VEGF Mediates Tumor Growth and Metastasis by Affecting the Expression of E-Cadherin and N-Cadherin Promoting Epithelial to Mesenchymal Transition in Gastric Cancer

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ABSTRACT

BACKGROUND: Gastric cancer (GC) is the fifth leading cancer in the world, and there is a high mortality rate in China. Exploring the relationship between the prognosis of GC and the expression of related genes is helpful to further understand the common characteristics of the occurrence and development of GC and provide a new method for the identification of early GC, so as to provide the best therapeutic targets.

METHODS: Vascular endothelial growth factor (VEGF) and markers of epithelial-mesenchymal transition (EMT) were investigated immunohistochemically using tumor samples obtained from 196 GC tissues and adjacent tumor tissues. The correlation of the expression level with histopathologic features and survival was investigated.

RESULTS: Here, we show that VEGF and EMT markers expression were significantly correlated with depth of tumor invasion and GC stage ($P < .05$), degree of differentiation and lymph node metastasis ($P < .001$). We found that the rate of VEGF positivity in GC tissues was 52.05%, which was significantly higher than that in adjacent cancer tissues (16.84%). In GC, the association between VEGF and E-cadherin was negative ($r = -0.188$, $P < .05$), whereas VEGF and N-cadherin were positively correlated ($r = 0.214$, $P < .05$). Furthermore, the Kaplan-Meier analysis and a Cox regression model were used to analyze the effect of VEGF and EMT marker expression on the survival of the patients. We found that the overall survival of GC patients was correlated with VEGF ($P < .001$), N-cadherin ($P < .001$), E-cadherin ($P = .002$) expression, and some histopathologic features.

CONCLUSIONS: Vascular endothelial growth factor and EMT markers exist side by side and play a part together in the development of GC, which provides new ideas for evaluating the prognosis of GC and researching targeted drugs.

KEYWORDS: Vascular endothelial-derived growth factor, gastric cancer, epithelial-mesenchymal transition

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Introduction

Gastric cancer (GC) is one of the most common malignancies worldwide and is the fourth leading cause of cancer-related death in China. Gastric cancer has a high morbidity and mortality.[1] In 2020, the GC morbidity ranked fifth worldwide, and there were approximately 1 million new cases; in addition, the GC mortality ranked fourth, and there were approximately 769,000 deaths.[2] Gastric cancer affects twice as many men as women and is the most common cancer in men. In recent years, with the improvement of medical treatment and quality of life, the diagnosis rate of early GC patients in China has increased significantly, but the prognosis of patients is less than ideal.[3,4] Studies have discovered a variety of novel molecular markers that can be used to effectively evaluate the prognosis of GC patients and contribute to the exploration of new treatment options.

Vascular endothelial growth factor (VEGF), a proangiogenic protein isolated from bovine pituitary follicular cells, was discovered in experimental studies.[5,6] In 1971, Judah Folkman proposed the hypothesis that tumor tissue can secrete "tumor angiogenic factors" (TAFs) to induce the formation of new blood vessels.[7,8] Currently, angiogenesis is still an important part of tumor research. Studies have shown that most malignant tumors develop in tissues with high blood vessel density, such as the lung and liver, and highly vascularized malignant tumor tissues are more prone to lymphatic and hematogenous metastasis.[9,10] When tumor tissue grows beyond the oxygen supply and nutrient requirements of the blood vessels in the area, the tumor tissue secretes angiogenic factors that enable

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the tumor to continue growing. It has been confirmed that esophageal cancer, lung cancer, breast cancer, renal cell carcinoma, and colorectal cancer show abnormal expression of VEGF and other angiogenic factors.[11-15] In this study, the function of VEGF in GC was further explored.

Epithelial-mesenchymal transition (EMT) is a cellular process in which cells lose their epithelial characteristics and acquire mesenchymal features.[16,17] Under the action of some physiological or pathological factors, intercellular interactions are weakened, and the tight connection and adhesion characteristics of epithelial cells disappear, which enhances the infiltration and migration ability of the cells.[18,19] EMT is regulated by multiple transcription factors, such as Snail, Twist, Slug, Zeb, and Fox, which block the expression of E-cadherin and upregulate the expression of N-cadherin protein.[20-22] In this study, immunohistochemical detection of the expression of these 2 proteins in GC tissues was used as evidence of the occurrence of EMT in GC.

The goals of this study were to examine the expression of VEGF and markers of EMT in GC and to evaluate whether VEGF and EMT marker expression levels are correlated with each other and with clinicopathological parameters and prognosis. The aim of these goals was to reveal the role of VEGF in the occurrence and development of GC to provide new therapeutic targets for patients.

Material and Methods

Sample collection

Between October 2017 and October 2018, a total of 196 patients with GC underwent radical gastrectomy. All patients met the following inclusion criteria: (1) all patients were initially diagnosed with GC and (2) all patients underwent surgical treatment and were confirmed to have GC by pathological assessment. None of the patients received radiotherapy or chemotherapy before surgery. The following patients were excluded: (1) patients who had been diagnosed with GC and were being readmitted to the hospital; (2) patients with tumors in other sites; (3) patients with other organ failure; and (4) patients with incomplete clinical data. Patients were selected according to the inclusion and exclusion criteria, and the following relevant data were collected: (1) clinical data during hospitalization: sex, age, clinical stage of the tumor and surgical pathological diagnosis were collected through the permanent electronic medical record system and (2) survival data: postoperative survival data of patients were collected through telephone follow-up. In addition, paraffin-embedded surgical specimens of cancer and adjacent tissues were collected, and immunohistochemical staining was performed.

Reagents

Rabbit monoclonal antibodies against VEGF (AB39638), E-cadherin (AB76011), and N-cadherin (AB76011) were purchased from Abcam Company, USA, and SP immunohistochemical kits and diaminobenzidine (DAB) chromogen kits were purchased from Fuzhou Mai Xin Company, China.

Immunohistochemical staining

Immunohistochemical analysis of VEGF and EMT marker expression was performed on formalin-fixed paraffin-embedded sections of surgical specimens. Sections 2 μm thick were prepared and deparaffinized by xylene, and epitope demasking was performed with 10 mM sodium citrate buffer (pH = 6) in a pressure cooker at 120°C for 10 seconds. The primary antibody was applied (1:50) in Ventana antibody dilution buffer and incubated overnight at 4°C in a humidified box.

Scoring systems

The slides were assessed by 2 pathologists with minimal interobserver variability and observed differences were resolved by simultaneous reevaluation. Yellow or yellow-brown particles in the cell membrane/cytoplasm/nucleus were considered positive immunohistochemical staining. Immunohistochemical scores included scores reflecting the staining intensity and percentage of positive cells. Grading according to staining intensity was as follows: 0 points for no staining, 1 point for light yellow staining (+), 2 points for yellow staining (++), and 3 points for tan staining (+++). The abundance of positive cells was graded from 0 to 4: 0: less than 5% of cells were positive; 1: 5% to 25% of cells were positive; 2: 26% to 50% of cells were positive; 3: 51% to 75% of cells were positive; and 4: 76% to 100% of cells were positive. The total score = staining intensity × the abundance of positive cells. Five observation sections with different visual fields were randomly selected, and the integral of the 5 visual fields was taken to calculate the average value as the final score of the section. The total possible score of each section was 12 points and was divided into 2 grades: I, negative, 0~4 points; II, positive, 5~12 points.

Follow-up

Patients underwent continuous follow-up until May 2020. No patient was lost to follow-up. The median follow-up interval was 31 months.

Statistical analysis

Statistical analysis was performed using SPSS software (version 26.0). The correlation between 2 variables was evaluated using the Spearmen correlation coefficient and differences in the immunohistochemical results between groups were analyzed using the chi-square test. Statistical significance was defined as $P < .05$. The postoperative survival rate was visualized with Kaplan-Meier (K-M) curves, and a Cox proportional risk regression model was used to analyze univariate and multivariate factors affecting the survival rate of patients with GC.

Ethics statement

A total of 196 GC tissues and adjacent tumor tissues were obtained from patients undergoing radical gastrectomy. The

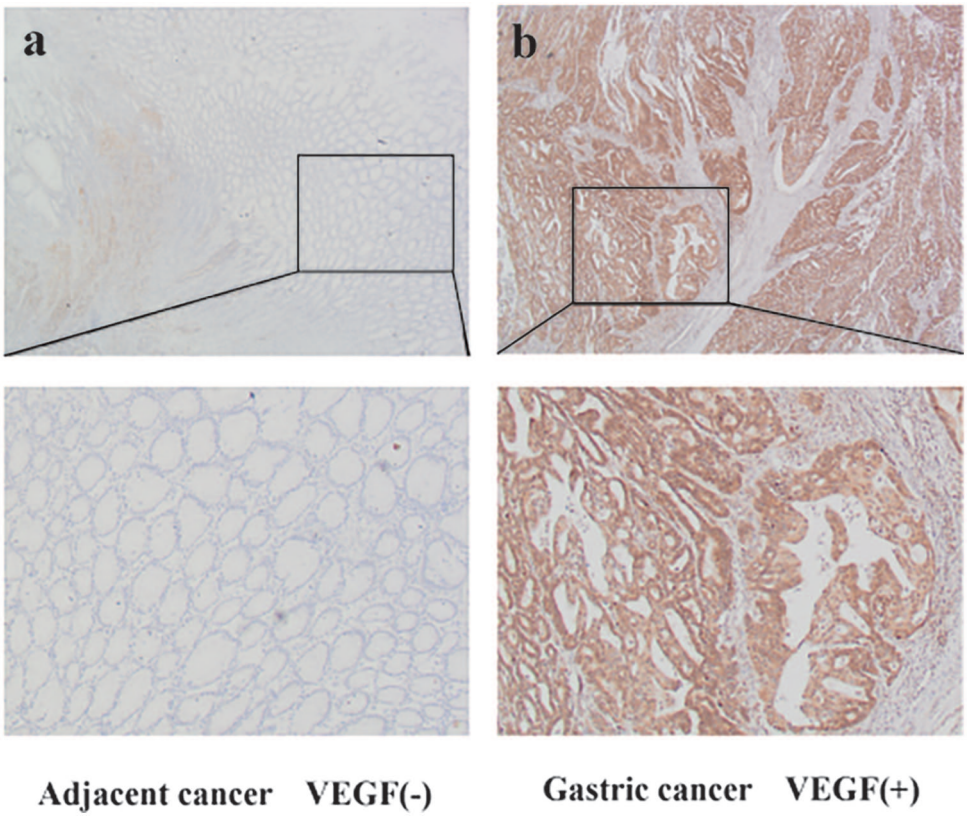

Figure 1. Immunohistochemistry. (A) VEGF staining was negative in adjacent tissues (×40). (B) VEGF expression was positive in gastric cancer tissues (×40).
VEGF indicates vascular endothelial growth factor.

Table 1. Expression level of VEGF protein in gastric cancer tissues and adjacent tissues.

FACTORS	VEGF			
	+	−	*P*	*R*
Gastric cancer	102 (52.05%)	94 (47.95%)	<.001***	−0.250
Adjacent tissues	33 (16.84%)	163 (83.16%)		

Abbreviation: VEGF, vascular endothelial growth factor.
*$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$.

samples were collected between October 2017 and October 2018 at the First Affiliated Hospital of Bengbu Medical College (Anhui, PR China) after obtaining informed consent and the approval of the Clinical Research Ethics Committee of the First Affiliated Hospital of Bengbu Medical College (2017 020). The research conformed to the principles of the Helsinki Declaration. The article confirming that informed consent was obtained from all subjects.

Results

Expression of vascular endothelial growth factor, N-cadherin, and E-cadherin

The expression of VEGF and N-cadherin was mainly detected in the cytoplasm of cancer cells, whereas E-cadherin expression was mainly detected in the cell membrane. Vascular endothelial growth factor expression was positive in 102 of 196 GC samples (52.05%) and negative in the remaining 94 samples (47.95%). Conversely, VEGF expression was positive in only 33 of 196 adjacent samples (16.84%) (Figure 1). The difference in VEGF expression between cancer and control samples was statistically significant ($P \leqslant .05$) (Table 1). E-cadherin expression was positive in 50/196 (25.5%) samples, and N-cadherin expression was positive in 99/196 (50.5%) samples (Figure 2).

Correlation of molecular markers and clinicopathological features

The correlations of VEGF and N-cadherin and E-cadherin expression with clinical features in patients with GC are shown

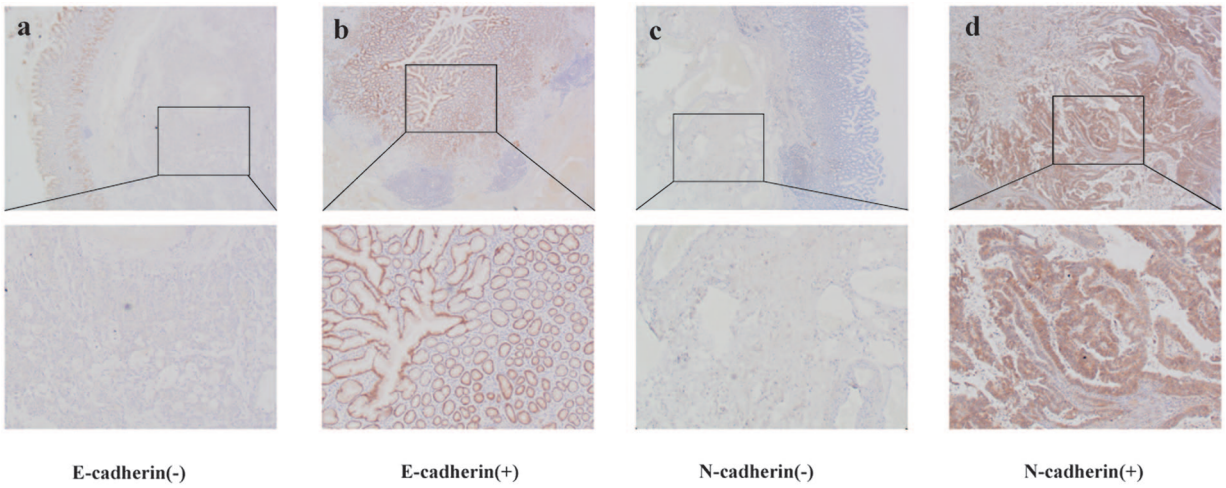

Figure 2. E-cadherin and N-cadherin expression in gastric tissues was determined by immunohistochemistry (×40). (A) E-cadherin expression as low in gastric tissues. (B) E-cadherin staining was positive in gastric tissues. (C) N-cadherin expression was low in gastric tissues. (D) N-cadherin staining was positive in gastric tissues.
VEGF indicates vascular endothelial growth factor.

in Table 2. The frequency of positive VEGF staining was significantly higher at age >50 ($P = .007$), tumor invasion to T1-T2 ($P = .008$), low differentiation ($P = .030$), and the presence of lymph node metastasis ($P < .001$). The high expression of N-cadherin was correlated with tumor location at the cardia ($P = .035$), low differentiation ($P = .036$), tumor invasion to T1-T2 ($P < .001$), and the presence of lymph node metastasis ($P < .001$). The expression of E-cadherin showed an opposite trend with VEGF and N-cadherin, namely, the high expression of E-cadherin in GC tissues was tumor size ≤5 ($P = .001$), high differentiation ($P < .001$), tumor invasion to T3-T4 ($P < .001$), and no lymph node metastasis ($P < .001$) but not with other parameters.

Associations between VEGF, N-cadherin, and E-cadherin expression

E-cadherin was detectable in 18 of 102 (17.65%) samples with positive VEGF expression and in 32 of 94 (34.04%) samples with negative VEGF expression, and N-cadherin was detectable in 62 of 102 (60.78%) samples with positive VEGF expression and in 37 of 94 (36.36%) samples with negative VEGF expression (Table 3). The association between VEGF and E-cadherin expression was weakly negative ($r = -0.188$, $P = .008$), whereas VEGF expression was weakly positive correlated with N-cadherin expression ($r = .214$, $P = .003$).

Univariate and multivariate Cox proportional risk regression models of the survival of gastric cancer patients

Data of 196 patients with GC were analyzed with a Cox univariate regression model (Table 4). The results showed that the overall survival of GC patients was directly proportionally associated with differentiation ($P = .001$) and E-cadherin ($P = .002$) expression, simultaneously inversely associated with tumor size ($P = .007$), depth of infiltration ($P < .001$), lymph node metastasis ($P < .001$), VEGF ($P < .001$), and N-cadherin ($P < .001$). The Cox multivariate regression model analysis results showed that the overall survival of GC patients was associated with differentiation ($P = .026$), lymph node metastasis ($P = .016$), and N-cadherin expression ($P < .001$), suggesting that these features significantly increase the risk of death in GC patients.

Effect of vascular endothelial growth factor, E-cadherin, and N-cadherin expression on the prognosis of patients with gastric cancer

The median survival of patients with high VEGF expression in GC tissues (26.5 months) was significantly shorter than that of patients with low VEGF expression (36 months) ($P < .05$). The median survival time of patients with high expression of N-cadherin was 15 months, which was lower than that of patients with low expression of N-cadherin (34 months). In contrast, the median survival time of patients with high expression of E-cadherin was longer than that of patients with low expression of E-cadherin (high: 34.5 months, low: 29.5 months), and the difference was statistically significant ($P < .05$) (Figure 3).

Discussion

Gastric cancer occurrence is a process involving many factors, among which angiogenesis plays a key role in the development of tumors. Angiogenesis is a normal physiological process in the body.[23] Angiogenesis is a unique and complex process, inflammation, tumors, and restenosis may lead to the activation of angiogenesis.[24] As active cellular proteins that specifically act on the vascular endothelium, VEGF family members are secreted, dimeric glycoproteins of approximately 40 kDa. In mammals, the VEGF family consists of 5 members, VEGFA,

Table 2. Association of VEGF, E-cadherin, and N-cadherin expression level with the clinicopathological parameters.

FEATURES	*N*	VEGF				E-CADHERIN				N-CADHERIN			
		+	−	χ^2	*P*	+	−	χ^2	*P*	+	−	χ^2	*P*
Sex													
Men	135	73	62	0.719	.4420	33	102	0.259	.724	70	65	0.312	.644
Women	61	29	32			17	44			29	32		
Age													
⩽50	29	9	20	8.164	.007**	7	22	0.034	.854	14	15	0.068	.842
>50	167	93	74			43	124			85	82		
Tumor Size													
⩽5	144	69	75	3.699	.074	46	98	11.825	.001***	66	78	4.749	.036*
>5	52	33	19			4	48			33	19		
Location													
Cardia	68	37	31	0.235	.655	15	53	0.653	.493		41	4.862	.035*
Others	128	65	63			35	93			72	56		
Differentiation													
Low	116	68	48	4.930	.030*	19	97	12.469	<.001**	65	51	3.470	.081
Middle and high	80	34	46			31	49			34	46		
Infiltration degree													
T1-T2	57	21	36	7.439	.008**	28	29	23.583	<.001***	16	41	16.191	<.001***
T3-T4	139	81	59			22	117			83	56		
Lymph node metastasis													
No	65	21	44	15.173	<.001***	27	38	13.148	<.001***	16	49	26.087	<.001***
Yes	131	81	50			23	108			83	48		

Abbreviation: VEGF, vascular endothelial growth factor.
*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Table 3. The correlation between VEGF, E-cadherin, and N-cadherin in gastric cancer.

VEGF	E-CADHERIN				N-CADHERIN			
	+	−	*R*	*P*	+	−	*R*	*P*
+	18	84	−0.188	.008**	62	40	0.214	.003**
−	32	62			37	57		

Abbreviation: VEGF, vascular endothelial growth factor.
*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

VEGFB, VEGFC, VEGFD, and placental growth factor (PLGF).[25] Vascular endothelial growth factor binding to its corresponding receptor stimulates cell proliferation and promotes the formation of new blood vessels and lymphatics.[26,27] According to a series of studies, the expression of VEGF is high in solid tumors and promotes malignant tumor behavior. These findings inspired us to perform this study of VEGF expression in GC tissues and carcinoma-adjacent tissues. The differences in expression of VEGF between196 samples of cancer tissues and carcinoma-adjacent tissues from patients diagnosed with GC were determined by immunohistochemical methods. The results showed that the expression level of VEGF in GC tissues was significantly higher than that in adjacent tissues, and the expression of VEGF protein in GC

Table 4. Univariate and multivariate analyses of survival of the gastric cancer patients.

VARIABLES	OVERALL SURVIVAL		
	HR	95% CI	*P*
Univariate analysis			
Sex	0.952	0.615-1.473	.825
Age	1.091	0.608-1.960	.770
Tumor size	1.801	1.176-2.758	.007**
Location	0.917	0.602-1.395	.684
Differentiation	0.477	0.307-0.743	.001**
Infiltration degree	3.109	1.761-5.488	<.001***
Lymph node metastasis	4.409	2.452-7.928	<.001***
N-cadherin	5.691	3.491-9.276	<.001***
E-cadherin	0.425	0.245-0.739	.002**
VEGF	2.133	1.396-3.260	<.001***
Multivariate analysis			
Tumor size	1.002	0.635-1.584	.929
Differentiation	0.593	0.374-0.940	.026*
Infiltration degree	1.326	0.726-2.422	.359
Lymph node metastasis	2.153	1.154-4.016	.016*
N-cadherin	4.589	2.761-7.5629	<.001***
E-cadherin	0.545	0.308-0.966	.038*
VEGF	1.356	0.884-2.078	.163

Abbreviations: CI, confidence interval; HR, hazard ratio; VEGF, vascular endothelial growth factor.
*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

tissues was positively correlated with depth of tumor invasion, degree of differentiation, and lymph node metastasis ($P < .05$). These results suggest that VEGF plays the same role in GC as it does in other tumors: VEGF not only maintains the proliferation and growth of tumor cells but also promotes the infiltration of GC tissues into the serous layer and lymphatic metastasis. Therefore, patients with higher VEGF protein expression levels will have tumors with a higher degree of malignancy and faster disease progression.

Studies have found that VEGF, E-cadherin, and metalloproteinases are abnormally expressed in GC. E-cadherin is usually expressed in epithelial cells, which mediates cell-cell and cell-matrix adhesion plays an important role in the process of establishing and maintaining epithelial integrity of ECM (extracellular matrix). Downregulation of E-cadherin is the first step of tumor cell migration and metastasis. Matrix metalloproteinase-9 (MMP-9) is a family of extracellular zinc-dependent neutral endopeptidases capable of degrading all extracellular matrix components and promoting tumor cell metastasis and invasion. Upregulation of MMP-9 and VEGF and downregulation of E-cadherin have an important effect on the development of GC. This study further demonstrated that the process of EMT is loss of E-cadherin and the gain of N-cadherin, which indicate the conversion of tumor cells into a metastatic phenotype and that EMT is correlated with GC progression and metastasis. We detected the occurrence of EMT by measuring the changes in E-cadherin and N-cadherin protein expression levels in tumor tissues. The data analysis results indicated that the expression levels of E-cadherin and N-cadherin in GC tissues were negatively correlated; N-cadherin was positively related to the degree of tumor differentiation, invasion depth, and lymph node metastasis, whereas E-cadherin played a negative role. Studies have confirmed that in breast cancer, Slug, as a key transcription factor in the EMT process, can not only regulate the protein levels of EMT-related markers such as E-cadherin and N-cadherin but also promote the expression of VEGFR2 in breast cancer tissues by inhibiting DLL4-Notch signaling.[28] Another transcription factor, FOX, can inhibit the invasion and metastasis of breast cancer by regulating miRNAs and inhibiting the VEGF-A/NRP1 signaling pathway.[29] These results suggest that EMT may be involved in the metastasis and invasion of GC and play a synergistic role with VEGF. In conclusion, it is speculated that VEGF may promote vascular formation by mediating the occurrence of EMT and promoting cancer cell acquisition of invasion and migration abilities. The mechanism by which VEGF participates in the occurrence and development of GC needs to be further explored.

In this study, the data analysis showed that the expression of VEGF protein in tumor tissues was related to the expression of EMT-related proteins. In tumor tissues with high VEGF expression levels, the expression level of N-cadherin protein was also higher, whereas the expression level of E-cadherin protein was lower, and the association between VEGF and E-cadherin and N-cadherin was statistically significant. In addition to previous studies, this study further suggests that VEGF plays a role in promoting angiogenesis and tumor metastasis through the EMT pathway. In addition, patient case and survival data were collected in this study. The data analysis showed that the expression levels of VEGF, E-cadherin and N-cadherin were closely correlated with the overall survival time of patients. Cox multivariate regression analysis showed that the expression levels of VEGF, E-cadherin and N-cadherin, the depth of tumor invasion, the degree of differentiation, and lymph node metastasis were independent risk factors affecting the survival and prognosis of patients. The survival analysis showed that the survival of patients with high expression of the VEGF and N-cadherin proteins was significantly shorter than that of patients with low expression. However, this article has the following limitations; the follow-up time span was long, and most relatives of patients could not provide accurate tumor recurrence times; the number of samples was insufficient, and the sample size should be further expanded. Due to limited

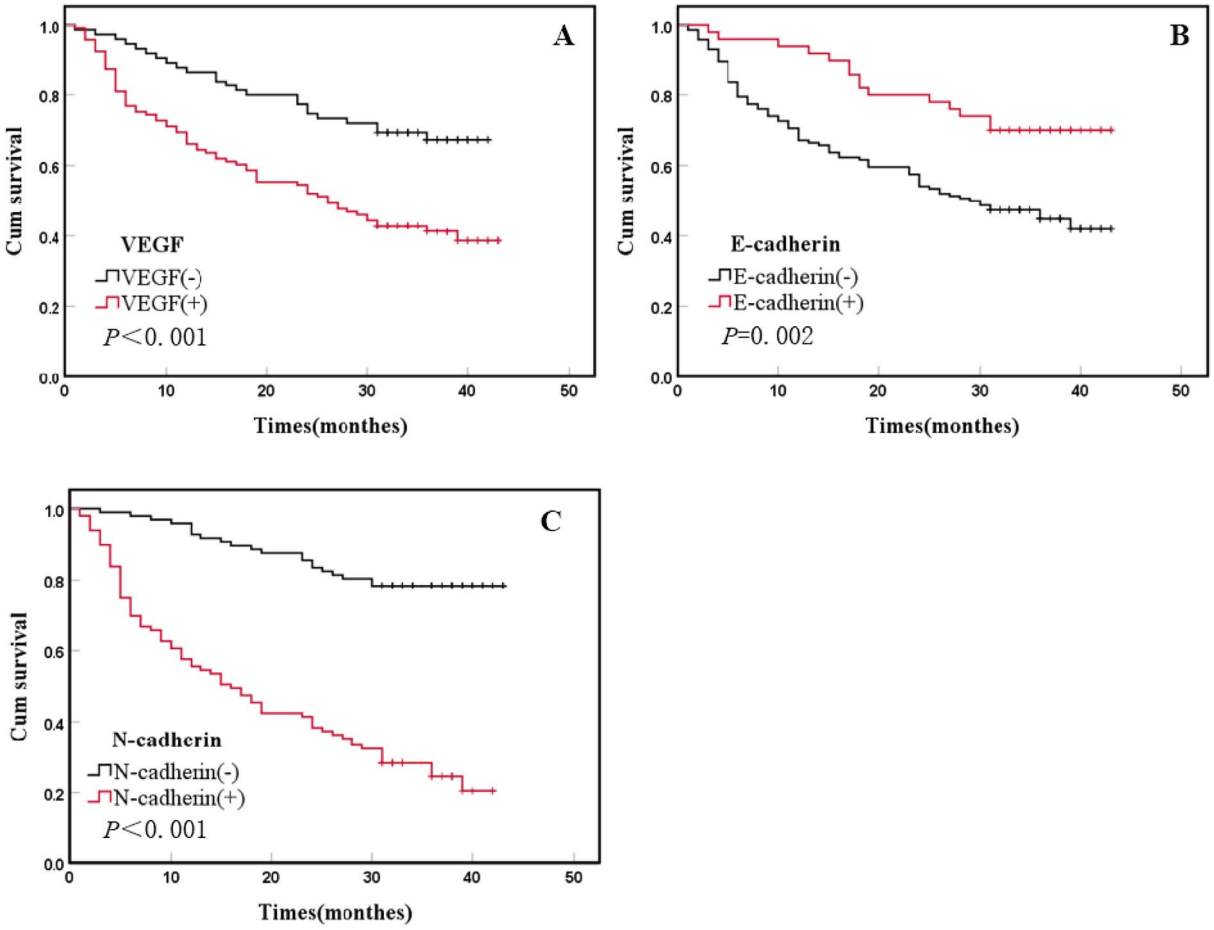

Figure 3. KM survival curve analysis between groups. (A) and (C) Survival analysis showed that the survival period of patients with positive VEGF and N-cadherin expression was shorter than that of patients with negative VEGF and N-cadherin expression ($P < 0.001$), (B) while patients with positive E-cadherin expression showed longer survival than those with negative expression ($P = 0.002$).
K-M indicates Kaplan-Meier; VEGF, vascular endothelial growth factor.

preparation time and lack of basic research, further studies on the mechanism of VEGF mediated EMT occurrence and related pathways should be conducted in the future.

Conclusions

Overall, VEGF and EMT play an important role in the occurrence and development of malignant tumors and are expected to become new indicators for clinical prognosis assessment. These findings lay a strong foundation for clinical diagnosis, identification of high-risk patients, and clinical therapy and will aid the understanding of tumor pathological processes. An understanding of the specific mechanisms of VEGF and EMT in colon cancer will contribute to the development of new combined targeted drug therapies for GC.

Author Contributions

YZ and FS designed the experiments. YW performed the immunohistochemistry experiments. MZ performed the computational experiments. All authors contributed to the analysis and interpretation of data. YZ drafted the initial version of the manuscript. All authors contributed to the formulation of the final manuscript, which they read and approved.

Ethics Statement

A total of 196 gastric cancer tissues and adjacent tumor tissues were obtained from patients undergoing radical gastrectomy. The samples were collected between October 2017 and October 2018 at the First Affiliated Hospital of Bengbu Medical College (Anhui, PR China) after obtaining informed consent and the approval of the Clinical Research Ethics Committee of the First Affiliated Hospital of Bengbu Medical College (ID: 2017020). The research conformed to the principles of the Helsinki Declaration.

Data Availability

All data generated or analyzed during this study are included in this published article and the raw data could be provided from the corresponding author on reasonable request.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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