Original Article

Correlation among *VEGFR3* gene promoter methylation, protein overexpression, and clinical pathology in early gastric cancer

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Background: The occurrence and development of gastric cancer is a multi-factor, multi-stage, multi-gene abnormal accumulation process. Both genetic and epigenetic mechanisms play an important role in the molecular mechanism of gastric cancer. DNA methylation is one of the most studied epigenetic expression mechanisms. To study the correlation between gene promoter methylation status and protein expression of vascular endothelial growth factor receptor 3 (VEGFR3), as well as their association with clinicopathological features in early gastric cancer (EGC) cases.

Methods: Immunohistochemical analysis and methylation-specific PCR (MSP) were used to detect the expression of VEGFR3 protein and methylation status of the VEGFR3 promoter in 50 cases of EGC and their paired normal gastric mucosa tissues. The level of DNA methylation of the VEGFR3 promoter, in situ VEGFR3 protein expression, and the clinicopathological characteristics of EGC patients were statistically analyzed.

Results: The positive rate of VEGFR3 protein expression in EGC tumor tissue (60%) was significantly higher than that in the normal gastric mucosa (10%). The detectable methylation frequency of VEGFR3 promoter in EGC tumor tissue (48%) was significantly lower than that in the normal gastric mucosa (85%). As anticipated, the methylation level of the *VEGFR3* gene promoter was negatively associated with the overexpression of VEGFR3 protein. In addition, methylation status of the *VEGFR3* gene promoter was positively correlated with lymph node metastasis in EGC patients ($P<0.05$), but was not linked to patients' gender, age, tumor size, degree of differentiation, or tumor invasion depth ($P>0.05$).

Conclusions: Hypomethylation of the *VEGFR3* gene promoter is one of the major mechanisms underlying *VEGFR3* gene overexpression in EGC tumor tissues and is related to lymph node metastasis in EGC patients. DNA methylation of VEGFR3 is expected to become a molecular diagnostic and prognostic biomarker for EGC.

Keywords: Early gastric cancer (EGC); vascular endothelial growth factor receptor 3 (VEGFR3); gene promoter; DNA methylation

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Introduction

Gastric cancer, one of the most prevalent malignant tumors and the third leading cause of cancer mortality worldwide (1), is divided into early gastric cancer (EGC) and advanced gastric cancer. EGC is characterized as gastric cancer with limited aggressiveness within the submucosa, and accounts for major incident gastric cancer in Eastern Asia. Although the 5-year postoperative survival rate of EGC exceeds 90%, a poor prognosis is correlated with lymph node metastasis. Therefore, a better understanding of the role of molecular biomarkers in EGC metastasis is important. Vascular endothelial growth factor receptor 3 (VEGFR3) is a lymphatic endothelial biomarker also found in blood vessels and malignant tumor cells, indicating its involvement in cancer lymphangiogenesis and metastasis (2,3). The occurrence and development of EGC are cumulative processes of multiple factors, stages, and genes. Both genetic and epigenetic mechanisms play important roles in the molecular mechanism of gastric cancer. DNA methylation is one of the most extensively studied epigenetic processes and the methylation degree of certain genes can be an efficient biomarker for early detection, evaluating prognosis and disease recurrence, and even targeted therapy (4-6). Digestive tract cells have a high degree of abnormal methylation and tumor susceptibility (7).

In this study, methylation status of the VEGFR3 gene promoter and protein overexpression in the EGC tissue of 50 patients were examined by methylation-specific PCR (MSP) and immunohistochemistry. We also investigated the correlation between *VEGFR3* gene methylation and its protein expression, as well as the correlation between abnormal methylation of the *VEGFR3* gene promoter and clinicopathological factors of EGC. The results of this study provide insights into whether the VEGFR3 epigenetic biomarker can be used for early diagnosis and prognosis in EGC patients.

Methods

Sample collection

This study included specimens of 50 EGC cases from patients admitted to Qilu Hospital of Shandong University (Shandong, China) between 2013 and 2014. The study population included 27 males and 23 females, with a median age of 54 years (14 patients ≥60 years of age, 36 patients <60 years of age). Two senior pathologists reviewed and interpreted the routine pathological sections according to the 2006 World Health Organization tumor classification and diagnostic criteria (8). Among these 50 cases, there were 20 cases of highly and moderately differentiated adenocarcinoma and 30 cases of poorly differentiated adenocarcinoma including signet ring cell carcinoma and mucinous adenocarcinoma; 30 cases of intramucosal carcinoma and 20 cases of submucosal carcinoma; 12 cases with lymph node metastasis and 38 cases without lymph node metastasis. At the same time, 20 cases of normal gastric mucosal tissues at the incised margin of EGC were selected as the control group. None of the patients received anti-tumor treatment such as radiotherapy and/or chemotherapy before surgery, and the clinical data were complete. This study was approved by the Regional Ethics Committee of Qilu Hospital of Shandong University. Because this was a retrospective study based on the medical records and tumor slides assessment, the written informed consent requirement was waived.

Immunohistochemistry staining

In each case, the primary and gastric mucosal tissue of the incised margin were excised, embedded into wax blocks, and sectioned into 3 μm thick slides. After being pretreated with 3% hydrogen peroxide at room temperature for 5–10 min, the slides were placed in antigen repair solution (ZLI-9064; Zsbio Commerce Store, Beijing, China), followed by microwave incubation. Then the sections were incubated with mouse anti-human VEGFR3 monoclonal antibody (1:100, ZM-0277; Zsbio Commerce Store, Beijing, China) overnight at 4 ℃ after being blocked in goat serum. The slides were further washed and incubated with biotinylated secondary antibody and horseradish-labeled streptavidin working solution (SP-9001, SP-9002; Zsbio Commerce Store, Beijing, China) at room temperature for 2 h. The stained tissues were finally developed in DAB colorant (ZLI-9018; Zsbio Commerce Store, Beijing, China) and the slides were counterstained with hematoxylin.

Methylation-specific polymerase chain reaction

For DNA extraction, about 10 pieces of each tissue wax blocks of 3 μm were sliced and placed in a 1.5 mL Eppendorf tube. The DNA of paraffin-embedded tissue samples was extracted with the paraffin genomic DNA extraction kit (QIAGEN Hilden, Germany). Sulfite

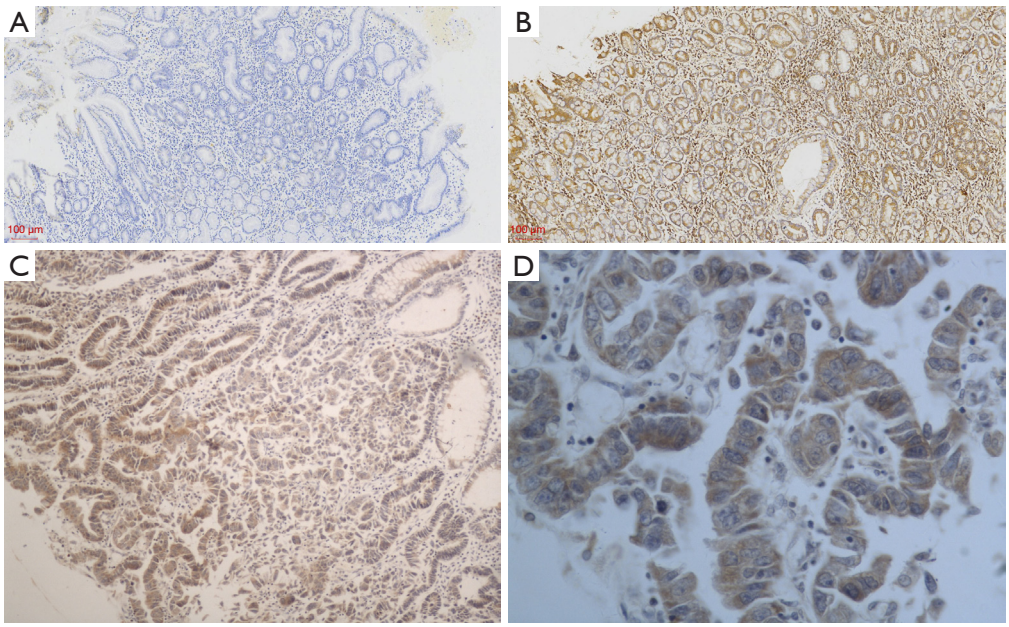

Figure 1 Immunohistochemical analysis of VEGFR expression in EGC. Representative photographs show (A) normal gastric tissue with no detectable VEGFR3 expression (100×); (B) weak expression in normal gastric tissue (100×); (C) overexpression in EGC tissue (100×); (D) overexpression in EGC tissue (400×). EGC, early gastric cancer.

modification of quantified DNA was conducted with a methylation modification kit (Merck Millipore, Hayward, CA, USA). VEGFR3 methylation-specific primer pairs (5'-GTC GGT TAT TTC GGG TGT TTC-3' and 5'-AAT ATC GAC GAA CAA TAT CGA CG-3') and non-methylation primer pairs (5'-GGG TTG GTT ATT TTG GGT TT-3' and 5'-ACA CAA TAT CAA CAA ATA TCA ACA-3') were synthesized by Shanghai Sangon Biological Company (Shanghai, China). PCR reactions were performed under the following conditions: 95 ℃ pre-denaturation for 10 min, 94 ℃ denaturation for 30 s, 45 ℃ annealing for 30 s, 72 ℃ extension for 45 min, and 72 ℃ extension for 10 min. Electrophoresis was performed and a photograph was taken using a gel imaging system (JY04S-3C; Beijing Junyi, Beijing, China).

Data analysis

For immunohistochemistry analysis, positive cells with brown-yellow substances in the cytoplasm were observed and counted under a light microscope. The sections with <10% positive cells were defined as negative (–), 10–25% positive cells were defined as weakly positive (+), 26–50% positive cells were defined as medium positive (++), and >50% positive cells were defined as strongly positive (+++). SPSS 11.0 statistical software was used to perform the chi-square test and Fisher's exact probability test. $P<0.05$ was considered statistically significant.

Results

Expression of VEGFR3 in EGC

Positive VEGFR3 expression in granules in EGC was stained brown-yellow and predominantly found in the tumor cell cytoplasm (*Figure 1A,B,C,D*). The positive expression of VEGFR3 in 50 EGC tissues was 60%, which was significantly higher than that in normal gastric mucosal tissues (10%) ($P<0.05$; *Table 1*). In addition, VEGFR3 was overexpressed in 11 of 12 tumors with lymph node metastasis, which was statistically different from the 19 of 38 tumors without lymph node metastasis ($P<0.05$). In contrast, no statistical difference in VEGFR3 expression was found among cases with different gender, age, tumor size, differentiation degree, or depth of tumor infiltration ($P>0.05$). Taken together, these results suggest that VEGFR3 expression is significantly correlated with the presence of EGC malignant tissue with lymph node

Table 1 Correlation between VEGFR3 expression and clinicopathological characteristics

Clinical parameters	Case	Present	Absent	P
Sex				
Male	27	16	11	
Female	23	14	9	>0.05
Patient age				
<60	36	20	16	
≥60	14	10	4	>0.05
Tumor size				
≤2 cm	21	10	11	
>2 cm	29	20	9	>0.05
Tumor				
High-middle grade	20	11	9	
Low grade	30	19	11	>0.05
Depth of invasion				
Mucosal	30	14	16	
Submucosal	20	16	4	<0.05
Lymph node metastasis				
Without	38	19	19	
With	12	11	1	<0.05

metastasis.

Methylation status of the VEGFR3 promoter in EGC

The MSP method was used to detect DNA methylation in the promoter of the *VEGFR3* gene using genomic DNA template modified with sodium bisulfite. The electrophoresis results are shown in *Figure 2*. To analyze the MSP results, the electrophoretic bands of the samples were compared with the DNA ladder bands and the obviously weaker bands were described as negative. Methylation was detected in 24 of the 50 EGC patients (48.0%) and 17 of the 20 normal gastric mucosa (85%). The methylation frequency of *VEGFR3* gene in gastric cancer was significantly lower than that in the normal gastric mucosa (P<0.05; *Table 2*).

Correlation between methylation of VEGFR3 gene promoter and protein expression in EGC

As shown in *Table 2*, among the 30 cases of gastric cancer with positive VEGFR3 expression, 20 had no methylation detected by MSP (66.67%). In addition, among the 20 cases of gastric cancer with negative VEGFR3 expression, 14 had MSP detectable methylation (70%). These results indicate that overexpression of VEGFR3 protein is closely related to hypomethylation of the *VEGFR3* gene promoter in EGC tumor cells.

Correlation between methylation status of VEGFR3 gene promoter and clinicopathological characteristics of EGC patients

As shown in *Table 3*, 22 of the 38 EGC tumor samples without lymph node metastasis showed detectable methylation, whereas 2 of the 12 samples with lymph node metastasis showed hypomethylation. There was a statistical difference between the methylation detectable rate of *VEGFR3* gene and lymph node metastasis of EGC cases (P<0.05). By contrast, no statistical difference in VEGFR3 promoter methylation status was found among cases with different gender, age, differentiation degree, or depth of tumor infiltration (P>0.05). Taken together, these results suggest that hypomethylation of *VEGFR3* gene promoter is significantly correlated with the presence of EGC malignant tissue with lymph node metastasis.

Discussion

VEGFRs are members of the receptor tyrosine kinase superfamily. They contain an extracellular ligand-binding domain consisting of seven immunoglobulin-like folds, a single transmembrane region, and a split tyrosine kinase domain. VEGFRs activate the mitogen-activated protein kinase and AKT signaling pathways in the cytoplasm to promote cell proliferation and migration (9-12). As a lymphangia-specific growth factor receptor, in normal adult tissues, VEGFR3 is mainly expressed in lymphoid endothelial cells, but not in vascular endothelial cells. Previous studies have revealed that the growth of new lymphatic vessels in tumors is mainly achieved through the regulation of VEGFR3 located on lymphatic endothelial cells. Vascular endothelial growth factor C (VEGFC) secreted by tumor cells specifically binds to its receptor VEGFR3, which causes the lymphatic vessel germ around the tumor to grow into tumor tissue. Studies have shown that the VEGF-C-VEGFR3/Flt4 axis regulates the growth and metastasis of breast tumors in an autocrine manner (13). The overexpression of VEGFR3 is also known to be

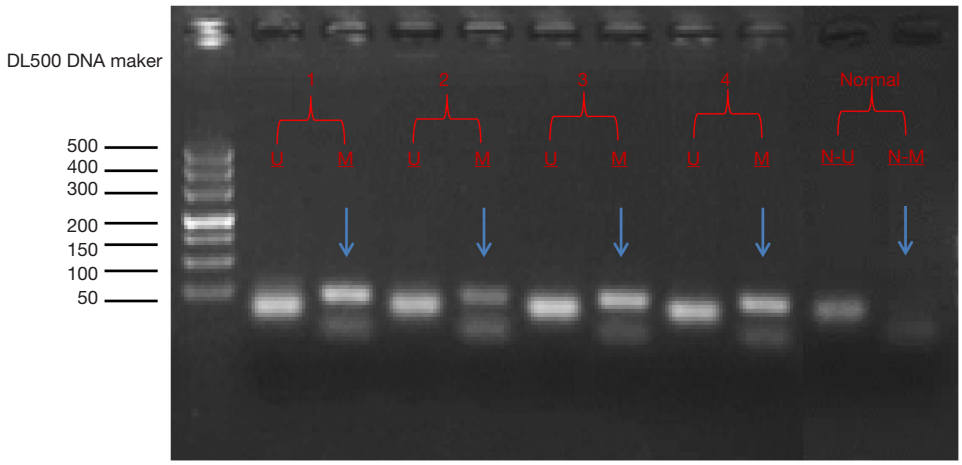

Figure 2 Representative electrophoresis imaging photo showing the MSP products of *VEGFR3* gene promoter in EGC and normal samples. The DNA ladder was DL500 DNA marker. U is unmethylated products of tumor tissue; M is methylated products of tumor tissue. NU and NM are from normal tissues. MSP, methylation-specific PCR; EGC, early gastric cancer.

Table 2 Protein expression of the *VEGFR3* gene and promoter methylation status

Promoter methylation	Cases	VEGFR3		P
		+	–	
Unmethylation	26	20	6	
Methylation	24	10	14	<0.05

associated with the prognosis of a variety of malignancies (14,15). The relationship between VEGFR3 and lymph node metastasis in gastric cancer has been reported. For example, Han *et al.* (16) confirmed that the expression of VEGFC and VEGFR3 in gastric cancer is related to lymph node metastasis. Choi *et al.* (17) found that VEGFR3-positive lymphatic density is significantly increased in gastric cancer and correlates with the primary tumor size, lymphatic invasion, and lymph node metastasis. The results of our experiment are consistent with previous studies confirming that VEGFR3 protein expression is related to tumor invasion depth and lymph node metastasis, but not to patients' gender, age, tumor size, general typing and degree of differentiation (18,19), suggesting that VEGFR3 is involved in the occurrence and development of EGC and is correlated with tumor invasion and metastasis. In addition, studies on tumor biotherapy have shown that blocking the VEGFR3-mediated signal transduction pathway can effectively inhibit tumor lymphangiogenesis (20), suggesting that VEGFR3 is also a promising molecular target for aggressive EGC.

DNA methylation is a biochemical modification process catalyzed by DNA methyltransferase, which transfers methyl to a specific base with s-adenosine methionitrate as a methyl donor. In mammals, DNA methylation mainly occurs on the 5-carbon atom of the pyrimidine ring of cytidine nucleoside, and forms CpG with its 3-terminal guanine, appearing in double-chain symmetry. These pieces of DNA-rich CpG sequences are called CpG islands (21,22). Abnormal DNA methylation is one of the common mechanisms of inactivation or activation of key factors leading to cancer. For example, through the detection of methylation-related genes in the process of colorectal cancer carcinogenesis, researchers have found that the hypermethylation of eight genes is related to a reduction of transcription level, whereas the hypomethylation of three genes is related to an increase in transcription level (23). In recent years, through analysis of the cancer genome, gene expression profile, and gene methylation profile, an increasing number of differentially methylated genes related to tumor development and prognosis have been found (24-27). Therefore, the detection of gene methylation can be an effective biomarker for the early diagnosis and prognosis of various tumors including gastric cancer, central nervous system tumor, lung adenocarcinoma, uterine tumor, thyroid papillary carcinoma, and even targeted therapy (5,28-31).

Hypermethylation of tumor suppressor gene promoters and hypomethylation of global DNA are common in cancer tissues. In the VEGFR3 promoter, proximal GC-

Table 3 Correlation of VEGFR3 promoter methylation status and clinicopathological characteristics

Clinical parameters	Case	VEGFR3 methylation		P
		Present	Absent	
Sex				
Male	27	16	11	
Female	23	8	15	>0.05
Patient age				
<60	36	18	18	
≥60	14	6	8	>0.05
Tumor size				
≤2 cm	21	11	10	
>2 cm	29	13	16	>0.05
Tumor				
High-middle grade	20	9	11	
Low grade	30	15	15	>0.05
Depth of invasion				
Mucosal	30	14	16	
Submucosal	20	10	10	>0.05
Lymph node metastasis				
Without	38	22	16	
With	12	2	10	<0.05

rich elements interact with Sp1 and Sp3 to activate transcription, and these CpG islands are also associated with the epigenetic silencing of gene expression in many cancer cell lines (32,33). For instance, the methylation status of three *VEGFR* gene promoters in 128 head and neck squamous cell carcinoma patients and their correlation with clinical characteristics were systematically examined in a previous study, and it was found that methylation of the VEGFR3 promoter was associated with poor prognosis (34). In this study, the methylation status of *VEGFR3* gene promoter in EGC tissues and normal tissues was detected by MSP, suggesting that hypomethylation of *VEGFR3* gene promoter is one of the main reasons for the upregulated expression of this gene in the development of EGC. Further analysis of the correlation between the methylation status of *VEGFR3* gene promoter and the clinicopathological factors of EGC revealed that the frequency of methylation of *VEGFR3* gene promoter was significantly correlated with the presence of lymph node metastasis (P<0.05). Therefore, detection of the methylation status of the *VEGFR3* gene promoter is expected to be one of the biomarkers for the molecular diagnosis and disease stage assessment of this malignant disease.

Conclusions

In summary, our results indicate that VEGFR3 is closely related to the occurrence and development of EGC, and promoter methylation is one of the major mechanisms underlying inhibition of VEGFR3 overexpression in normal cells. To validate the methylation biomarkers for diagnosis and prognosis, clinical studies with larger sample sizes are needed that use different methylation detection methods such as Sanger sequencing and deep sequencing, to optimize the accuracy and reliability of the laboratory results. At present, several genes with promising experimental results are expected to be applicable for the clinical diagnosis and treatment of gastric cancer (35-39). Due to the continually evolving technologies for detecting methylation, detection of DNA methylation may be clinically applied in the future.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2020.03.74). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was approved by the Regional Ethics Committee of

Qilu Hospital of Shandong University [No. KYLL-2017(KS)-399]. Because this was a retrospective study based on the medical records and tumor slides assessment, the written informed consent requirement was waived.

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