Expression and clinical significance of VEGF and markers of EMT in gastric cancer

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Article

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Abstract

To explore the association of the levels of vascular endothelial growth factor (VEGF) and markers of epithelial – mesenchymal transition (EMT) in gastric cancer with histopathological features and long-term prognosis. Immunohistochemistry was used to quantitatively assess the expression of VEGF in gastric cancer tissues and adjacent tissues and to detect the expression level of EMT markers in gastric cancer tissues. Here we show that VEGF and EMT markers expression were significantly correlated with depth of tumor invasion and gastric cancer stage ($P < 0.05$), degree of differentiation and lymph node metastasis ($P < 0.001$). We found that the rate of VEGF positivity in gastric cancer tissues was 52.05%, which was significantly higher than that in adjacent cancer tissues (16.84%). In gastric cancer, the association between VEGF and E-cadherin was negative (r=-0.188, $P < 0.05$), whereas VEGF and N-cadherin were positively correlated ($r = 0.214$, $P < 0.05$). Furthermore, 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 gastric cancer patients was correlated with VEGF ($P < 0.001$), N-cadherin ($P < 0.001$), E-cadherin ($P = 0.002$) expression and parts of histopathological features. VEGF and EMT markers exist side by side and play a part together in the development of gastric cancer, which provides new ideas for evaluating the prognosis of gastric cancer and researching targeted drugs.

1. Introduction

Gastric cancer 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 gastric cancer morbidity ranked fifth worldwide, and there were approximately 1 million new cases; in addition, the gastric cancer 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 gastric cancer 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 gastric cancer 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 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 dense blood vessels, 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 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, 12–14, 15]. In this study, the function of VEGF in gastric cancer 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, 21, 22]. In this study, immunohistochemical detection of the expression of these two proteins in gastric cancer tissues was used as evidence of the occurrence of EMT in gastric cancer.

The goals of this study were to examine the expression of VEGF and markers of EMT in gastric cancer 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 gastric cancer to provide new therapeutic targets for patients.

2. Result

2.1 Expression of VEGF, 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. VEGF expression was positive in 102 of 196 gastric cancer 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%) (Fig. 1). The difference in VEGF expression between cancer and control samples was statistically significant ($P \leq 0.05$) (Table 1) (Raw data in supplementary material). E-cadherin expression was positive in 50/196 (25.5%) samples, and N-cadherin expression was positive in 99/196 (50.5%) samples (Fig. 2).

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%)	<0.001***	-0.250
Adjacent tissues	33(16.84%)	163(83.16%)		

2.2 Correlation of molecular markers and clinicopathological features

The correlations of VEGF and N-cadherin and E-cadherin expression with clinical features in patients with gastric cancer are shown in Table 2 (Raw data in supplementary material). VEGF expression was correlated with age, differentiation, infiltration degree and lymph node metastasis (all $P < 0.05$) but not with other clinicopathological features (all $P > 0.05$). The expression of E-cadherin was correlated with tumor size, differentiation, infiltration degree and lymph node metastasis (all $P < 0.05$). The expression of N-cadherin was correlated with tumor location, differentiation, infiltration degree and lymph node metastasis (all $P < 0.05$).

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

Fetures	n	VEGF				E-cadherin				N-cadherin			
		+	-	X^2	*P*	+	-	X^2	*P*	+	-	X^2	*P*
Gender				o.719	0.4420.0			0.259	0.724			0.312	0.644
Male	135	73	62			33	102			70	65		
Female	61	29	32			17	44			29	32		
Age				8.164	0.007**			0.034	0.854			0.068	0.842
≤ 50	29	9	20			7	22			14	15		
> 50	167	93	74			43	124			85	82		
Tumor Size				3.699	0.074			11.825	0.001***			4.749	0.036*
≤ 5	144	69	75			46	98			66	78		
> 5	52	33	19			4	48			33	19		
Location				0.235	0.655			0.653	0.493			4.862	0.035*
Cardia	68	37	31			15	53			27	41		
Others	128	65	63			35	93			72	56		
Differentiation				4.930	0.030*			12.469	<0.001**			3.470	0.081
Low	116	68	48			19	97			65	51		
Middle and High	80	34	46			31	49			34	46		
Infiltration degree				7.439	0.008**			23.583	<0.001***			16.191	<0.001***
Below the serosa	57	21	36			28	29			16	41		
Up to serosa	139	81	59			22	117			83	56		
Lymph node metastasis				15.173	<0.001***			13.148	<0.001***			26.087	<0.001***
No	65	21	44			27	38			16	49		
Yes	131	81	50			23	108			83	48		

2.3 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 62 of 94 (65.96%) samples with negative VEGF expression, N-cadherin was detectable in 62 of 102 (60.78%) samples with positive VEGF expression and 57 of 94 (60.63%) samples with negative VEGF expression (Table 3) (Raw data in supplementary material). The association between VEGF with E-cadherin expression was negative, whereas VEGF expression was positively correlated with N-cadherin expression.

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

VEGF	E-cadherin				N-cadherin			
	+	-	*r*	*P*	+	-	*r*	*P*
+	18	84	-0.188	0.008**	62	40	0.214	0.003**
-	32	62			37	57		

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

Data of 196 patients with gastric cancer were analyzed with a Cox univariate regression model (Table 4) (Raw data in supplementary material). The results showed that the overall survival of gastric cancer patients was inversely associated with tumor size ($P = 0.007$), differentiation ($P = 0.001$), depth of infiltration ($P < 0.001$), lymph node metastasis ($P < 0.001$), and VEGF ($P < 0.001$), N-cadherin ($P < 0.001$) and E-cadherin ($P = 0.002$) expression. The Cox multivariate regression model analysis results showed that the overall survival of gastric cancer patients was associated with differentiation ($P = 0.026$), lymph node metastasis ($P = 0.016$) and N-cadherin expression ($P < 0.001$), suggesting that these features significantly increase the risk of death in gastric cancer patients.

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

Variables	**Overall survival**		
	HR	**95%CI**	***p***
Univariate analysis			
Gender	0.952	0.615–1.473	0.825
Age	1.091	0.608–1.960	0.770
Tumor size	1.801	1.176–2.758	0.007**
Location	0.917	0.602–1.395	0.684
Differentiation	0.477	0.307–0.743	0.001**
Infiltration degree	3.109	1.761–5.488	<0.001***
Lymph node metastasis	4.409	2.452–7.928	<0.001***
N-cadherin	5.691	3.491–9.276	<0.001***
E-cadherin	0.425	0.245–0.739	0.002**
VEGF	2.133	1.396–3.260	<0.001***
Multivariate analysis			
Tumor size	1.002	0.635–1.584	0.929
Differentiation	0.593	0.374–0.940	0.026*
Infiltration degree	1.326	0.726–2.422	0.359
Lymph node metastasis	2.153	1.154–4.016	0.016*
N-cadherin	4.589	2.761–7.5629	<0.001***
E-cadherin	0.545	0.308–0.966	0.038*
VEGF	1.356	0.884–2.078	0.163

2.5 Effect of VEGF, E-cadherin, and N-cadherin expression on the prognosis of patients with gastric cancer

The median survival of patients with high VEGF expression in gastric cancer tissues (26.5 months) was significantly shorter than that of patients with low VEGF expression (36 months) ($P < 0.05$). 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 ($P < 0.05$) (Fig. 3).

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]. If the balance of angiogenesis in the body is upset, it may lead to inflammation, tumors and restenosis [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 five members, VEGFA, VEGFB, VEGFC, VEGFD and placental growth factor (PLGF) [25]. VEGF 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 gastric cancer tissues and carcinoma-adjacent tissues. The differences in expression of VEGF between196 samples of cancer tissues and carcinoma-adjacent tissues from patients diagnosed with gastric cancer were determined by immunohistochemical methods. The results showed that the expression level of VEGF in gastric cancer tissues was significantly higher than that in adjacent tissues, and the expression of VEGF protein in gastric cancer tissues was positively correlated with depth of tumor invasion, degree of differentiation, and lymph node metastasis ($P < 0.05$). These results suggest that VEGF plays the same role in gastric cancer as it does in other tumors: VEGF not only maintains the proliferation and growth of tumor cells but also promotes the infiltration of gastric cancer 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.

The process of EMT involves downregulation of the expression level of E-cadherin protein, a marker of epithelial cells, and upregulation of N-cadherin protein, a marker of mesenchymal cells. 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 gastric cancer tissues were negatively correlated and were closely related to the degree of tumor differentiation, invasion depth and lymph node metastasis. 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 gastric cancer 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 gastric cancer 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, while 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. 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 gastric cancer.

4. Material And Methods

4.1 Sample collection

Between October 2017 and October 2018, a total of 196 patients with gastric cancer underwent radical gastrectomy. All patients met the following inclusion criteria: all patients were initially diagnosed with gastric cancer; and all patients underwent surgical treatment and were confirmed to have gastric cancer by pathological assessment. None of the patients received radiotherapy or chemotherapy before surgery. The following patients were excluded: patients who had been diagnosed with gastric cancer and were being readmitted to the hospital; patients with tumors in other sites; patients with other organ failure; and 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 was collected through telephone follow-up. In addition; surgical wax block samples of cancer and adjacent tissues were collected, and immunohistochemical staining was performed.

4.2 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.

4.3 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 sec. The primary antibody was applied (1:50) in Ventana antibody dilution buffer and incubated overnight at 4°C in a humidified box.

4.4 Scoring systems

The slides were assessed by two 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: 5–25% of cells were positive; 2: 26–50% of cells were positive; 3: 51–75% of cells were positive; and 4: 76–100% of cells were positive. The total possible score of each section was 12 points and was divided into 4 grades: I, negative, 0 ~ 1point; II, weak positive, 2 ~ 4 points; III, positive, 5 ~ 8 points; and IV, strong positive, 9 ~ 12 points.

4.5 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.

4.6 Statistical analysis

All data generated or analysed during this study are included in this published article and its supplementary information files. Statistical analysis was performed using SPSS software (version 26.0). The correlation between two variables was evaluated using Pearson's chi-square and Fisher's exact test. Statistical significance was defined as $P < 0.05$. 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 gastric cancer.

4.7 Ethics statement:

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 [2017] No.020. The research conformed to the principles of the Helsinki Declaration. The article confirming that informed consent was obtained from all subjects.

Declarations

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data generated or analysed during this study are included in this published article and its supplementary information files.

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Figures

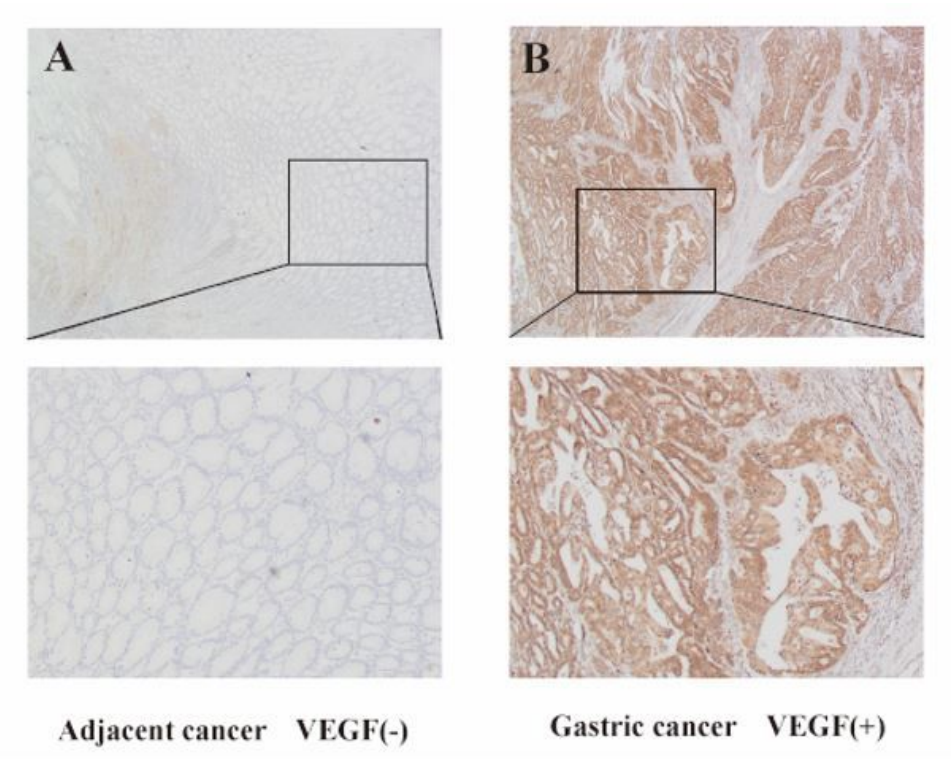

Figure 1

Immunohistochemistry. A: VEGF staining was negative in adjacent tissues (x40). B: VEGF expression was positive in gastric cancer tissues (x40).

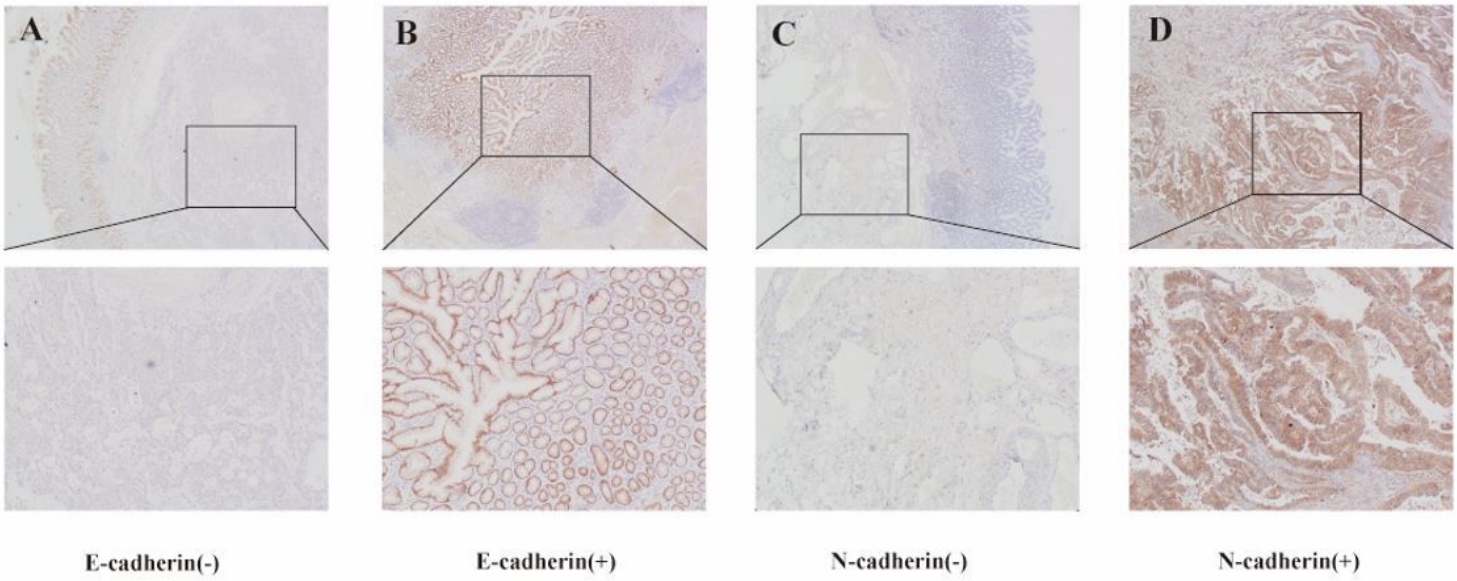

Figure 2

E-cadherin and N-cadherin expression in gastric tissues was determined by immunohistochemistry (x40). 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.

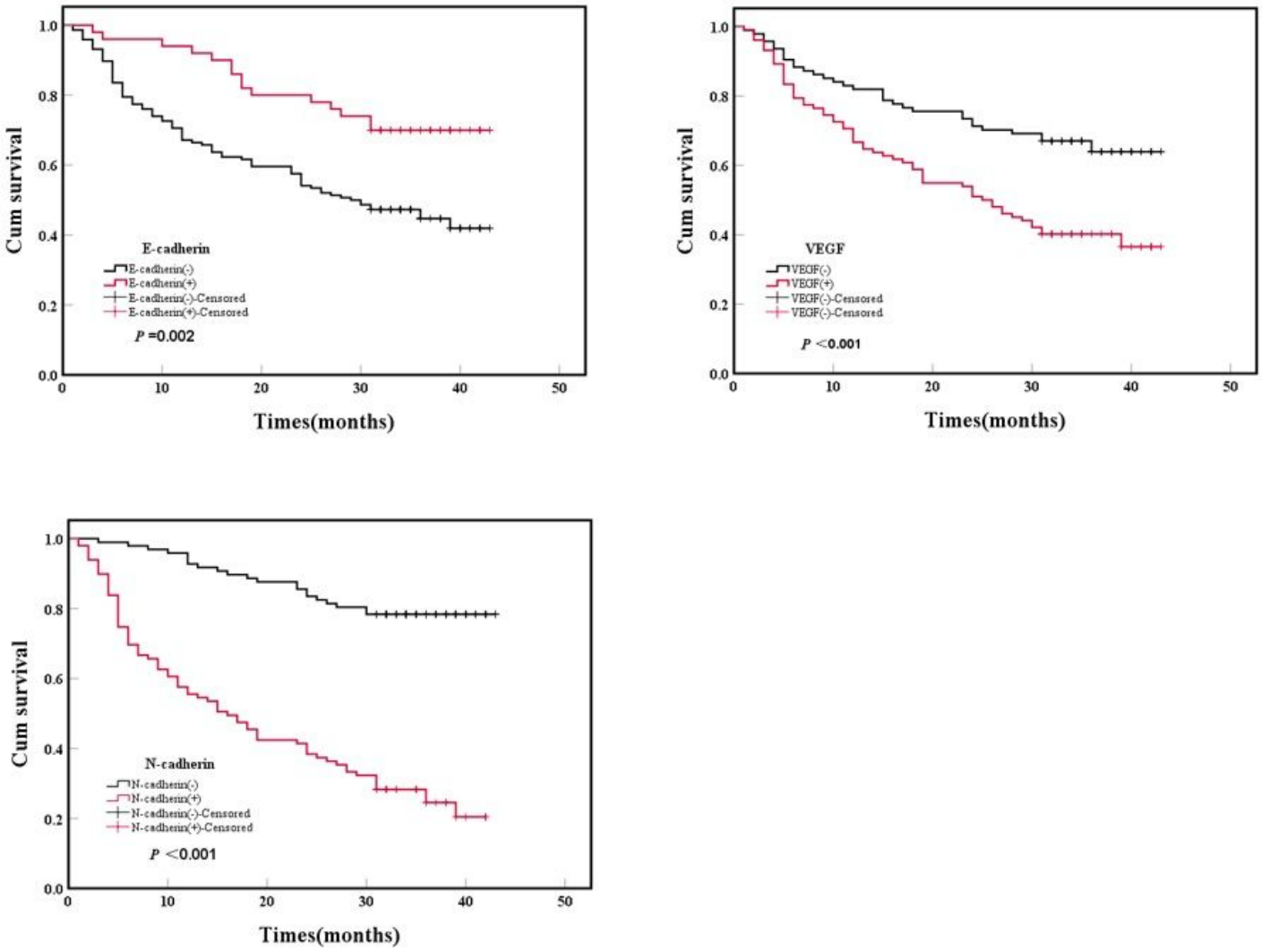

Figure 3

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), while patients with positive E-cadherin expression showed longer survival than those with negative expression (P =0.002).

Supplementary Files

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