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Vascular endothelial growth factor-C expression predicts lymph node metastasis of human gastric carcinomas invading the submucosa

T. Amioka[a], Y. Kitadai[b,*], S. Tanaka[b], K. Haruma[c], M. Yoshihara[a], W. Yasui[d], K. Chayama[a]

[a]*First Department of Internal Medicine, Hiroshima University School of Medicine, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan*
[b]*Department of Endoscopy, Hiroshima University School of Medicine, Hiroshima, Japan*
[c]*Division of Gastroenterology, Department of Medicine, Kawasaki Medical School, Kurashiki, Japan*
[d]*First Department of Pathology, Hiroshima University School of Medicine, Hiroshima, Japan*

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Abstract

We examined the relationship between vascular endothelial growth factor (VEGF)-C expression and lymph node metastases in gastric carcinomas invading the submucosa. Of the six human gastric carcinoma cell lines, two constitutively expressed *VEGF-C* mRNA. In three of 12 gastric biopsy specimens (25%), *VEGF-C* mRNA was detected in tumour tissues, but not in corresponding normal mucosa by reverse transcriptase-polymerase chain reaction (RT-PCR). Of the 139 resected gastric carcinomas, 44 (32%) showed intense cytoplasmic VEGF-C immunoreactivity in many cancer cells at the invading edge. VEGF-C immunoreactivity was associated with greater depth of tumour invasion, lymphatic invasion and lymph node metastases. In addition, vessel count was also significantly higher in the VEGF-C immunoreactive tumours than in other tumours. These results suggest that VEGF-C may be involved in the progression of human gastric carcinoma, particularly via lymphangiogenesis. VEGF-C expression at the invading edge of a gastric carcinoma may be a sensitive marker for metastasis to the lymph nodes.

Keywords: Gastric cancer; Submucosal invasion; Lymph node metastases; Endoscopic mucosal resection; Vascular endothelial growth factor-C; mRNA expression; Immunohistochemistry

1. Introduction

Recent advances have enabled the early detection of gastric carcinoma by endoscopy and treatment by means of endoscopic mucosal resection (EMR) [1,2]. However, even when the lesion is completely resected by EMR, additional surgery is necessary when lymph node metastases appear likely. In cancers with invasion of the submucosa, the risk of lymph node metastases ranges from 10 to 25% [3,4]. The deepest portion of a tumour has a higher malignant potential than other areas, this is the part of the tumour that ultimately will invade, spread locally and metastasise. Risk factors for nodal metastasis in submucosally invasive gastric carcinoma include an undifferentiated histological type, massive invasion of the submucosal layer and invasion of lymphatic vessels [5]. However, which patients with successful EMR of a submucosally invasive carcinoma should undergo subsequent regional lymph node dissection remains unclear. Thus, a reliable marker for lymph node metastases that could be evaluated in EMR specimens would be very helpful.

Vascular endothelial growth factor (VEGF), now termed VEGF-A, belongs to the platelet-derived growth factor family and is the most important known inducer of angiogenesis and vessel permeability [6,7]. Five additional forms VEGF, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and the placental growth factor (PIGF) have been characterised [8]. VEGF-C is a ligand at the VEGF receptor (VEGFR)-3, also designated Flt-4. This is a tyrosine kinase receptor that is expressed predominantly in the endothelium of lymphatic vessels [9]. Alitalo and colleagues [10] reported that VEGF-C is a

* Corresponding author. Tel.: +81-82-257-5193; fax: +81-82-257-5194.
E-mail address: ykitadai@hiroshima-u.ac.jp (Y. Kitadai).

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lymphoangiogenic factor that can selectively induce hyperplasia of the lymphatic vasculature.

In this study, we examined the expression of VEGF-C in submucosally invasive gastric carcinoma to determine whether expression is associated with lymph node metastases. We found that VEGF-C expression in gastric carcinoma tissues could serve as a useful predictor of lymph node metastases.

2. Materials and methods

2.1. Cell cultures

Six cell lines established from human gastric carcinomas were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (Nissui, Tokyo, Japan) with 10% fetal bovine serum (MA Bioproducts, Walkersville, MD, USA). The TMK-1 cell line was established in our laboratory from a poorly differentiated adenocarcinoma [11]. The other five gastric carcinoma cell lines (MKN-1, from an adenosquamous carcinoma; MKN-7, MKN-28 and MKN-74, from well differentiated adenocarcinomas; and MKN-45, from a poorly differentiated adenocarcinoma) were provided by Dr. T. Suzuki (Fukushima Medical College, Fukushima, Japan).

2.2. Patients and tumour specimens

Paired biopsy specimens of gastric carcinoma and normal gastric mucosa were obtained from 12 patients, snap-frozen in liquid nitrogen, and stored at −80 °C until RNA extraction for reverse transcription-polymerase chain reaction (RT-PCR) analysis. Informed consent was obtained from all of the patients. Paraffin-embedded archival specimens from 139 consecutive patients with submucosally invasive gastric carcinoma who underwent surgical resection at Hiroshima University Hospital were studied using immunohistochemistry (IHC). Pathology reports and clinical histories at the time of surgery were reviewed to determine the tumour stage. Definitions of stages and criteria for histological classification followed those proposed by the Japanese Research Society for gastric cancer [12]. Histological grade, tumour stage and depth of invasion in these cases are summarised in Table 1.

Table 1
Relationship between clinicopathological findings and VEGF-C expression

Clinicopathological findings	No. of cases	VEGF-C-positive cases (%)	*P* value
Macroscopic type			
I	9	2 (22)	NS
II	10	6 (60)	
IIa + IIc	28	8 (29)	
IIc	92	26 (28)	
Histological grade			
pap, tub1, tub2	114	38 (33)	NS
sig, por	25	4 (16)	
Depth of invasion			
sm1	32	2 (6)	$P<0.05$
sm2	50	17 (34)	
sm3	57	23 (40)	
Lymphatic invasion			
−	93	20 (22)	$P<0.05$
+	46	22 (48)	
Venous invasion			
−	123	27 (30)	NS
+	16	5 (31)	
Lymph node metastases			
−	117	27 (23)	$P<0.05$
+	22	15 (68)	
Infiltrating growth patterns			
α	46	10 (22)	NS
β	65	22 (34)	
γ	28	10 (36)	

I, protruded type; IIa, superficial elevated type; IIc, superficial depressed type; pap, papillary adenocarcinoma; tub1, well-differentiated adenocarcinoma; tub2, moderately differentiated adenocarcinoma; sig, signet-ring cell carcinoma; por, poorly differentiated adenocarcinoma; NS, non significant; VEGF, vascular endothelial growth factor.

2.3. RT-PCR

Total RNA was extracted from human gastric carcinoma cell lines using RNAzol B (Cinna/Biotex, Houston, TX, USA) according to the manufacturer's instructions. Two pairs of oligomers were synthesised based on the reported sequences for human *VEGF-C* (5′-AGTT TTGCCAATTCACACTTCCTG-3′ and 5′-GTCATTG GCAGAAAACCAGTCTT-3′) and *VEGFR-3* (5′-AG CCATTCATCAACAAGCCT-3′ and 5′-GGCAACA GCTGGATGTC-ATA-3′) [13, 14]. RT-PCR was performed using the extracted RNA and the oligomers as templates and primers, respectively [15]. The cDNA was amplified by 30 PCR cycles of denaturation for 2 min at 94 °C; annealing for 2 min at 55 °C; and extension for 3 min at 72 °C. After the reaction, the mixtures were loaded onto a 5% non-denaturing polyacrylamide gel in Tris–borate-ethylene diamine tetra acetic acid (EDTA) buffer. RT-PCR reaction without reverse transcriptase showed no specific bands.

2.4. Northern analysis

Polyadenylated mRNA was extracted from gastric carcinoma cell lines using a FastTrack mRNA isolation kit (Invitrogen, San Diego, CA, USA). This mRNA was electrophoresed on a 1% denaturing formaldehyde/agarose gel, electrotransferred at 0.6 A to a GeneScreen nylon membrane (Dupont, Boston, MA, USA), and cross-linked with ultraviolet (UV) light at 120 000 mJ/cm^2 using a UV Stratalinker 1800 (Stratagene, La Jolla, CA, USA). Hybridised membranes were washed

at 65 °C with 30 mM NaCl, 3 mM sodium cirate (pH 7.2), and 0.1% sodium dodecyl sulphate (SDS, w/v).

The probe for the *VEGF-C* transcripts in these analyses was a 1.2-kb cDNA kindly provided by Dr. K. Alitalo (University of Helsinki, Finland). The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA probe was purchased from Clontech (Palo Alto, CA, USA). Each cDNA fragment was purified by agarose gel electrophoresis, recovered using GeneClean (BIO 101, La Jolla, CA, USA), and radiolabelled using a random-primer technique with [^{32}P]-labelled deoxyribonucleotide triphosphates [16].

2.5. In situ *mRNA hybridisation*

Formalin-fixed, paraffin-embedded sections from the biopsy samples were prepared for *in situ* hybridisation as described in Ref. [17]. *In situ* mRNA hybridisation was carried out by using the microprobe manual staining system (Fisher Scientific, Pittsburgh, PA, USA). A specific antisense oligonucleotide DNA probe was designed with a sequence complementary to that of the *VEGF-C* mRNA transcript based on a reported cDNA sequence [13]. The probe sequence was 5′-GCTCGT GCTGGTGTTCATGCACTGGAGC-3′. A poly d(T)$_{20}$ oligonucleotide was used to verify the integrity and lack of degradation of mRNA in each sample.

2.6. *Immunohistochemical staining*

Consecutive 4-μm sections were cut from each tissue block. Following trypsinisation, sections were immunostained for VEGF-C, by an immunoperoxidase technique. Primary antibodies used were a goat polyclonal anti-VEGF-C antibody (N-19, Santa Cruz Biotechnology, CA, USA) at a 1:100 dilution and a mouse monoclonal antibody (Nichirei, Tokyo, Japan) against CD34. In negative control sections, non-specific IgG was substituted for the primary antibody. IHC was carried out using the catalyzed signal amplification (CSA) system (Dako, Glostrup, Denmark). Staining of cells was evaluated by two independent observers blinded to the patient's status. Positivity was defined as the presence of VEGF-C immunoreactivity in at least 30% of the cancer cells.

2.7. *Vessel counting*

Vessel counting was carried out by light microscopy of anti-CD34-immunostained sections in areas at the invasive edge of the tumour containing the highest numbers of capillaries and small venules. These highly vascular areas were identified by scanning tumour sections at low magnifications (×40 and ×100). After the six areas with greatest neovascularisation were identified, vessels were counted in a ×200 field (×20 objective and ×10 ocular), and the mean count for the six fields was calculated. As in the study of Weidner and colleagues [18], identification of a lumen was not required for a structure to be defined as a vessel. The vessels were counted by two investigators who had no knowledge of the VEGF-C expression in the tumours.

2.8. *Statistical analysis*

Statistical significance was evaluated using the Chi-squared test and the Mann–Whitney *U* test. The significance level was set at a 5% value of *P* for each analysis.

3. Results

3.1. *Expression of* VEGF-C *and* VEGFR-3 *in gastric carcinoma cell lines*

Of the six cell lines, two constitutively expressed *VEGF-C* mRNA, of the latter, TMK-1 cells expressed *VEGF-C* mRNA at a high level. The results of the RT-PCR analysis (Fig. 1a) agreed well with those of

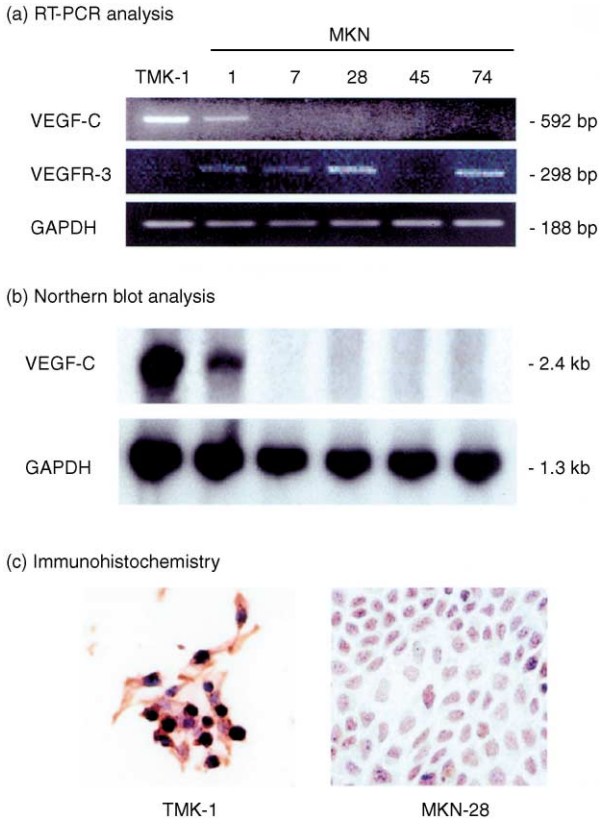

Fig. 1. Expression of *VEGF-C* and *VEGFR-3* mRNA in gastric carcinoma cell lines. *VEGF-C* mRNA expression was analysed by RT-PCR (a) and northern analysis (b). *GAPDH* was used as an internal control. With IHC for VEGF-C protein in the gastric carcinoma cell lines (c), VEGF-C immunoreactivity is evident within the cytoplasm of TMK-1 cells, but not MKN-28 cells.

Northern analysis (Fig. 1b). Expression of VEGF-C was confirmed at the protein level by IHC, VEGF-C protein was demonstrated in the cytosol of the TMK-1 cells (*VEGF-C* mRNA-positive), but not in MKN-28 cells (*VEGF-C* mRNA-negative; Fig. 1c). In four out of the six cell lines, *VEGFR-3* mRNA could be detected by RT-PCR (Fig. 1a). The findings included co-expression of VEGF-C and its receptor by MKN-1 cells.

3.2. *Expression of* VEGF-C *and* VEGFR-3 *mRNA in the tumour tissues*

Twelve gastric carcinoma tissue samples paired with samples of normal mucosa were examined to verify *VEGF-C* and *VEGFR-3* mRNA expression. RT-PCR was performed using primers for *VEGF-C* and *VEGFR-3*, with primers for *GAPDH* as an internal control. Fig. 2 shows a representative gel indicating *VEGF-C* and *VEGFR-3* mRNA expression. In three of the 12 tumour biopsy specimens (25%), *VEGF-C* mRNA was detected by RT-PCR. The signals indicating *VEGF-C* expression were detected in the cytoplasm of the tumour cells by *in situ* hybridisation (Fig. 3d). Expression of *VEGF-C* mRNA in normal mucosa was not detectable. In contrast, *VEGFR-3* mRNA expression was detected in all gastric carcinomas and normal mucosa tissues, although *VEGFR-3* mRNA showed more abundant expression in the tumour tissues than in the normal tissues.

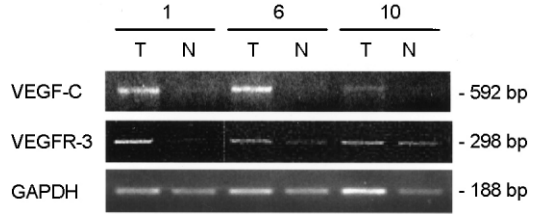

Fig. 2. Expression of *VEGF-C* and *VEGFR-3* mRNA in biopsy specimens. Expression of mRNA for *VEGF-C* and *VEGFR-3* was analysed by RT-PCR. T, tumour tissue; N, corresponding normal mucosa. *GAPDH* was used as an internal control. *VEGF-C* and *VEGFR-3* mRNA expression was greater in the tumour tissues than in the normal tissues.

3.3. *Expression of VEGF-C protein in the tumour tissues*

Expression of VEGF-C protein in gastric carcinoma invading the submucosa was examined by IHC. Histological diagnoses of immunoreactivity for VEGF-C in tumour cells in these cases are summarised in Table 1. Moderate to strong immunoreactivity for VEGF-C was seen in the cytoplasm of the carcinoma cells in the resection specimens (Fig. 3b). VEGF-C immunoreactivity at the site of deepest penetration of the invasive tumour was more intense than in the superficial

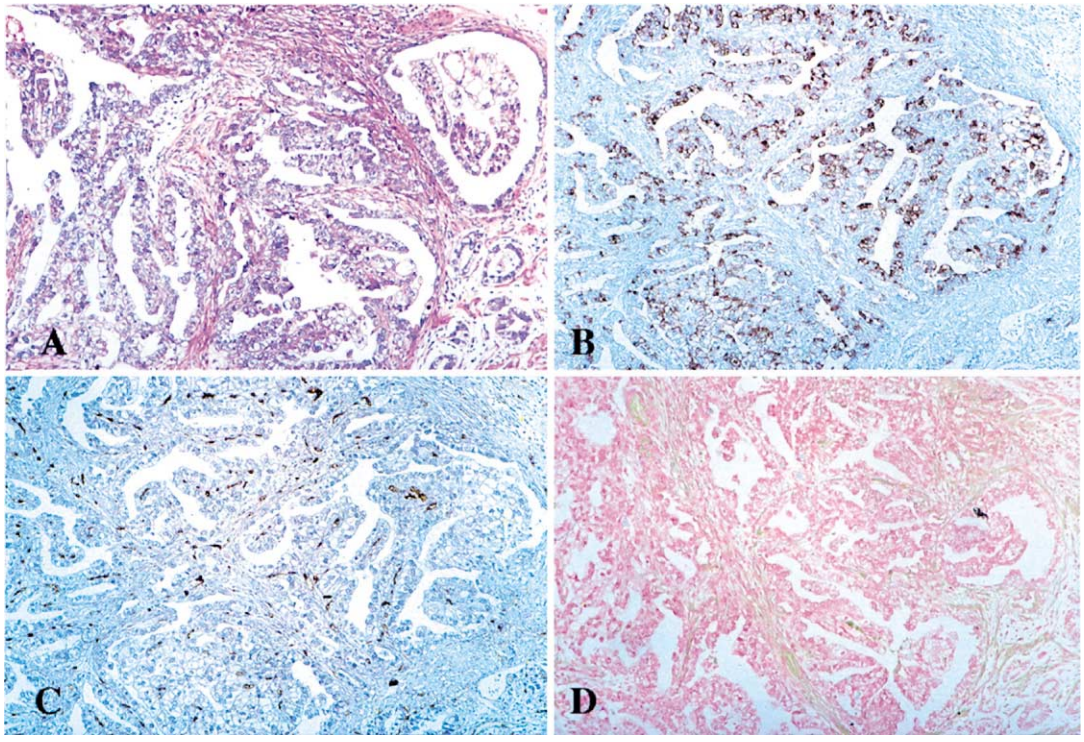

Fig. 3. Immunohistochemical staining for VEGF-C (b) and CD34 (c) in human gastric carcinoma (case No. 6). (a) Haematoxylin and eosin (H&E) stain. Intense VEGF-C immunoreactivity is present in the cytoplasm of the tumour cells (b). CD34 staining in the same VEGF-C-positive carcinoma is shown in (c). Expression of VEGF-C by tumour cells was confirmed at the mRNA level by *in situ* hybridisation (d). VEGF-C mRNA was detected in the cytoplasm of the tumor cells.

Table 2
Univariate and multivariate analysis of factors related to lymph node metastases

Factors	Univariate analysis		Multivariate analysis	
	P value	Odds ratio (95% CI)	*P* value	Odds ratio (95% CI)
Macroscopic type (IIc, IIa + IIc versus I, IIa)	NS		NS	
Depth of sm invasion (sm2, 3 versus sm1)	NS		NS	
Histological grade (por, sig versus tub1, tub2, pap)	NS		NS	
Lymphatic invasion (+ versus −)	0.0004	3.48 (2.22–15.95)	0.0253	3.48 (1.17–10.40)
Venous invasion (+ versus −)	NS		NS	
INF (γ versus α, β)	0.0442	2.77 (1.03–7.48)	NS	
VEGF-C (+ versus −)	0.0002	4.18 (2.42–17.51)	0.0116	4.18 (1.38–12.70)

INF, infiltrating growth pattern; CI, confidence interval; NS, non significant.

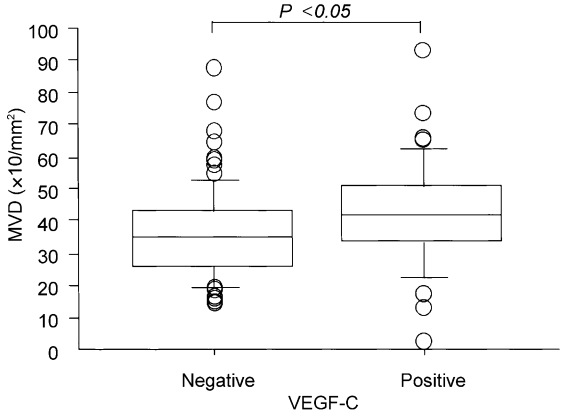

Fig. 4. Relationship between small-vessel counts obtained by staining for CD34 and VEGF-C immunoreactivity in tumour cells.

portions. Of the 139 gastric carcinomas, 44 (32%) showed intense VEGF-C immunoreactivity in the cytoplasm of numerous cancer cells. Normal gastric mucosa showed no cytoplasmic staining for VEGF-C (data not shown).

3.4. Correlation between VEGF-C expression and clinicopathological features

We next sought to identify associations between VEGF-C expression, vessel count and clinicopathological features. As shown in Table 1, immunohistochemical VEGF-C positivity in cancer cells was significantly related to depth of invasion, lymphatic invasion, and lymph node metastases. No relationship was seen between VEGF-C immunoreactivity and histological grade. Vessels were counted in tumours aided by IHC staining using an anti-CD34 antibody, a representative section is shown in Fig. 3c. Although the range of microvessel density (MVD) showed substantial overlapping between VEGF-C-positive and -negative tumours, it was significantly higher in VEGF-C-positive tumours than in VEGF-C-negative tumours (Fig. 4).

3.5. Univariate and multivariate analyses of factors related to lymph node metastases

In the univariate analysis, the infiltrating growth pattern (INF), lymphatic invasion and VEGF-C-positivity correlated significantly with lymph node metastases. In the multivariate analysis (Table 2), the significant risk factors were lymphatic invasion ($P = 0.0253$) and VEGF-C-positivity ($P = 0.0116$).

4. Discussion

Ideally, patient selection for curative EMR of gastric carcinoma should be based on the exclusion of lymph node metastases and distant metastases [19]. In practice, however, the absence or presence of lymph node metastases is often difficult to determine in patients undergoing EMR. Unfortunately, routine histopathological examination of primary gastric carcinoma specimens cannot always identify the patients at the highest risk. New ways to accurately determine when to anticipate the presence of lymph node metastases are required.

The VEGF family is a group of growth factors that regulate the proliferation of endothelial cells. VEGF-A is known to play an important role in tumour angiogenesis [20]. Four additional members of the VEGF family, VEGF-B, VEGF-C, VEGF-D, and PIGF, have recently been discovered [13,21–23]. VEGF-C and VEGF-D are ligands for VEGFR-3, which is expressed on the endothelial cells of lymphatic vessels [13]. The expression of VEGF-C is associated with the development of lymphatic vessels. A significant correlation between lymph node metastasis and VEGF-C expression has been reported for several tumours [24–27]. To date, relatively little has been reported about VEGF-C expression in gastric carcinoma [14,28,29]. Yonemura and colleagues previously examined the expression of VEGF-C in human gastric carcinomas, finding that VEGF-C expression was correlated to VEGFR-3 expression and that patients with high VEGF-C expression had

a significantly poorer prognosis than those with low VEGF-C expression [14,28]. Kabashima and colleagues recently reported that lymphatic invasion was significantly increased in VEGF-C-positive early gastric carcinoma [29].

Because no metastasis is seen in gastric carcinomas limited to the mucosal layer, nodal metastasis from the submucosally invading cancer appears to be the earliest metastatic phenomenon in gastric cancer. A small number of cancer cells in the submucosal layer presumably give rise to these lymph node metastases. Therefore, we focused on lymph node metastases in early, superficially invasive gastric cancers and excluded advanced cases.

In our study, expression of VEGF-C was examined in gastric carcinoma cell lines, as well as in clinical specimens. *VEGF-C* mRNA was expressed constitutively by two of the six cell lines. In surgical specimens of gastric carcinoma, VEGF-C was expressed mainly by the tumour cells. A heterogeneous staining pattern was observed in most cases. In 32 of the 44 cases of a VEGF-C-positive tumour, VEGF-C immunoreactivity at the deepest penetration site of the invasive tumours was more intense than in the superficial portion. Analyses using semi-quantitative RT-PCR showed expression of *VEGF-C* mRNA in tumour tissues to be higher than expression in normal mucosa from the same patient. More interestingly, we found that VEGF-C overexpression was significantly associated with lymphatic infiltration and lymph node metastases. Therefore, VEGF-C may be responsible for lymphatic dissemination in human gastric carcinoma.

VEGF-C is a ligand for KDR (VEGF receptor-2) and VEGFR-3 [13]. KDR is expressed on endothelial cells of both blood vessels and lymphatics. Cao and colleagues recently demonstrated that VEGF-C is a potent angiogenic factor *in vivo*, signalling through both KDR and VEGFR-3 [30]. Valtola and colleagues demonstrated that expression of VEGFR-3 is upregulated in the endothelium of blood vessels that participate in angiogenesis in breast cancer, and that VEGF-C secreted by the intraductal carcinoma cells acts predominantly as an angiogenic growth factor for blood vessels [31]. In agreement with their findings, we observed a positive correlation between VEGF-C expression in tumour cells and the vessel count in gastric tumours. However, in cases of colorectal carcinoma, Akagi and colleagues observed no significant difference in microvessels numbers between VEGF-C-positive and VEGF-C-negative tumours [25]. Additional study will be needed to clarify whether VEGF-C directly stimulates tumour angiogenesis in gastric carcinomas.

In a recent study [32], the combination of anti-PAL-E and CD31 immunostaining was useful for the identification of lymphatic endothelial cells (PAL-E-negative/CD31-positive), VEGFR-3 expression was observed mostly in the lymphatic endothelial cells. We also tried to measure lymphatic vessel numbers using VEGFR-3 IHC because it had been considered the only specific marker for lymphatic endothelial cells. However, we could not obtain specific immunoreactivity for VEGFR-3 in archival paraffin sections under our experimental conditions, so as yet no versatile specific marker for the identification of lymphatic endothelial cells is available (at least in our hands). When we examined *VEGFR-3* mRNA expression in human gastric carcinomas using RT-PCR, the expression was higher in the tumour tissues than in the corresponding normal mucosa. In addition, we found that several gastric carcinoma cell lines constitutively expressed *VEGFR-3* mRNA. Determining whether VEGF-C acts as an autocrine factor *in vivo* would be of great interest.

In conclusion, this study demonstrated a correlation between VEGF-C expression and lymph node metastases in submucosally invasive gastric carcinoma. These findings suggest that VEGF-C expression may be an important factor facilitating lymphatic invasion and metastases in early stages of gastric carcinoma. Further investigation with large patient groups is required to clarify the reliability of VEGF-C expression as an indicator for additional surgery after EMR for submucosally invasive gastric carcinoma.

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References

1. Oohara T, Johjima Y, Sadatsuki H, Kondo Y. Conservative surgery for early gastric cancer. *Gastroenterol Surg* 1985, **8**, 15–19.
2. Ohta H, Noguchi K, Takagi M, Nishi T, Kajitani Y, Kato Y. Early gastric carcinoma with special reference to macroscopic classification. *Cancer* 1987, **60**, 1099–1106.
3. Maehara Y, Orita H, Okuyama T, *et al*. Predictors of lymph node metastasis in early gastric cancer. *Br J Surg* 1992, **79**, 245–247.
4. Inoue K, Tobe T, Kan N, Nio Y, *et al*. Problems in the definition and treatment of early gastric cancer. *Br J Surg* 1991, **78**, 818–821.
5. Nishida T, Tanaka S, Haruma K, Yoshida M, Koji S, Kajiyama G. Histological grade and cellular proliferation at deepest invasive portion correlate with the high malignancy of submucosal invasive gastric cancer. *Oncology* 1995, **52**, 340–346.
6. Berse B, Brown LF, Van de Water L, Dvorak HF, Senger DR. Vascular permeability gene is expressed differentially in a normal tissue, macrophages, and tumors. *Mol Biol Cell* 1992, **3**, 211–220.

7. Ferrara N, Houck KA, Jakeman LB, Leung DW. Molecular and biological properties of the vascular endothelial growth factor family of proteins. *Endocr Rev* 1992, **13**, 18–32.
8. Veikkola T, Alitalo K. VEGFs, receptors and angiogenesis. *Semin Cancer Biol* 1999, **9**, 211–220.
9. Kaipainen A, Korhonen J, Mustonen T, van Hinsbergh VM, Fang G-H, Dumont DJ, Breiman M, Alitalo K. Expression of the fms-like tyrosine kinase FLT4 gene becomes restricted to endothelium of lymphatic vessels during development. *Proc Natl Acad Sci USA* 1995, **92**, 3566–3570.
10. Jeltsch M, Kaipainen A, Joukov V, *et al*. Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. *Science* 1997, **76**, 1423–1425.
11. Ochiai A, Yasui W, Tahara E. Growth-promoting effect of gastrin in human gastric carcinoma cell line TMK-1. *Jpn J Cancer Res* 1985, **76**, 1064–1071.
12. Japanese Research Society for Gastric Cancer. *Japanese Classification of Gastric Carcinoma*. Tokyo, Kanehara, 1999.
13. Joukov V, Pajusola K, Kaipainen A, *et al*. A novel vascular endothelial growth factor VEGF-C is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *EMBO J* 1996, **15**, 290–298.
14. Yonemura Y, Endo Y, Fujita H, *et al*. Role of vascular endothelial growth factor C expression in the development of lymph node metastasis in gastric cancer. *Clin Cancer Res* 1999, **5**, 1823–1829.
15. Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor, New York, Cold Spring Harbor Laboratory, 1989.
16. Feiberg AP, Vogelstein B. A technique for radiolabeling DNA restriction endonuclease fragment to high specific activity. *Anal Biochem* 1983, **132**, 6–13.
17. Kitadai Y, Bucana CD, Ellis LM, Anzai H, Tahara E, Fidler IJ. In situ mRNA hybridization technique for analysis of metatasis related genes in human colon carcinoma cells. *Am J Pathol* 1995, **147**, 1238–1247.
18. Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis: correlates with metastasis in invasive brest carcinoma. *N Engl J Med* 1991, **324**, 1–8.
19. Oya M, Yao T, Nagai E, Tsuneyoshi M. Metastasizing intramucosal gastric carcinomas. *Cancer (Phillia)* 1995, **75**, 926–935.
20. Shweiki D, Neeman M, Itin A, Keshet E. Induction of vascular endothelial growth factor expression by hypoxia and by glucose deficienty in multicell spheroids: implications for tumor angiogenesis. *Proc Natl Acad Sci USA* 1995, **92**, 768–772.
21. Olofsson B, Pajusola K, Kaipainen A, *et al*. Vascular endothelial growth factor B, a novel growth factor for endothelial cells. *Proc Natl Acad Sci USA* 1996, **93**, 2576–2581.
22. Yamada Y, Nezu J, Shimae M, Hirata Y. Molecular cloning of a novel vascular endothelial growth factor, VEGF-D. *Genomics* 1997, **42**, 483–488.
23. Achen MG, Jeltsch M, Kukk E, *et al*. Vascular endothelial growth factor D (VEGF-D) is a ligand for tyrosine kinases VEGF receptor 2 (flk1) and VEGF receptor 3 (flt4). *Proc Natl Acad Sci USA* 1988, **95**, 548–553.
24. Tsurusaki T, Kanda S, Sasaki H, *et al*. Vascular endothelial growth factor-C expression in human prostatic carcinoma and its relationship to lymph node meatsatsis. *Br J Cancer* 1999, **80**, 309–313.
25. Akagi K, Ikeda Y, Miyazaki M, *et al*. Vascular endothelial growth factor-C expression in human colorectal cancer tissues. *Br J Cancer* 2000, **83**, 887–891.
26. Ohta Y, Shridhar V, Bright RK, *et al*. VEGF and VEGF type C play an important role in angiogenesis and lymphangiogenesis in human malignant mesothelioma tumors. *Br J Cancer* 1999, **81**, 54–61.
27. Kurebayashi J, Otsuki T, Kunisue H, *et al*. Expression of vascular endothelial growth factor family members in breast cancer. *Jpn J Cancer Res* 1999, **90**, 977–981.
28. Yonemura Y, Fushida S, Bando E, *et al*. Lymphangiogenesis and the vascular endothelial growth factor receptor (VEGFR)-3 in gastric cancer. *Eur J Cancer* 2001, **37**, 918–923.
29. Kabashima A, Maehara Y, Kakeji Y, Sugimachi K. Overexpression of vascular endothelial growth factor C is related to lymphogenous metastasis in early gastric carcinoma. *Oncology* 2001, **60**, 146–150.
30. Cao Y, Linden P, Farnebo J, *et al*. Vascular endothelial growth factor C induces angiogenesis *in vivo*. *Proc Natl Acad Sci USA* 1998, **95**, 14389–14394.
31. Valtola R, Salven P, Heikkila P, *et al*. VEGFR-3 and its ligand VEGF-C are associated with angiogenesis in breast cancer. *Am J Pathol* 1999, **154**, 1381–1390.
32. Jussila L, Valitalo R, Partanen TA, *et al*. Lymphatic endothelium and Kaposi's sarcoma spindle cells detected by antibodies against the vascular endothelial growth factor receptor-3. *Cancer Res* 1998, **58**, 1599–1604.