriously threatens to the quality of life. The mechanism of GC is still unclear, leading to a difficulty in the treatment. CD44V6 plays an important role in tumorigenesis and progression, while its role in GC still needs further elucidation.

PATIENTS AND METHODS: GC tissue and para-carcinoma tissue were collected from patients in different tumor-mode-metastasis (TNM) stages. CD44V6 and vascular endothelial growth factor (VEGF) expressions were detected by real-time PCR and Western blot. Their correlations with the clinicopathological characteristics of GC were analyzed. GC cell line SGC-7901 was cultured in vitro and divided into control, scramble group, and CD44V6 small interfering RNA (siRNA) group. Cell proliferation was assessed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay. Cell apoptosis was evaluated by caspase 3 activity assay.

RESULTS: CD44V6 and VEGF protein expressions significantly increased in GC tissue compared with adjacent normal control ($p < 0.05$). CD44V6 expression was correlated with differentiation, lymph node metastasis, and TNM staging ($p < 0.05$). CD44V6 was positively correlated with VEGF ($p < 0.05$). CD44V6 siRNA reduced CD44V6 and VEGF expressions in SGC-7901, inhibited cell proliferation, and enhanced caspase 3 activity compared with control ($p < 0.05$).

CONCLUSIONS: CD44V6 participates in GC occurrence and development by up-regulating VEGF expression. Targeting CD44V6 regulates GC progression through inhibiting VEGF expression, promoting cell apoptosis, and restraining cell proliferation.

Key Words:

Gastric carcinoma, CD44V6, VEGF, Apoptosis, Proliferation.

Introduction

Gastric carcinoma (GC) is a common digestive cancer that occurs in gastric epithelium¹. As one of the most common malignancies around the world, GC is featured as regional. Because of dietary habit and environmental factor, GC occurs frequently in our country, especially in northwest and eastern coastal areas^{2,3}. GC occurs mainly in the elderly over 50 years old and mostly in the male patients. It shows a younger trend in newly diagnosed cases⁴. GC brings serious impact on the social labor force and production, and also makes great economic burden to family and society⁵.

Cell adhesion molecules are involved in the interaction between cells and extra-cellular matrix. CD44 is closely related to cell adhesion force, which is mainly composed of 10 constitution exons and 10 variant exons⁶. As a variant of CD44, CD44V is proved to be related to various tumors' occurrence and development, such as breast cancer, liver cancer, and colon cancer^{7,8}. It was reported^{9,10} that the activation or excessive expression of CD44V6 could lead to the occurrence of precancerous lesions of GC. However, the regulatory role of CD44V6 on GC has not been clarified. This study aimed to investigate the correlation and mechanism of CD44V6 in GC occurrence and progress.

Patients and Methods

Patients

A total of 56 cases of GC patients who received surgery in Hongqi Hospital Affiliated to Mudanjiang Medical University between Jan 2016 and Dec 2016 were enrolled. There were 37 males and 19 females with a median age of 58 (36-77) years old. All the subjects were diagnosed as primary GC by pathology. The patients received ultrasonic cardiogram, electrocardiogram, and chest X-ray examination before the operation. The cases were staged based on tumor-node-metastasis (TNM) staging criteria published by Union for International Cancer Control (UICC) in 2012. There were 10 cases in stage I, 12 cases in stage II, 17 cases in stage III, and 17 cases in stage IV. There were 9 cases in well differentiation, 12 cases in moderate differentiation, 21 cases in poor differentiation, and 14 cases in un-differentiation. There were 29 patients with invasion to serosa layer and 27 cases without it. A total of 36 patients appeared lymph node metastasis and 20 cases without. The tumor tissue and para-carcinoma tissue apart from the lesion for at least 5 cm were collected and stored at -80°C . This study was approved by the Ethics Committee of Hongqi Hospital Affiliated to Mudanjiang Medical University, and all the subjects signed the informed consent.

Inclusion and Exclusion Criteria

Inclusion criteria: primary GC diagnosed by pathology, without chemotherapy or radiotherapy before surgery^{5,6}.

Exclusion criteria: recurrent GC, surgery history, chemotherapy or radiotherapy, combination of other diseases, such as infectious disease, malignant tumor, severe liver and renal disease, pulmonary fibrosis, bone metabolic disease, secondary renal hypertension, and immune disease, cannot or unwilling coordinate this study and follow-up^{5,6}.

Clinical Information

The clinical information was collected and recorded, including age, family history, GC family history, body mass index (BMI), pathological differentiation, and clinical stage.

Main Materials and Instruments

GC cell line SGC-7901 was purchased from the cell bank, Chinese Academy of Sciences. Dulbecco Modified Eagle Medium (DMEM)

medium, fetal bovine serum (FBS), ethylenediaminetetraacetic acid (EDTA), and penicillin-streptomycin were purchased from Hyclone (Logan, UT, USA). Dimethyl sulphoxide (DMSO) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) were obtained from Gibco BRL Co. Ltd. (Grand Island, New York, USA). Trypsin-EDTA was derived from Sigma-Aldrich (St. Louis, MO, USA). Caspase 3 activity detection kit and polyvinylidene difluoride (PVDF) membrane were got from Pall Life Sciences (Covina, CA, USA). Western blot related reagents were provided by Beyotime Biotech. (Shanghai, China). Enhanced chemiluminescence (ECL) reagent was purchased from Amersham Biosciences (Piscataway, NJ, USA). Rabbit anti human vascular endothelial growth factor (VEGF) and CD44V6 monoclonal antibodies, and mouse anti rabbit horse radish peroxidase (HRP) labeled IgG secondary antibody were purchased from Cell Signaling Technology (Beverly, MA, USA). RNA extraction kit and reverse transcription kit were purchased from Axygen (Tewksbury, MA, USA). CD44V6 siRNA and negative control were synthesized by GenePharma (Shanghai, China). Lipo2000 was obtained from Invitrogen/Life Technologies (Carlsbad, CA, USA). TaqMan microRNA reverse transcription kit was got from Thermo Electron Corp (Waltham, MA, USA). Lab system version 1.3.1 micro-plate reader was provided by Bio-Rad Laboratories (Hercules, CA, USA). ABI 7700 Fast real-time PCR amplifier was derived from ABI (Foster City, CA, USA). SW-CJ benchtop was provided by Sutai purification equipment engineering co., Ltd (Suzhou, China). DNA amplifier PCR System 2400 was obtained from PE Gene Applied Biosystems. (Foster City, CA, USA). Other reagents were purchased from Sangon Biotech. Co. Ltd. (Shanghai, China).

Methods

GC Cell Line SGC-7901 Cell Culture and Grouping

SGC-7901 cells conserved in liquid nitrogen were resuscitated and centrifuged at 1000 r/min for 3 min. Next, the cells were re-suspended in 1 ml medium and cultured at 37°C and 5% CO_2 for 24-48 h. SGC-7901 cells were seeded in a six-well plate at $1 \times 10^5/\text{cm}^2$ and cultured in high glucose DMEM medium containing 10% FBS,

100 U/ml penicillin, and 100 µg/ml streptomycin. The 2nd-8th generation SGC-7901 cells in logarithmic phase were used for experiments. The cells were divided into control, scramble group, and CD44V6 small interfering RNA (siRNA) group.

Cd44v6 Sirna Lipofectamine Transfection

CD44V6 siRNA and negative control were transfected with the SGC-7901 cells. CD44V6 siRNA, 5'-GCUUCGUGCUCAAATAUAT-3', 5'-UAUACUAGGGAAUGCCTT-3'. CD44V6 siRNA negative control, 5'-AUGCCAUCCU-GAGGUAGGAUG-3', 5'-ACAGGGAUGUAUA-GUCGCGUG-3'. CD44V6 siRNA and negative control were added to 200 µl serum free DMEM medium and mixed at room temperature for 15 min, respectively. Next, lipo2000 was mixed with CD44V6 siRNA and negative control at room temperature for 30 min. Then, they were added to the cells and cultured at 37°C and 5% CO₂ for 6 h. The cells were further cultured for 48 h for the following experiments.

Real-time PCR

Total RNA was extracted from the tissue by TRIzol and reverse transcribed to cDNA. The primers were designed using PrimerPremier 6.0 software and synthesized by Invitrogen (Table I). Real-time PCR was performed at 56°C for 1 min, followed by 35 cycles of 92°C for 30 s, 58°C for 50 s, and 72°C for 35 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as an internal reference. The relative expression of mRNA was calculated by 2^{-ΔCt} method.

Western Blot

The tissues and cells were added with RIPA and cracked on ice for 15-30 min. Next, the samples were treated by ultrasound at 5 s for 4 times and centrifuged at 10000 × g for 15 min. The protein was transferred to new Eppendorf (Ep) tube and quantified by Bradford method. The protein was separated by 10% sodium lauryl sulfate-polyacrylamide gel electrophoresis (SDS-

PAGE) and transferred to PVDF membrane at 100 mA for 1.5 h. After blocked by 5% skim milk for 2 h, the membrane was incubated in CD44V6, NF-κB, and iNOS monoclonal antibodies (1:1000, 1:2000, and 1:1500, respectively) at 4°C overnight. Then, the membrane was incubated in goat anti rabbit secondary antibody (1:2000) at room temperature for 30 min. Next, the membrane was treated by the developer for 1 min and exposed to observe the result. The film was scanned by Quantity One software and analyzed by the protein image processing system. Each experiment was repeated for four times.

MTT Assay

SGC-7901 cells in logarithmic phase were seeded in 96-well plate at 5×10³ cells/well. After 24 h incubation, the cells were divided into three groups, including control, scramble group, and CD44V6 siRNA group. After 48 h, a total of 20 µl sterile MTT was added to each well for 4 h. Then, the plate was treated with 150 µl dimethyl sulfoxide (DMSO) for 10 min and tested at 570 nm to calculate proliferation rate. Each experiment was repeated for three times.

Caspase 3 Activity Assay

Caspase 3 activity was tested according to the manual. The renal tissue was digested by trypsin and centrifuged at 600 × g and 4°C for 5 min. Next, the cells were added with 2 mM Ac-DEVD-pNA and detected at 405 nm to calculate caspase 3 activity.

Statistical Analysis

All data analyses were performed by SPSS11.5 software (SPSS Inc. Chicago, IL, USA). All data were presented as mean ± standard deviation and compared by Student's *t*-test or one-way ANOVA. Tukey's post-hoc test was used for comparing measurement data between groups. Enumeration data were compared by chi-square test. Pearson was selected for correlation analysis. *p* < 0.05 was depicted as statistically significant.

Table I. Primer sequences.

Gene	Forward, 5'-3'	Reverse, 5'-3'
GAPDH	AGTACCAGTCTGTTGCTGG	TAATAGACCCGGATGTCTGGT
CD44V6	ACCTCTCTAGATCTGCCCAT	TAGAATGGTGTAAACCTCTT
VEGF	TCTCTCTAGATCCTG	TGGTGTATAGACTCT

Results

CD44V6 Expression in GC Tissue and Para-Carcinoma Tissue

Real-time PCR and Western blot were used to test CD44V6 mRNA and protein expressions in GC tissue and para-carcinoma tissue. CD44V6 mRNA and protein levels were significantly enhanced in GC tissue compared with the para-carcinoma tissue ($p < 0.05$, Figure 1).

VEGF Expression in GC Tissue and Para-Carcinoma Tissue

Real-time PCR and Western blot were used to test VEGF mRNA and protein expressions in GC tissue and para-carcinoma tissue. VEGF mRNA and protein levels were significantly enhanced in GC tissue compared with the para-carcinoma tissue ($p < 0.05$, Figure 2).

Relationship Between CD44V6 Expression and Clinicopathological Characteristics

CD44V6 exhibited no correlation with gender, age, and family history of GC patients. CD44V6 expression was correlated with differentiation, lymph node metastasis, and TNM staging ($p < 0.05$, Table II). CD44V6 was positively correlated with VEGF ($p < 0.05$).

Regulation of CD44V6 on CD44V6 Expression in GC Cells

Real-time PCR was selected to test the effect of CD44V6 siRNA transfection on the CD44V6 mRNA expression. CD44V6 siRNA transfection significantly inhibited CD44V6 mRNA expression in SGC-7901 cells compared with the control ($p < 0.05$). On the other side, CD44V6 siRNA negative control transfection failed to affect

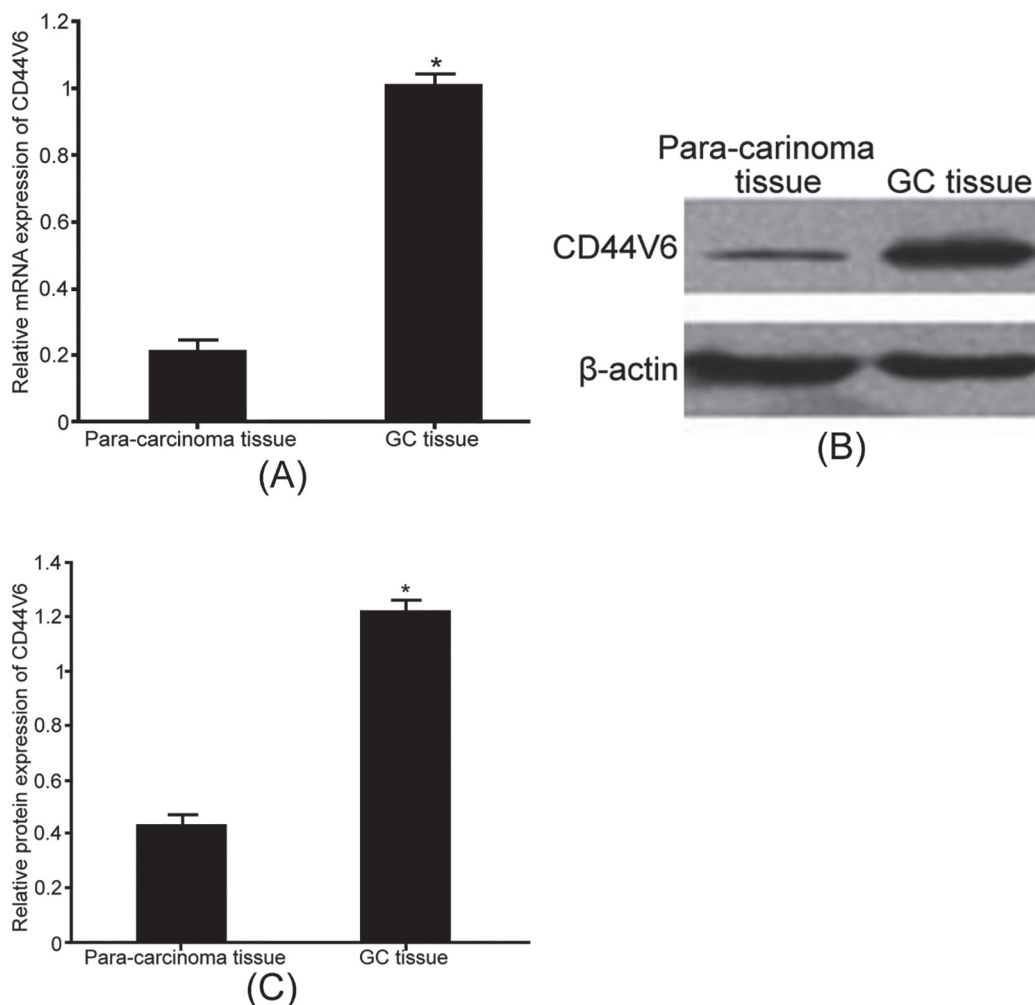


Figure 1. CD44V6 expression in GC tissue and para-carcinoma tissue. **A**, CD44V6 mRNA expression. **B**, CD44V6 protein expression. **C**, CD44V6 protein expression analysis. * $p < 0.05$, compared with para-carcinoma tissue. GC: gastric carcinoma.

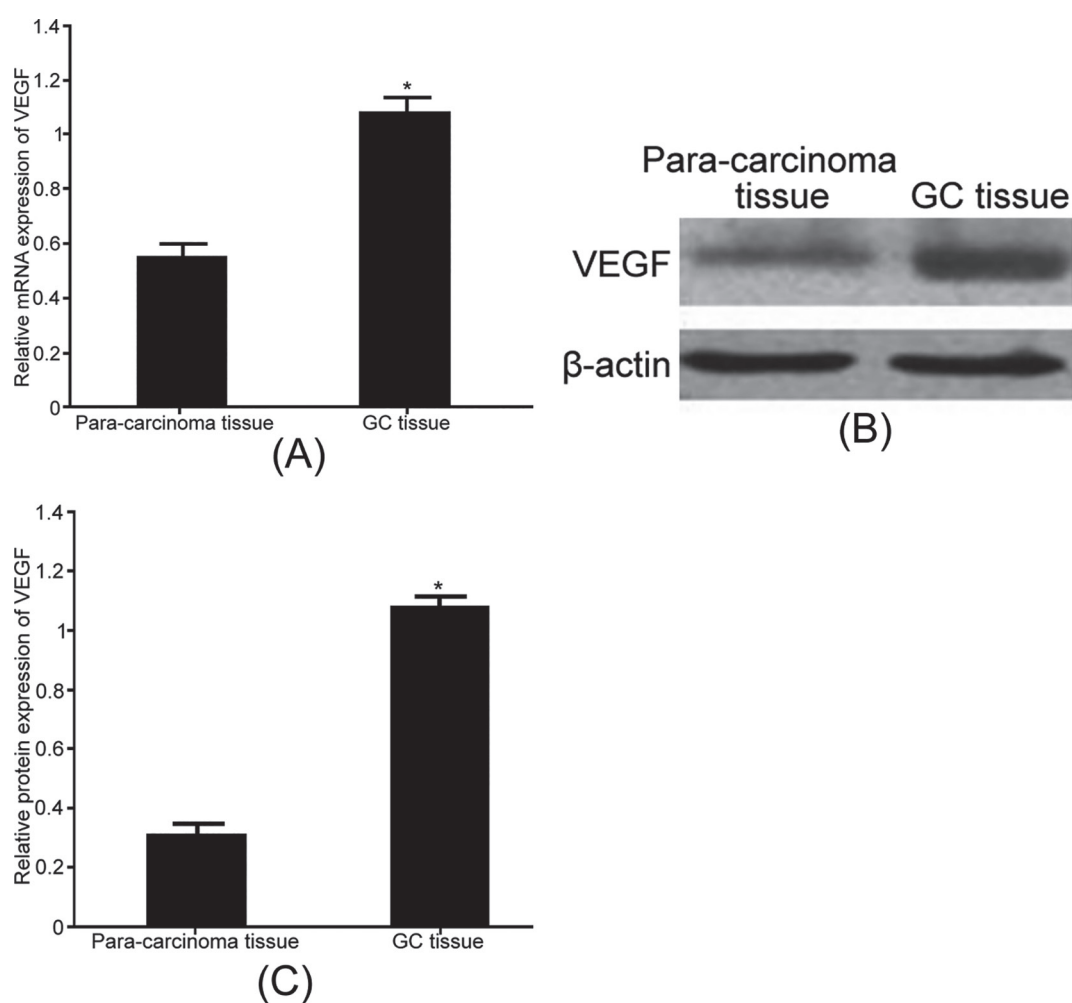


Figure 2. VEGF expression in GC tissue and para-carcinoma tissue. **A**, VEGF mRNA expression. **B**, VEGF protein expression. **C**, VEGF protein expression analysis. * $p < 0.05$, compared with para-carcinoma tissue. GC: gastric carcinoma, VEGF: vascular endothelial growth factor.

CD44V6 mRNA expression in GC cells ($p > 0.05$, Figure 3).

Effects of CD44V6 on SGC-7901 Cell Proliferation

MTT assay was applied to assess the impact of CD44V6 on SGC-7901 cell proliferation. It was shown that CD44V6 siRNA transfection significantly suppressed SGC-7901 cell prolifer-

ation compared with control ($p < 0.05$, Figure 4). It suggested that CD44V6 may affect GC cell proliferation.

Effects of CD44V6 on Caspase 3 Activity in GC Cells

Caspase 3 activity detection kit was used to evaluate the influence of CD44V6 on caspase 3 activity in GC cells. CD44V6 siRNA transfec-

Table II. The relationship between CD44V6 expression and clinicopathological characteristics.

		Age	BMI	Family history	TNM stage	Tumor size	Lymph node metastasis	Differentiation	VEGF
CD44V6 gene	r value	0.013	0.127	0.011	0.672	0.521	0.823	0.615	0.531
CD44V6 protein	r value	0.011	0.115	0.025	0.681	0.721	0.641	0.691	0.457
	p	> 0.05	> 0.05	> 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05

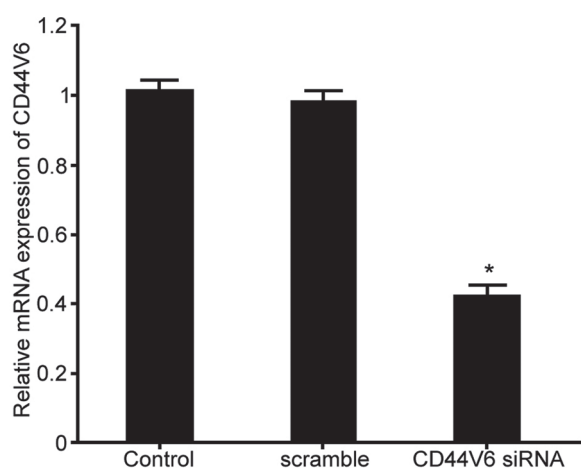


Figure 3. Regulation of CD44V6 on CD44V6 expression in GC cells. * $p < 0.05$, compared with control. GC: gastric carcinoma.

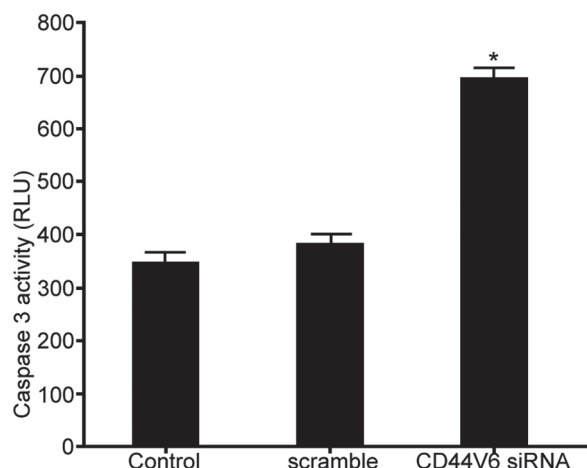


Figure 5. The influence of CD44V6 on caspase 3 activity in GC cells. * $p < 0.05$, compared with control.

tion significantly enhanced caspase 3 activity in SGC-7901 cells compared with control ($p < 0.05$, Figure 5). It indicated that the down-regulation of CD44V6 elevated caspase 3 activity and promoted GC cell apoptosis.

Effects of CD44V6 on VEGF Expression in GC Cells

Western blot was performed to determine the effect of CD44V6 on VEGF expression in GC cells. CD44V6 siRNA transfection significantly restrained VEGF expression in SGC-7901 cells compared with control ($p < 0.05$, Figure 6). It demonstrated that CD44V6 regulated GC occurrence and development by influencing VEGF.

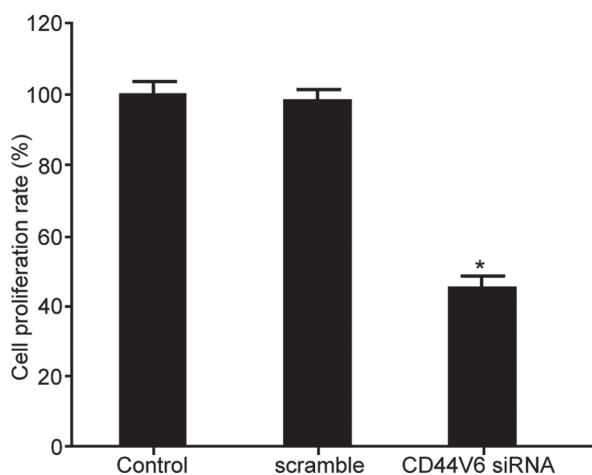
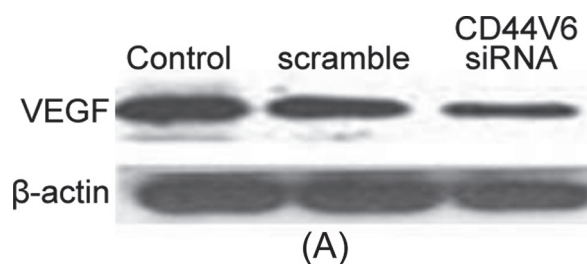


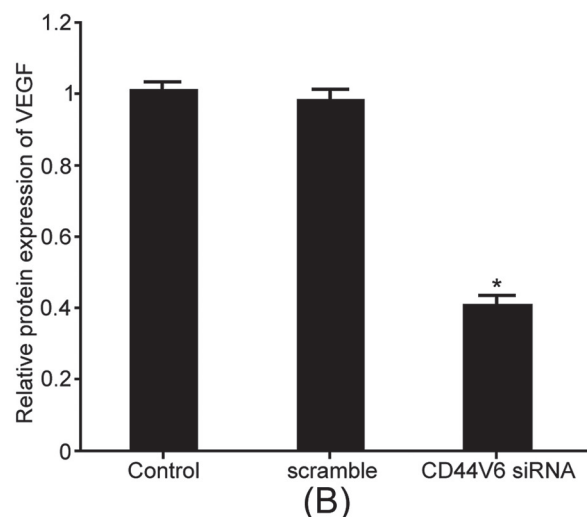
Figure 4. The effect of CD44V6 on SGC-7901 cell proliferation. * $p < 0.05$, compared with control.

Discussion

Numerous factors may affect the pathogenesis of GC, such as genetic factor, and many other



(A)



(B)

Figure 6. The effect of CD44V6 on VEGF expression in GC cells. **A**, VEGF protein expression. **B**, VEGF protein expression analysis. * $p < 0.05$, compared with control. GC: gastric carcinoma, VEGF: vascular endothelial growth factor.

factors, environment, diet, cold and wet, *Helicobacter pylori* (HP) infection, and carcinogenic substances in foods including aflatoxin and nitrite^{11,12}. The early symptoms of GC are concealed, resulting in the patients in an advanced stage when diagnosed, which brings great difficulty for the clinical treatment. GC patients in late stage may appear stomach pain, weight loss, anemia, emaciation, malnutrition, and even cachexia performance¹³. GC treatment is comprehensive composed of operation, adjuvant chemotherapy, radiotherapy, and immunotherapy¹⁴. Although the incidence and mortality of GC declined following the improvement of living standard and medical technology, it is still not optimistic in our country. GC has featured as high incidence, poor prognosis, and short survival that seriously affect the quality of life^{15,16}. Up to now, the mechanism of GC pathogenesis is still unclear, leading to great difficult to its treatment. Thus, investigating the target is in favor of regulating GC incidence and development.

The process of normal gastric mucosa epithelial cell transforming into tumor cell is characterized as polygenes, multi-factor, and multi-step¹⁷. Cell adhesion molecule CD44 participates in its regulation¹⁸. CD44V6 plays an important role in mediating cell adhesion, lymphocytes homing, and activation. Meanwhile, it is also involved in cell migration^{19,20}. CD44V6 participates in multiple cancers' occurrence and development, such as breast cancer and liver cancer^{7,8}. However, regulatory role of CD44V6 on GC has not been clarified. This study revealed that CD44V6 mRNA and protein levels significantly enhanced in GC tissue compared with para-carcinoma tissue. It was correlated with differentiation, lymph node metastasis, and pathological staging, suggesting that CD44V6 plays an important role in GC occurrence and development. We further applied siRNA to interference CD44V6 expression in GC cells. Down-regulation of CD44V6 suppressed GC cell proliferation and enhanced caspase 3 activity. As the strongest member of apoptosis family, caspase 3 enhancement induces cancer cell apoptosis²¹. VEGF can stimulate tumor cell mitosis and promote angiogenesis through autocrine and paracrine. It is a strong vascular growth factor with the highest specificity. Moreover, as a key cytokine for tumor local growth and metastasis, it can facilitate vascular endothelial cell differentiation^{22,23}. This work observed that VEGF elevated in GC tissue. CD44V6 was positively correlated with VEGF. CD44V6 siRNA reduced

VEGF expression in GC cells, so as to inhibit cell proliferation and accelerate cell apoptosis. The specific mechanism of CD44V6 in regulating GC still needs further in-depth investigation.

Conclusions

CD44V6 participates in GC incidence and development by up-regulating VEGF expression. Targeting CD44V6 regulates GC progression through inhibiting VEGF expression, promoting cell apoptosis, and restraining cell proliferation.

Acknowledgements

This work was supported by Natural Science Foundation of Heilongjiang provincial science and Technology (No. H20160213).

Conflict of Interest

The Authors declare that they have no conflict of interests.

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