

A study of the correlation between vascular endothelial growth factor isoform189 expression and intratumoral vessel count with lymph node involvement in advanced gastric cancer

Yi-Cheng CHEN, Kazuhiko KASUYA, Kazushige ITO, Yuiti NAGAKAWA,
Keiji SUZUKI, Yu TAKAGI, Tatuya AOKI, Yasuhisa KOYANAGI

Department of Surgery, Tokyo Medical University

ABSTRACT

Angiogenesis is essential for tumor growth and metastasis, and depends on the production of angiogenic factors by host and/or tumor cells. We examined the role of angiogenesis and vascular endothelial growth factor (VEGF) isoforms expression in metastasis and recurrence of advanced gastric carcinoma. Surgical specimens of 39 advanced gastric carcinomas from January 1997 to June 1999 were examined. We evaluated lymph node involvement, CD34 immunostained stromal microvessels, hematogeneous spread and VEGF isoform expression by RT-PCR. The results are: (1) Microvessel screening showed 23 patients with low vessel counts and 16 patients with high vessel counts. (2) VEGF165 expression was increased in all 39 cases, and VEGF189 was increased in 27 cases (69.2%). (3) Lymph node involvement was found in 17 of 23 cases (73.9%) of low and 14 of 16 cases (87.5%) of high vessel counts, and in 22 of 27 cases (81.5%) with increased VEGF189 expression and 9 of 12 cases (75%) without increased VEGF189. (4) Tumors with both increased VEGF189 expression and a high vessel count showed a high frequency of lymph node metastasis (91.7%). We concluded that the increased expression of VEGF isoform189 and the presence of highly proliferated stromal microvessels in advanced gastric carcinoma has a tendency to correlate with a high frequency of lymph node involvement.

INTRODUCTION

The prognosis of gastric cancer depends on the clinicopathologic stage of disease such as the depth of tumor invasion, the presence of hematogeneous metastasis, peritoneal dissemination, and localized nodal recurrence, but the pathophysiology of these events is unclear. Most advanced gastric cancers are grossly of a depressed type. Histologically, these tumors show inflammatory reactions to direct invasion by tumor cells, and usually areas of healing as well. Extracellular matrix or leaked serum components induced by factors secreted from cancer cells may cause inflammation or capillary proliferation. Fibrotic changes may protect

the host¹⁾, but this is controversial. Angiogenic reactions are essential for tumor growth and metastasis²⁾. We have also noticed that increasing angiogenic factors act to increase both the vessel permeability and the incidence of tumor metastasis^{6,8,10,12)}.

Vascular endothelial growth factor (VEGF) is an endothelial cell mitogen and a strong angiogenic factor, also known as heparin-binding growth factor, with a molecular weight of 45 kD³⁾. VEGF mRNA consists of four isoforms: VEGF121, VEGF165, VEGF189 and VEGF206, which contain 121, 165, 189 and 206 amino acids, respectively. These exist in vivo due to alternative mRNA splicing and different biological activities^{4,5)}. VEGF121 and VEGF165 bind heparin weakly and are

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Reprint requests to: Yi-Cheng CHEN, Department of Surgery, Tokyo Medical University 6-7-1 Nishi-shinjuku, Shinjuku-ku, Tokyo, 160-0023, Japan

the most abundantly expressed variants in cells and tumors⁶). In contrast, VEGF189 and VEGF206 exist in the extracellular matrix strongly bound to heparan sulfate. VEGF165 is the predominantly expressed isoform in cells, and VEGF189 is closely associated with tumor progression⁷. Some studies have demonstrated a relation between VEGF mRNA expression and tumor prognosis, but there are no studies of VEGF mRNA isoform expression in gastric cancer. This study evaluated VEGF mRNA expression as an angiogenic factor inducing neovascularization and whether there is a relationship between VEGF expression and the spread of advanced gastric cancer.

MATERIALS AND METHODS

Thirty-nine advanced gastric cancers are evaluated. All samples were surgical materials from 1997 to 1999 at Tokyo Medical University Hospital. Clinicopathologic characteristics are shown in Table 1. Surgical materials were fixed in 10% buffered formalin and embedded in paraffin for routine histopathologic analysis. Cases with one or more lymph nodes involved were classified as lymph node positive (LN+). Hepatic metastasis was defined on preoperative screening or at post-operative follow-up by either abdominal echography or CT scan within 1 year (H+).

Evaluation of intratumoral and/or stromal microvessels was performed as follows: Immunostaining for CD34 was done on consecutive hematoxylin eosin sections. We performed CD34 staining to show the endothelial cells using En Vision plus kit (DAKO Japan, Kyoto, Japan) and anti-human CD34 mouse monoclonal antibody (Nichirei, Tokyo, Japan). And we used Victoria blue staining to show the elastic elements of vessels. The vessel count was assessed in five intratumoral and adjacent stroma regions randomly for each high-power field ($\times 100$) by light microscopy. Only the ones which enclosed by positively stained cells were counted to be vessels. The partial noticed vessels around the margins of microscopic field were not counted (Fig. 1).

Evaluation of VEGF mRNA included the following:

Table 1 Clinicopathologic characteristics

Age: 41 to 81 years; average 64.2 ± 10.7 years	
Sex: male: 28; female: 11	
Macroscopic type:	Type 1: 4 ; Type 2: 10 Type 3: 17 ; Type 4: 6 IIa+IIc like: 1; IIc like: 1
Histologic type: (dominant)	por2(sig): 5; por2: 7; por1: 11 tub2: 9 ; tub1: 4 pap: 2 ; muc: 1

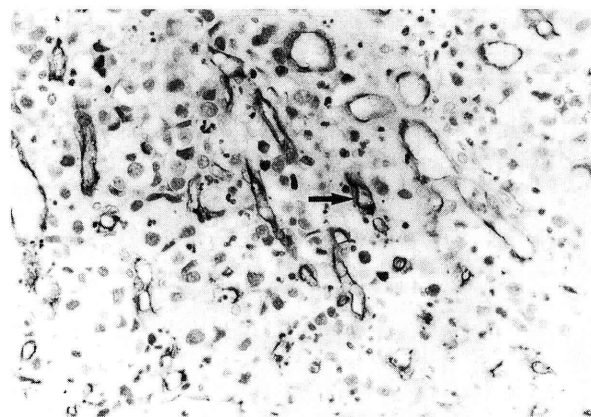


Fig. 1 Advanced gastric cancer with high vessel proliferation (high vesselcount group) (arrow, vascular endothelial cell of microvessels). CD34 immunostain, $\times 100$

Samples of 5 mm³ were prepared from neoplastic areas without necrosis, then rapidly frozen and stored at -80°C . We evaluated VEGF mRNA isoforms by reverse transcription polymerase chain reaction (RT-PCR) according to Berkman et al⁸. The sense primer-1 was 5'-AAGCCATCCTGTGTGCCCCTGATG-3', and the anti-sense primer-1 was 5'-GCGAATTCCTCC-TGCCCGGCTCAC-3'. The inner PCR sense primer-2 5'-CGGATCAAACCTCACCAAGGCC-3' and anti-sense primer-2 5'-CTTTCTCCGCTCTGAGCAAGG-C-3' detect VEGF165 and VEGF189 (Fig. 2). RT-PCR products were electrophoresed on a 1% agarose gel and stained with ethidium bromide. DNA extracted at 204 bp and 132 bp from the agarose gel was ligated in pGEM-T easy vector (Promega Mandison, WI, USA) and transformed to E. coli XLI blue nRF' to confirm the sequence. Statistic analysis was performed using the χ^2 test or Fisher's exact test for correlations between VEGF expression and the number of vessels. Prognostic significance was assessed using the Kaplan-Meier method and the generalized Wilcoxon test. The Statview for windows of Microsoft was used for statistic analysis and the accepted level of significance was $p < 0.05$.

RESULTS

The averaged vessel counts ranged from 5.2 to 29.4 per field. The mean vessel count for all cases was 14.9 per field with 7.1 as the standard deviation. We therefore divided these into a high vessel count group (≥ 15) and a low vessel count group (< 15). The 39 advanced gastric cancers were divided into two groups, 16 (41%) with high vessel counts and 23 (59%) with low vessel counts using CD34 staining. The density of microvessels was sometimes heterogeneous, occasionally with patchy areas adjacent to the tumor margin showing a high degree of vessel proliferation. VEGF165 expression was detected in all 39 tumors. Twenty-seven

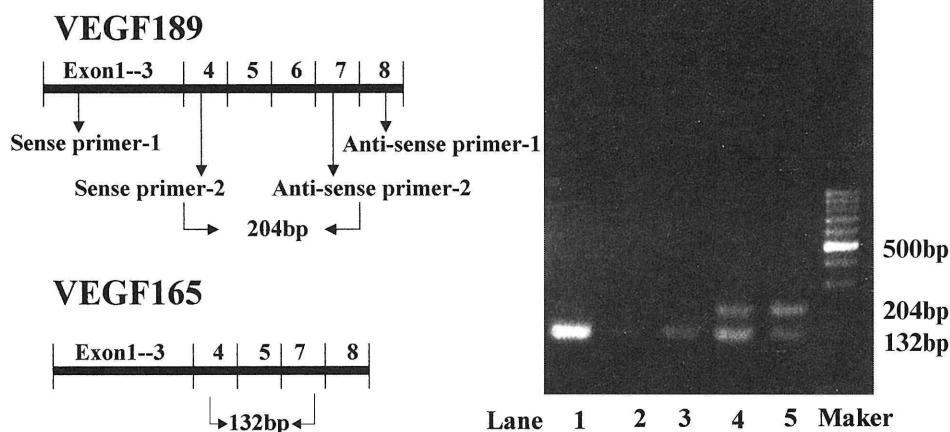


Fig. 2 RT-PCR of VEGF isoforms. Lane 1: VEGF189(+), VEGF165(++); Lane 2: VEGF189(-), VEGF165(+); Lane 3: VEGF189(-), VEGF165(+); Lane 4: VEGF189(+), VEGF165(+); Lane 5: VEGF189(+), VEGF165(+).

(69.2%) tumors expressed VEGF189. We confirmed VEGF165 and the 72 bp insertion for VEGF189 from RT-PCR products by sequencing in two cases. Neither the vessel count nor VEGF189 expression correlated with patient age, tumor size, tumor location, gross appearance, depth of tumor invasion, disease staging or histologic type by the χ^2 test. Twelve (75%) of the 16 cases with high vessel count showed VEGF189 expression, as did 15 (65.2%) in the low vessel count group. There was no statistical correlation between VEGF189 expression and the number of microvessels in the tumor or adjacent area. Thirty-one (79.5%) of the 39 tumors were accompanied by lymph node disease: 14 (87.5%) in the high vessel count group and 17 (73.9%) in the low vessel count group. High vessel count tumors with positive VEGF189 expression showed a greater incidence of lymph node involvement (11/12, 91.7%) than other combinations: positive VEGF189 with low vessel count (11/15, 73.3%), negative VEGF189 with high vessel count (3/4, 75%) and negative VEGF189 with low vessel count (6/8, 75%). However, these differences were not significant ($p=0.49$) (Fig. 3). The follow-up periods of patients ranged from 37 days to 841 days (averaged 376 days). Five patients died post-operatively and the others were following in our out-patient department. The median survival time in the positive VEGF189 with high vesselcount group was 134 days, 285 days in the positive VEGF189 with low vessel count group, 215 days in the negative VEGF189 with high vessel count group and 194 days in the negative VEGF189 with low vessel counts. There was shorter survival in the positive VEGF189 with high vessel count group compared to other combinations. The generalized Wilcoxon test showed a $p=0.20$ in the comparison of the positive VEGF189 with high vessel count group to the other three groups (Fig. 4). All of the hepatic

metastatic cases were positive for VEGF189 expression. In these 5 cases of liver metastasis, 2 cases had high vessel counts and 3 had low vessel counts. One patient who died had positive VEGF189 expression and a high vessel count (Table 2).

DISCUSSION

Cancers accompanied by desmoplasia are disposed to distant metastasis. Martin et al⁹⁾ reported that anti-smooth muscle actin-positive fibroblasts around tumors were able to breach the basement membrane. There are also reports on the poor prognosis of colon carcinomas¹⁰⁾ and breast cancers¹¹⁾ with severe desmoplasia. The destruction of basement membrane occurs at invasive sites (marginal areas of tumor) as a result of fibroblast grooving and matrix proteinase secretion. It is well known that advanced gastric cancer shows fibrous changes due to peptic or cancerous ulcerations. Moreover, gastric cancer is derived from abundant stromal desmoplasia. Desmoplasia generally leads to inflammatory cell infiltration, capillary proliferation, and neovascularization necessary for tumor growth and progression. The same mechanisms participate in tumor metastasis²⁾.

VEGF is one of the most important factors which increase the permeability of the microvasculature³⁾. In other studies, VEGF was related to liver metastasis and/or hematogenous spreading. We hypothesized that the number or density of intratumoral vessels might increase as a result of enhanced VEGF mRNA expression which, in turn, might relate to hematogeneous dissemination or lymph node involvement. The latter is sometimes caused by venous inflow instead of lymph ductal routes. Thus, lymph node disease might be associated with the development of hematogenous invasion, similar to a study¹²⁾ which explored the role of lymphatic spread in

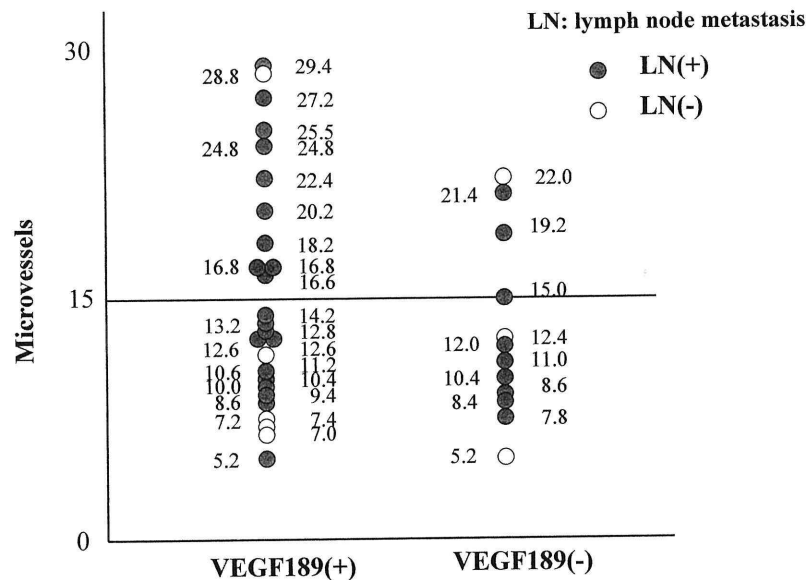


Fig. 3 Correlation between VEGF189 expressions with microvessel-counts and LN metastasis. VEGF189 Expression(+) with high microvessel-count (12 cases): LN(+) 11/12, 91.7%. VEGF189 Expression(-) with high microvessel-count (4 cases): LN(+) 3/4, 75%. VEGF189 Expression(+) with low microvessel-count (15 cases): LN(+) 11/15, 73.3%. VEGF189 Expression(-) with low microvessel-count (8 cases): LN(+) 6/8, 75%.

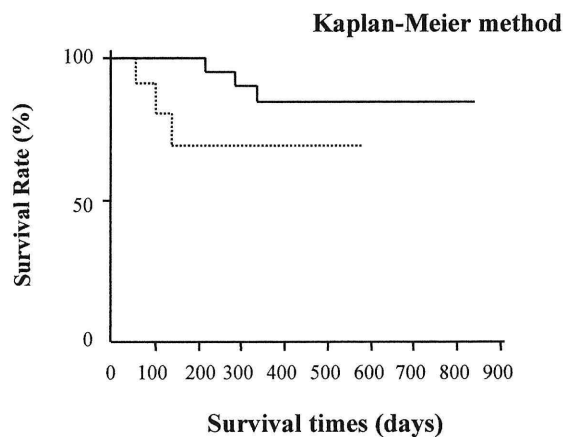


Fig. 4 Survival curves for 12 cases of VEGF189(+) with high vesselcount (dotted line), and for 27 cases of the other types (solid line). The estimated median survival days of the former was 126.5 ± 9.6 days, and the estimated median survival days of the latter was 326.6 ± 7.4 days.

Table 2 Correlation between liver metastasis, VEGF189 expression, and Vessel-count

Case	VEGF189	Vessel-count	outcome
1	(+)	high	died
2	(+)	high	alive
3	(+)	low	alive
4	(+)	low	alive
5	(+)	low	alive

liver metastasis.

The prognosis of gastric cancer depends on histologic type and disease staging. Generally, tumors with high vascularity are predisposed to hematogenous metastasis, while differentiated types metastasize to the liver occasionally and poorly differentiated types predominantly lead to peritoneal dissemination¹³. Other reports suggest a close relationship between tumor vascularity, prognosis, metastasis, and VEGF expression^{14~17}. Takahashi et al¹⁶ found that vessel count and VEGF protein expression were higher in differentiated (intestinal) types than in poorly differentiated (diffuse) types; metastasis in differentiated types was dependent on angiogenesis induced by VEGF expression in both gastric and colon cancer¹⁵. Maeda et al¹⁴ found that positive VEGF expression as a prognostic indicator correlated with vessel involvement, lymph node disease, and liver metastasis in gastric cancer patients. Saito et al¹⁷ also reported that VEGF is associated with hematogenous recurrence and is the strongest prognostic factor, following lymph node involvements in advanced gastric cancer.

Little is known about the angiogenic of VEGF mRNA isoforms. VEGF165 has been assumed to be the predominant form. Extracellular cleavage of VEGF189 is required for its mitogenic effects¹⁸. Cancer-associated thrombosis is accompanied by platelet aggregation and activation at metastatic sites and activated platelets release VEGF¹⁹. Up-regulation of VEGF189 causes increased angiogenesis in colon cancer

cell lines²⁰⁾. In the present study, we found a high frequency of lymph node disease and a shortening of survival in the group with increased VEGF189 expression and high vessel count. This finding supports the data from other reports. Tokunaga et al²¹⁾ found that VEGF189 was expressed in half of colon cancers. Moreover, Tomisawa et al²²⁾ found 100% VEGF189 expression in advanced pT3-4 renal tumors with vessel proliferation. Oshika et al⁷⁾ determined that the VEGF189 cell-associated isoform was closely associated with progression of non-small cell lung cancer.

There is no previous report about VEGF isoform in gastric cancer. Unfortunately no significant values were found among all those factors in this study. But still the increased expression of VEGF isoform189 and the presence of highly proliferated stromal microvessels has a tendency to correlate with a high frequency of lymph node involvement. Thus, VEGF may be responsible for distant metastasis of gastric cancer, similar to other cancers^{7,20~22)}.

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進行胃癌における VEGF isoform の増幅，腫瘍内血管数 とリンパ節転移の関連性の検討

陳 怡 誠 粕 谷 和 彦 伊 藤 一 成
永 川 裕 一 鈴 木 敬 二 高 木 融
青 木 達 哉

東京医科大学外科学第三講座
(指導：小柳泰久主任教授)

【背景】癌は浸潤に伴い，間質の繊維化や新生血管の増生がみられ，それらは癌や癌周囲の血管から流出する血漿成分や遊走された血球成分，癌細胞から分泌される因子が引き金となっている．我々は血管浸透性亢進および血管内皮細胞増殖因子として Vascular endothelial growth factor (VEGF) の増幅に注目した．VEGF は 4 つの splicing variants の内，ヘパリン親和性の強い VEGF189 は癌細胞に存在し，予後と関連するとの報告がある．【材料と方法】進行胃癌 39 例 (1997 年 1 月-1999 年 6 月) を対象とした．リンパ節転移は一つでも認められるものを陽性とした．腫瘍内微小血管は CD34 染色陽性脈管数を 100 倍率にて 5 視野カウントして平均し，15 未満 (低脈管群) と 15 以上 (高脈管群) に分けた (全例の平均 14.9)．VEGF isoform の発現は RT-PCR で isoform 165, 189 の variant band を検出した．【結果】1) 低脈管群 23 例，高脈管群 16 例であった．リンパ節転移は低脈群 17 例 (73.9%)，高脈管群 14 例 (87.5%) であった．2) 腫瘍内 VEGF165mRNA は全例で増幅があり，VEGF189mRNA は 27 例 (69.2%) でみられた．リンパ節転移は VEGF189 (+) 例で 22 例 (81.5%)，(-) 例で 9 例 (75.0%) にみられた．3) VEGF189 (+) の高脈管症例 12 例中 11 例 (91.7%) にリンパ節転移があり，VEGF189 (+) 低脈管群の 73.5%，VEGF189 (-) 高脈管群の 75%，VEGF189 (-) 低脈管群の 75% に比べ高率であった．4) VEGF189 (+) の高脈管群は他の群に比し，予後不良の傾向が認められた．【結論】VEGF189 が増幅し，腫瘍内の血管が増生している進行胃癌は，リンパ節転移の頻度が高く，予後不良の傾向がある．

キーワード：VEGF，胃癌，腫瘍内血管，リンパ節転移
