

Significance of vascular endothelial growth factor expression and its correlation with inducible nitric oxide synthase in gastric cancer

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Supported by Science and Technology Fund of Medicine and Health of Zhejiang Province, No.2000A116

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Received: 2003-10-15 **Accepted:** 2003-12-06

analysis showed the immunoreactive grades of VEGF were not associated with that of iNOS.

CONCLUSIONS: The expression of VEGF₁₆₅mRNA is well related with lymph node metastasis and TNM stages of UICC in gastric cancer, and is concerned with the invasiveness and metastasis of gastric cancer. The relationship can be observed between the expression of VEGF and iNOS in gastric cancer.

Song ZY, Wen SQ, Peng JP, Huang X, Qian KD. Significance of vascular endothelial growth factor expression and its correlation with inducible nitric oxide synthase in gastric cancer. *World J Gastroenterol* 2004; 10(9): 1250-1255

<http://www.wjgnet.com/1007-9327/10/1250.asp>

Abstract

AIM: To investigate the clinical significance of the expression of VEGF₁₆₅mRNA and the correlation with vascular endothelial growth factor (VEGF) protein and inducible nitric oxide synthase (iNO) in human gastric cancer.

METHODS: We tested VEGF₁₆₅mRNA expression in 31 cases of resected gastric cancer specimens and normal paired gastric mucosae by RT-PCR. Total RNA was extracted with TRIzol reagents, transcribed into cDNA with oligo (dT)₁₅ priming, inner controlled with β -actin expression and agarose gel isolated after PCR. VEGF expression was quantitated with IS1000 imaging system. Meanwhile we also examined expression levels of VEGF protein and iNOS in 85 cases of gastric cancer. All paraffin-embedded samples were immunohistochemically stained by streptavidin-peroxidase method (SP).

RESULTS: The mean expression of VEGF₁₆₅mRNA in gastric cancer was 1.125 ± 0.356 , significantly higher than that of normal paired mucosae, which was 0.760 ± 0.278 . The data indicated that the expression level of VEGF₁₆₅mRNA was well related to lymph node metastasis and TNM stages of UICC. The expression levels in patients with lymph node metastasis and without lymph node metastasis were 1.219 ± 0.377 and 0.927 ± 0.205 respectively ($P < 0.05$). The expression in stages I, II, III, IV was 0.934 ± 0.194 , 1.262 ± 0.386 respectively ($P < 0.01$). Further analysis showed the lymph node metastasis rate in the group with over-expression of VEGF was higher than that in the group with low expression of VEGF (83.3% vs 46.2%), and the ratio of stage III+IV in the group with over-expression of VEGF was also higher than that in the group with low expression with VEGF (77.8% vs 33.8%) ($P < 0.05$). The positive rates of expression of VEGF protein and iNOS in 85 cases of gastric cancer were 75.4% and 58.8% respectively, and 50.1% of the patients showed positive staining both for iNOS and VEGF, the correlation with the two factors was significant ($P = 0.018$). But more intensive

INTRODUCTION

Many observations have shown that angiogenesis plays an important role in the growth, progression and metastasis of solid tumors. Several potential angiogenic factors have been identified, vascular endothelial growth factor (VEGF) is known as one of the most powerful and specific molecules in vascularization. VEGF₁₆₅ is the major isoform among the five different VEGF species^[1,2]. Studies have demonstrated that tumor cells could produce and secrete VEGF periodically by themselves, which binding with the VEGF receptor in the vascular endothelial cells and then promote the angiogenesis in tumors^[3]. Inducible nitric oxide synthase (iNOS) is one of the isoforms of nitric oxide synthase that catalyzes the formation of nitric oxide, a regulator of vascular permeability. Expression of VEGF protein and iNO has been shown by immunohistochemistry in gastric cancer^[4-8]. However, the relationship has not been well evaluated between the VEGF₁₆₅mRNA and the clinical pathological features, and between VEGF and iNO, the two angiogenic regulators in gastric cancer.

The present study detected the expression of VEGF₁₆₅mRNA by RT-PCR and the expression of VEGF protein and iNO by immunohistochemistry in surgically resected human gastric cancer specimens, and characterized the correlation that mentioned above.

MATERIALS AND METHODS

Materials

Specimens of cancer tissue from 85 cases of gastric cancer were confirmed pathologically and at the second Affiliated Hospital of Medical College of Zhejiang University from August 1998 to January 2002. Of these, 54 patients were male, and 23 female, with a median age of 54 (31-77) years. All the cases were classified by WHO criteria histologically and TNM staging of UICC. Fresh cancerous and normal paired gastric mucosae obtained from 31 cases of them were frozen quickly by liquid nitrogen and stored at -80 °C until used for RT-PCR. All the specimens from 85 cases were fixed in 40 g/L buffered formaldehyde and embedded in paraffin for immunohistochemistry.

RT-PCR

Total RNA was extracted from 100 mg each frozen tissue using TRIzol reagents according to the manufacturer's instructions (GIBCO BRL) and the purity and quality were identified by an ultraviolet spectrometer and denatural gel electrophoresis.

First-strand cDNA was synthesized from 2 µg of total RNA in a 40 µL reaction volume by reverse transcription (RT) using 50 pmol oligo (dT)₁₅ 2 µL, RNasin 40 U, 10 mmol/L dNTP 2 µL, M-MLV reverse transcriptase 10 U (PROMEGA). The RNA template and oligo (dT)₁₅ were incubated at 65 °C for 10 min first, and were then added to the total reaction system at 37 °C for 1 h, stored at -20 °C for PCR. cDNA of 5 µL was amplified by PCR in a 25 µL reaction volume, containing 10 mmol/L dNTP 0.5 µL, Taq 1.5 U, per primer 10 pmol, and was inner controlled with β-actin. VEGF primers according to Tishcer's design^[9] were the upper primer: 5'-CAAGGATCCATGAAC TTTCTGCTGCTT-3', the lower primer: 5'-CTTAAGCTTGC TCCTTCC TCCTGCCCCGCG-3'. The upper primer of β-actin: 5'-TCGACAACGGCTCCGGCA-3', the lower primer of β-actin: 5'-CGTACATGGCTGGGGTGT-3'. The recombinant plasmid pGEM- T-VEGF₁₆₅ was used as positive control (provide by the Surgery Laboratory in our hospital). PCR condition of VEGF₁₆₅: pre-denaturation 94 °C for 5 min, then 30 cycles of amplification at 94 °C for 45 s, at 60 °C for 45 s, and at 72 °C for 45 s, extension at 72 °C for 10 min.

Aliquots of the PCR products (10 µL) were separated and visualized with ethidium bromide staining after electrophoresis in a 15 g/L agarose gel in Tris acetate ethylenediaminetetraacetic acid buffer at 100 V for 20 min, and quantitated with IS 1000 imaging system (Alpha Inotech). The expression of VEGF₁₆₅mRNA was expressed as follow: numeral value of VEGF₁₆₅=scanning data of VEGF₁₆₅ band/ scanning data of β-actin band. The value greater than 1 was regarded as higher expression of VEGF₁₆₅mRNA, and the value less than 1 was regarded as lower expression of VEGF₁₆₅mRNA. Statistical analysis was performed by means of SPSS10.0.

Immunohistochemical analysis

Paraffin-embedded samples were serially sectioned at 4 µm, and mounted onto the histosticked-coated slides. Sections were dewaxed in xylene, and dehydrated in ethanol, and then heated at 98 °C in EDTA retrieved solution for the antigens. Endogenous peroxidase was blocked by incubation of samples in hydrogen peroxide blocking reagent (Maixin Bio, Fuzhou). After washed with phosphate-buffered saline solution, the samples were incubated for 60 min with the primary antibodies. The anti-VEGF antibody is a polyclonal mouse anti-serum against VEGF of human origin and recognizes an amino-terminal epitope found in VEGF_{121,165,189}, and the anti-iNOS antibody is a monoclonal mouse anti-serum against the C-terminal domain of iNOS of human origin (The two antibodies were from Santa Cruz Biotechnology, Inc., CA). Location of the primary antibodies was achieved by subsequent application of a biotinylated anti-primary antibody, an avidin-biotin complex conjugated to horseradish peroxidase. The reaction products were visualized with diaminobenzidine. The slides were counter-stained by hematoxylin. Negative controls were established by replacing the primary antibody with PBS and normal rabbit serum. Known immunostaining-positive slides were used as positive controls. Immunoreactivity was diagnosed depending on the shade of cells staining assigned to 0-3 scores (0: negative reaction, 1: weak brown-color staining, 2: moderate brown-color staining, 3: strong brown-color staining), and the percent of positive staining cells, the average percentage of positive cells was determined in at least 5 areas

at ×400 and assigned to 0-3 scores (0: negative or equivocal staining, 1: weakly positive expression, cells were staining in 1-25%, 2: moderately positive expression, cells were staining in 25-50%, 3: strongly positive expression, the cells were stained over 50%). The diagnosed grades accorded to the sum of two scores, -: 0-1, +: 2, ++: 3-4, +++: >5. Statistical analysis was performed by means of SPSS10.0.

RESULTS

Expression of VEGF₁₆₅mRNA in gastric cancer

As shown by RT-PCR, the amplification products of VEGF and β-actin were 655 bp and 370 bp, respectively. Over-expression of VEGF₁₆₅ was detected in gastric cancer mucosae, and the over-expression rate was 58%, whereas 16% in normal paired mucosae. The mean value of the VEGF₁₆₅mRNA expression in gastric cancer tissues was significantly higher than that in normal paired tissues, which was 1.125 ± 0.356 and 0.760 ± 0.278 respectively (Figure 1 and Table 1).

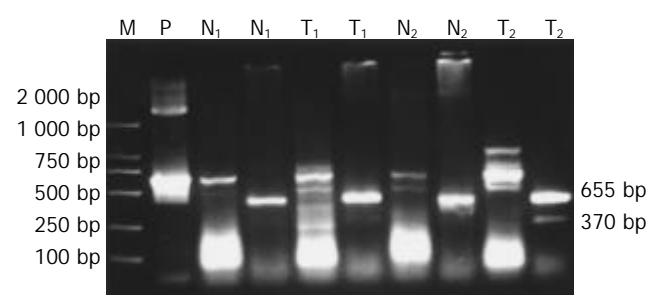


Figure 1 RT-PCR products of VEGF₁₆₅mRNA by 20 g/L agarose gel electrophoresis. M, marker; P, recombined plasmid of VEGF₁₆₅; T, gastric cancer mucosae; N, non-gastric cancer mucosae; β-actin. The product of VEGF₁₆₅ mRNA was 655 bp, and the product of β-actin was 370 bp.

Relationship between expression of VEGF₁₆₅mRNA and clinical pathological features of gastric cancer

As shown in Table 2, the expression level of VEGF₁₆₅mRNA in the group of positive lymph node metastasis was higher than that in the group of negative lymph node metastasis ($P < 0.05$), and the expression level in stages III and IV was also higher than that in stages I and II ($P < 0.05$). The expression levels had no correlation with age, sizes of tumors and degree of histological differentiation.

Another observation shown in Table 3 and Table 4 was that the rate of lymph node metastasis and the ratio of stages III and IV in the group with over-expression of VEGF₁₆₅ mRNA were also higher than those in the group with low-expression of VEGF₁₆₅ mRNA (83.3% vs 46.2%, 77.8% vs 30.8% respectively, $P < 0.05$).

Correlation between VEGF and iNOS in gastric cancer

Positive immunostaining for iNOS and VEGF was observed in cancer cells, and weakly positive or negative staining was observed in the normal gastric epithelial tissues. VEGF and iNOS immunoreactivity was located mainly in the cytoplasm and cell membrane (Figure 2). The positive expression rate of iNOS was 58.8% (50/85), and that of VEGF was 75.4% (64/85). There were 50.1% (43/85) patients showing positive staining both for iNOS and VEGF, 16.5% (14/85) showing negative staining both for iNOS and VEGF. Correlation with the two factors was significant ($P < 0.05$). But more intensive analysis by Kendall test showed that the immunoreactive grades of VEGF had no association with that of iNOS ($P > 0.05$, see Table 5).

Table 1 Expression of VEGF₁₆₅mRNA in gastric cancer and non-cancerous mucosa by RT-PCR

No.	Sex	Age (yr)	Histological type	Depth of invasion	Lympho node metastasis	Stage of TNM	VEGFmRNA	
							T	N
1	M	67	PD	musclaris	-	I	1.286	0.782
2	M	67	WD	extraserosa	+	III	1.568	1.086
3	F	41	PD	serosa	+	III	1.624	0.925
4	M	54	PD	extraserosa	+	IV	0.815	0.729
5	M	35	PD	extraserosa	+	III	1.204	0.627
6	M	50	MD	extraserosa	+	III	1.111	0.335
7	F	34	WD	extraserosa	+	III	1.347	0.845
8	M	46	MD	serosa	+	III	0.785	0.701
9	M	60	WD	submucosae	-	I	0.833	0.807
10	F	70	WD	serosa	-	I	0.627	0.525
11	M	62	WD	serosa	+	IV	0.850	0.646
12	F	31	WD	mucosae	-	I	0.815	1.136
13	M	55	MD	extraserosa	+	IV	1.545	0.872
14	M	70	WD	serosa	+	II	0.765	0.577
15	M	64	MD	serosa	-	III	1.082	0.842
16	M	66	MD	serosa	+	III	1.069	0.422
17	M	77	PD	serosa	+	IV	1.383	10.28
18	M	40	PD	serosa	+	II	1.156	1.381
19	F	50	PD	serosa	+	II	0.989	0.874
20	M	52	PD	serosa	+	III	1.895	0.356
21	F	38	PD	extraserosa	+	III	1.667	0.759
22	F	68	WD	mucosae	-	I	0.884	0.682
23	F	53	WD	extraserosa	+	III	1.456	0.682
24	M	61	MD	extraserosa	+	III	1.574	0.098
25	M	43	PD	serosa	-	I	1.198	0.429
26	M	43	PD	serosa	+	III	0.368	0.994
27	M	57	PD	serosa	+	II	1.052	0.661
28	F	44	PD	mucosae	-	I	0.952	0.793
29	M	71	WD	extraserosa	-	II	0.750	0.728
30	M	77	PD	extraserosa	+	IV	1.375	10105
31	F	50	PD	mucosae	-	I	0.842	0.794
Mean value							1.125	0.760

Table 2 Relationship between expression of VEGF₁₆₅mRNA and clinical pathological features in gastric cancer tissue

Variable	No	Expression of VEGF (mean±SD)	
Age (yr)			
<55	16	1.139±0.393	<i>P</i> >0.05
>55	15	1.139±0.393	
Sizes of tumor			
≤2 cm	2	0.993±0.271	<i>P</i> >0.05
2-3 cm	6		
>3 cm	23	1.171±0.375	
Histological type			
Well differentiated	10	0.979±0.339	<i>P</i> >0.05
Moderate or poorly differentiated	21	1.1937±0.350	
Lymph node metastasis			
Positive	21	1.219±0.377	<i>P</i> =0.03
Negative	10	0.927±0.205	
Stage of UICC			
I	8	0.934±0.194	<i>P</i> =0.009
II	5		
III	13	1.262±0.386	
IV	5		

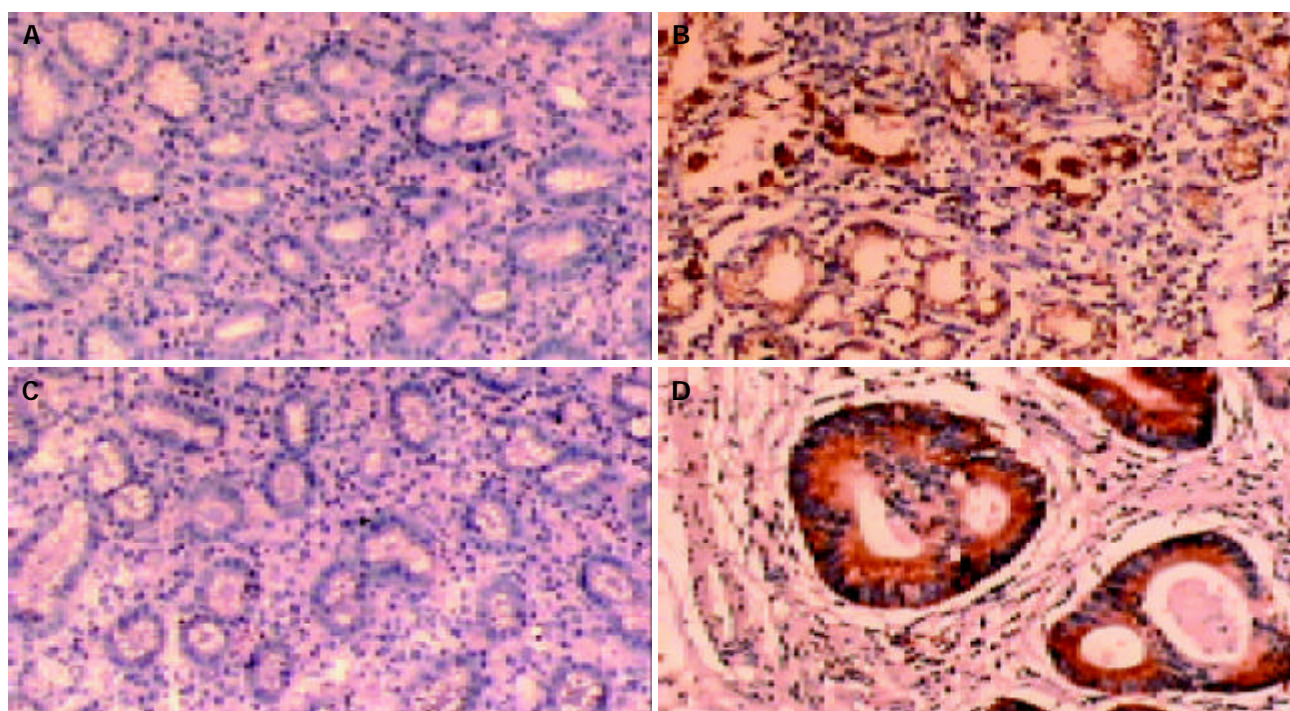


Figure 2 Immunostaining of VEGF and iNOS in gastric cancer and normal paired tissues. A: Negative staining of VEGF in normal paired gastric mucosae (×100), B: Positive staining of VEGF in gastric cancer (×100), C: Negative staining of iNOS in normal paired gastric mucosae (×100), D: Positive staining of iNOS in gastric cancer (×100).

Table 3 Relationship between level of VEGF₁₆₅mRNA expression and lymph node metastasis

Variable	Lymph node metastasis			Metastasis
	Positive cases	Negative cases	Total rate	
Over-expression	15	3	18	83.3%
Low-expression	6	7	13	46.2%
Total	19	10	31	

$$\chi^2=4.775, P=0.02.$$

Table 4 Relationship between level of VEGF₁₆₅mRNA expression and stages of UICC

Variable	Stages of UICC		Total	Ratio of III+IV
	I+II	III+IV		
Over-expression	4	14	18	77.8%
Low-expression	9	4	13	30.8%
Total	13	18	31	

$$\chi^2=6.85, P=0.009.$$

Table 5 Association with immunoreactive grades of VEGF protein and iNOS in gastric cancer

iNOS (N)	VEGF (N)				Total
	-	+	++	+++	
-	14	12	6	3	35
+	5	9	8	2	24
++	2	5	11	1	19
+++	0	2	3	2	7
Total	21	28	28	8	85

$$\chi^2=5.615, P=0.132.$$

DISCUSSION

VEGF is well characterized by its potent, specific angiogenesis-promoting effect. Native VEGF is a basic, heparin-binding, homodimeric glycoprotein of M_r 43 000–46 000. Five human VEGF mRNA species encoding VEGF isoforms of 121, 145, 165, 189, and 206 amino acids have been identified by alternative splicing of VEGFmRNA. An important biological property that distinguishes the different VEGF isoforms is their heparin and heparin-sulfate binding ability. VEGF₁₆₅ is the predominant form. VEGF has been found to be a highly specific mitogen for endothelial cells, and a prime regulator of angiogenesis and vasculogenesis, and could also contribute to the development of tumors because of its ability to induce permeabilization of blood vessels^[1,2,10,11]. Numerous studies have demonstrated that the high expression level of VEGF in many kinds of tumor cells^[12–14]. The same observations have also been seen in gastric cancer cells by *in vitro* and *in vivo*^[3–6,15,16]. We tested VEGF₁₆₅mRNA expression in 31 cases of resected gastric cancer specimens and normal paired gastric mucosae by RT-PCR, the results showing that the mean expression of VEGF₁₆₅mRNA in gastric cancer was significantly higher than that of normal paired mucosae. VEGF in high expression could combine with its receptors Flt-1 or KDR to exert its powerful function of promoting angiogenesis^[1,2,10]. Furthermore, some studies showed the high expression of Flt-1 and KDR in gastric tumor specimens by RT-PCR and immunohistochemistry, and exogenous VEGF₁₆₅ stimulated the growth of KDR positive carcinoma cells^[17,18]. These findings indicate there is a possible autocrine pathway for VEGF in gastric cancer, which is very important in the development of gastric cancer.

VEGF induces the formation of fenestrations in blood vessels and vesiculo-vacuolar organelles that form channels through which blood-borne proteins can extravasate. This could lead to the formation of an extravascular fibrin gel, which provides a matrix that supports the growth of endothelial cells and tumor cells and allows invasion of stromal cells into the development of tumors^[2]. A substantial number of studies have demonstrated a strong association between elevated tumor

expression of VEGF and advanced disease or poor prognosis in various cancers^[1,2]. In our study, the expression of VEGF₁₆₅mRNA was well related with lymph node metastasis and TNM stage, VEGF₁₆₅mRNA elevated in lymph node positive or stages III and IV patients, and was consistent with many immunohistochemical results. Kakeji *et al.*^[4] also revealed VEGF to be an independent prognostic factor and independent risk factor for liver metastasis. Karayiannakis *et al.*^[19] showed there was a significant association between serum VEGF levels and disease stage, as well as invasion depth of the tumor and the presence of distant metastasis. It was concerned with the invasiveness and metastasis of gastric cancer, and might be predictive of tumor status and prognosis in advanced gastric cancer patients, and could provide new prognostic information not afforded by conventional clinicopathologic prognostic indicators^[20-24].

Early gastric cancer patients have high survival rates, but there is lymph node metastasis in early gastric cancer, especially in submucosa, the rate of lymph node metastasis was about 20%, resulting in the decrease of 5-year survival of early gastric cancers. In our study, the expression of VEGF₁₆₅mRNA in the 5 cases of early gastric cancer had no difference with the normal paired mucosae, which the mucosae or submucosae were invaded, and lymph node metastasis was negative in the 5 cases. Recently Amioka *et al.* demonstrated VEGF was associated with lymphatic invasion and lymph node metastasis in early gastric cancer^[25-27]. The clinical significance of VEGF expression in early gastric cancer should be intensively studied.

VEGF production has been found to be regulated by growth factors, cytokines, oncogenes, anti-oncogenes and other extra cellular molecules, such as hypoxia, p53 gene, TGF- α ^[28-32]. But few investigations have been concerned with relationship between VEGF and iNOS. Nitric oxide produced through iNOS induction could enhance vasodilation, increase vascular permeability and accelerate nutrient supply of tumor tissues and promote neovascularization, thereby facilitating tumor growth^[7,8]. It has been shown that the expression of iNOS in most tumor tissues was higher than that in normal tissues. There were many reports concerning the high expression of iNOS in gastric cancer, which increased with the stage of the cancer and lymph node metastasis^[7,8,33]. We found the expression rate of iNOS in gastric cancer was 58.8%, and iNOS and VEGF had co-expressions in 50.1% of the patients, there was a correlation between the two factors. According to some reports^[34], VEGF could induce the release of NO from endothelial cells, on the other hand, endogenous NO enhanced VEGF synthesis in rat vascular smooth muscle cells; Host expression of NOS could contribute to induction of NOS in tumor and melanoma growth in mice, possibly by regulating the amount and availability of VEGF. The positive interaction between endogenous NO and VEGF might have implications for endothelial regeneration^[35]. Kisley *et al.*^[36] examined the effect of iNOS deficiency on VEGF protein concentration in mouse lung tumors, and showed VEGF concentration was lower than 54% in lung tumors isolated from iNOS(-/-)mice versus controls(wild-type +/+). NO enhanced the transcription of VEGF gene by inducing HIF-1 (hypoxia -inducible factor-1) binding activity in glioblastoma A-172 cells and hepatoma Hep3B cells. HIF was the best-characterized regulator of VEGF gene transcription^[37]. The high coincidental expression of iNOS and VEGF protein accumulation may be important events to enhance gastric carcinogenesis and poor clinical features. But in our present work, more intensive analysis showed that the immunoreactive grades of VEGF were no association with that of iNOS, indicating that many other factors may induce the production and angiogenesis of VEGF besides iNOS in gastric cancer.

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Edited by Zhao M and Wang XL Proofread by Xu FM