Expression of VEGF-C and VEGF-D mRNA Levels Using Real-Time Quantitative RT-PCR in Lymph Node Metastasis of Human Gastric Carcinomas

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ABSTRACT

This study was undertaken to determine whether expressions of the vascular endothelial growth factor C (VEGF-C) and D (VEGF-D) correlate with clinicopathological parameters, with particular reference to lymph node metastasis in gastric carcinoma. Based on the data, as a preliminary study, we investigated whether VEGF-C expression could be a predictor of lymph node metastasis. Twenty-two surgical specimens of gastric carcinomas with (n=14) or without (n=8) lymph node metastasis were studied. The mRNA levels of VEGF-C and VEGF-D were quantified using real-time quantitative (RTQ) RT-PCR, a new method of kinetic quantitative PCR. We then calculated the tumor:normal VEGF-C and VEGF-D mRNA level ratios for each specimen which were designated as the normalized mRNA value. The normalized mRNA values of VEGF-C in primary tumors correlated well with lymph node metastasis but that of VEGF-D did not. We then arbitrarily chose the cut-off point for the normalized mRNA values of VEGF-C based on the mRNA values of patients without lymph node metastasis (mean + SD); we examined the data to see if the values above the cut-off point would indicate the presence of lymph node metastasis. The sensitivity and specificity of the normalized mRNA values of VEGF-C for the diagnosis of lymph node metastasis were 85.2% and 75.0%, respectively. These results suggest that the VEGF-C may play an important role in lymph node metastasis in gastric carcinoma and that VEGF-C may be a useful diagnostic marker for lymph node metastasis.

Key words : VEGF-C, VEGF-D, Real-time PCR, Lymph node metastasis, Gastric carcinoma

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INTRODUCTION

Gastric carcinoma has been one of the leading causes of cancer deaths in Japan. Lymph node metastasis in gastric carcinoma has been recognized as one of the most important prognostic factors. Therefore, to elucidate the molecular biology of lymph node metastasis would be an invaluable aid in developing novel therapeutic strategies.

Patients with early gastric carcinoma without lymph node metastasis can be treated by minimally invasive treatments, such as endoscopic mucosal resection (EMR), and laparoscopic partial gastrectomy. In order to select the correct treatment method, it is necessary to determine precisely whether or not the primary tumor has lymph node metastasis. However, there are no satisfactory parameters for the preoperative assessment of lymph node metastasis.

VEGF-C and VEGF-D are novel members of the VEGF family that show some selectivity toward lymphatic endothelial cells. Experiments with transgenic mice have demonstrated that overexpression of VEGF-C in basal keratinocytes leads to the development of hyperplastic lymphatic vessels in the skin[1]. Furthermore, in the differentiated chick chorioallantoic membrane, purified VEGF-C induces the growth of lymphatic vessels but has very little effect on blood capillaries[2]. These studies strongly suggest a role for VEGF-C in lymphangiogenesis and lymphatic maintenance. VEGF-C is a ligand for VEGFR-2 and VEGFR-3. In adult tissues, VEGFR-2 localizes predominantly in blood vascular endothelial cells, whereas VEGFR-3 is expressed mainly in lymphatic endothelia[3]. In analyses using surgical specimens, expression of VEGF-C has been shown to correlate with the rate of metastasis to lymph nodes in breast[4], colorectal[5], gastric[6], thyroid[7,8], lung[9], and prostate[10] carcinomas. VEGF-D was isolated as a fos-inducible factor from mouse skin fibroblast and shows homology to VEGF-C through database searches for sequences[11]. VEGF-D is a ligand for VEGFR-2 and VEGFR-3 as well as VEGF-C[12]. Because of their similarity in sequences and common receptors, VEGF-C and VEGF-D are thought to have similar biological functions. Recent evidence indicates that VEGF-D stimulates both angiogenesis and lymphangiogenesis in experimental tumors and, furthermore, that VEGF-D expression is required for the growth and establishment of lymphatic vessels within tumors 13). On the basis of these data, it has therefore been suggested that VEGF-D expression promotes metastatic spread of tumors via the lymphatic route[13]. Interestingly, VEGF-D has been reported to be up-regulated in malignant melanoma as well as in melanoma cell lines[14] and in inflammatory breast carcinoma[4] although VEGF-D transcripts are down-regulated in adenocarcinoma of the lung with lymph node metastasis[15]. Therefore, it is necessary to investigate the correlation between VEGF-D expression and lymph node metastasis in other carcinomas.

In this study, we have examined the expressions of VEGF-C and VEGF-D in relation to lymph node metastasis and the other clinicopathological factors in gastric carcinoma.

MATERIALS AND METHODS

Patients and tumor samples

Tissue samples were obtained from 22 primary gastric carcinomas that were resected at the First Department of Surgery, Sapporo Medical University Hospital in 2000 and 2001. Informed consent was obtained from patients prior to their participation in this study. Normal mucosa was obtained from the non-tumorous portion of the gastrectomy specimen that was distant from the primary tumor. Tissues were quickly frozen in liquid nitrogen and stored at −80℃ until RNA extraction.

All of the resected primary tumors were histologically examined by H&E staining according to the TNM classification. Clinicopathological characteristics of the patients are summarized in Table 1.

RNA extraction and cDNA synthesis

Total RNA was isolated using TRIzol (Life

Table 1 Patient Characteristics

		n(−)	n(+)	p value
Age (years)		66±2.8	66±2.3	0.90
Gender	Men	6	11	0.77
	Women	2	3	
Tumor size (mm)		71±6.8	63±6.7	0.90
Histopathological grading	G1, G2	5	5	0.22
	G3, G4	3	9	
Depth of invasion	T1, T2	4	4	0.29
	T3, T4	4	10	
Lymphatic invasion	negative	3	1	0.11
	positive	5	13	
Venous invasion	negative	6	1	0.0023
	positive	2	13	
Liver metastasis	negative	8	12	0.39
	positive	0	2	

(mean±SE)

Technologies, Inc., Grand Island, NY, USA) according to the standard acid-guanidium-phenol-chloroform method, followed by DNase treatment (Wako, Tokyo, Japan) to avoid contaminating genomic DNA. The purity and concentration of the RNA were determined by spectrophotometry at 260nm. cDNA was generated with the SUPERSCRIPT First-Strand Synthesis System (GIBCO BRL) according to the protocol recommended by the manufacturer. Briefly, the prepared RNA (10μg) was preincubated with 50 ng of random hexanucleotide primer in 12μl of solution for 10 min at 70℃. After being chilled on ice, 2μl of 10-fold RT buffer [200mM Tris-HCl (pH 8.4), 500mM KCl], 2μl of 25mM $MgCl_2$, 1μl of each dNTP at 10mM, 2μl of 0.1M DTT, and 1 μl of SuperScript II reverse transcriptase (50 units/μl; GIBCO BRL) were added. The RT reaction was performed at 42℃ for 50 min, followed by heating at 70℃ for 15 min.

Real-time RT-PCR with LightCycler

Quantitative PCR was performed in a total reaction volume of 20μl per capillary for the LightCycler format. The reaction mixture contained Taq DNA polymerase, the dNTP mixture, and a buffer (LightCycler DNA Master hybridization probes, Roche); 3.0mM $MgCl_2$ for VEGF-C and GAPDH, or 4.0mM $MgCl_2$ for VEGF-D; each primer at 0.25mM, and 5μl of template cDNA. For cDNA amplification, 95℃ (5 min) for primer elongation was followed by 40 cycles of amplification at 95℃ (0 sec) for denaturation, 64℃ (10 sec) for VEGF-C, 67℃ (10 sec) for VEGF-D for annealing, and 72℃ (8 sec) for extension, with a temperature slope of 20℃/sec performed in the LightCycler. Real-time PCR monitoring was achieved by measuring the fluorescent signal at the end of the annealing phase for each cycle. For GAPDH amplification, the same profile was used except for the annealing step, which was at 55℃ (8 sec). The cDNA fragments corresponding to VEGF-C, VEGF-D and GAPDH were amplified using the following sets of primers:

VEGF-C
Sense
5'-TGCCGATGCATGTCTAACT-3'
Anti-sense
5'-TGAACAGGTCTCTTCATCCAGC-3'
VEGF-D
Sense
5'-GTATGGACTCTCGCTCAGCAT-3'
Anti-sense
5'-AGGCTCTCTTCATTGCAACAG-3'
GAPDH
Sense
5'-GAAGGTGAAGGTCGGAGT-3'
Anti-sense
5'-GAAGATGGTGATGGGATTTC-3'

By running serial dilutions of a reference cDNA sample, a standard curve was generated in each experiment. Quantification of cDNA in each sample was then performed automatically by reference to the standard curve constructed each time according to the LightCycler software. The reference cDNA samples used to generate standard curves were obtained from a breast cancer cell line, ZR. The results were normalized for GAPDH, the abundance of which was also determined by LightCycler software. We then calculated the tumor:normal VEGF-C and VEGF-D mRNA level ratios individually, which were designated as the normalized mRNA value. Negative controls lacking tem-

plate cDNA were always included in each experiment.

Statistical analysis

VEGF-C and VEGF-D expression levels were abnormally distributed, and therefore nonparametric tests were used. The Mann-Whitney U-test was used to evaluate the relationship between the clinicopathological characteristics and the mRNA levels of VEGF C and D. The results were considered to be statistically significant at $p<0.05$.

RESULTS

VEGF-C mRNA expression in gastric carcinoma

VEGF-C was expressed in all specimens, regardless of whether they were derived from tumors or normal tissues. The VEGF-C mRNA levels normalized for GAPDH in normal tissues and tumor ranged from 0.065 to 4.45 and from 0.18 to 12.78, respectively. The normalized mRNA value of VEGF-C ranged from 0.12 to 5.92. The normalized mRNA was significantly higher in the patients with lymph node metastasis than in those without it ($p = 0.0078$) (Figure 1a). The normalized mRNA value was higher in the patients with lymph invasion than in those without it, but this difference was not significant ($p = 0.20$) (Figure 1b). 18 patients had lymphatic invasion and 4 did not. We thought that comparison between these two groups might be statistically inadequate because the group without lymphatic invasion was small.

VEGF-D mRNA expression in gastric cancer

VEGF-D was expressed in all specimens as well as VEGF-C. The VEGF-D mRNA levels normalized for GADH of normal tissues and tu-

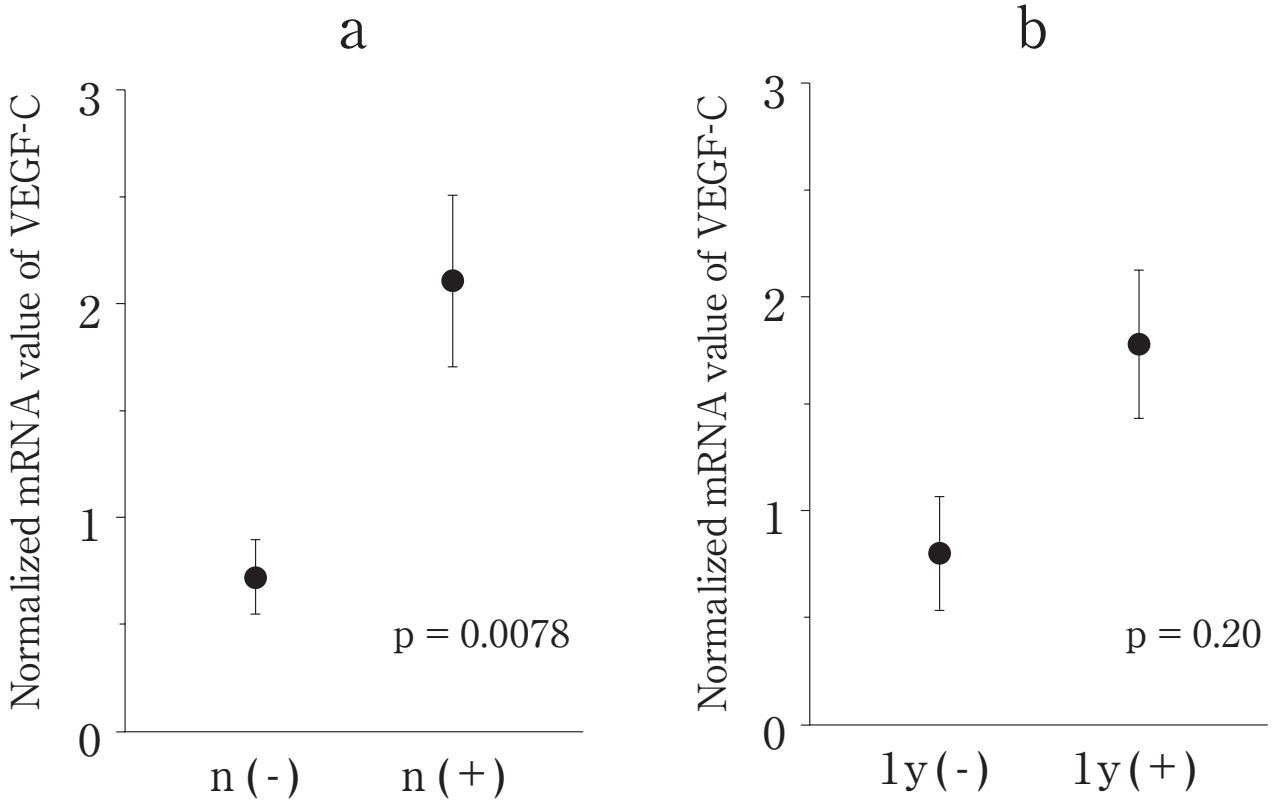

Fig. 1 The normalized mRNA value of VEGF-C determined by RTQ RT-PCR. (a) The normalized mRNA value of VEGF-C was significantly higher in tumors with lymph node metastasis than in those without it ($p = 0.0078$). n(−):,tumors without lymph node metastasis (n=8); n(+), tumors with lymph node metastasis (n=14). (b) No significant difference was observed between two groups ($p = 0.20$). ly(−),tumors without lymphatic invasion (n=4); ly(+),tumors with lymphatic invasion (n=18). Bars represent the mean ± SE.

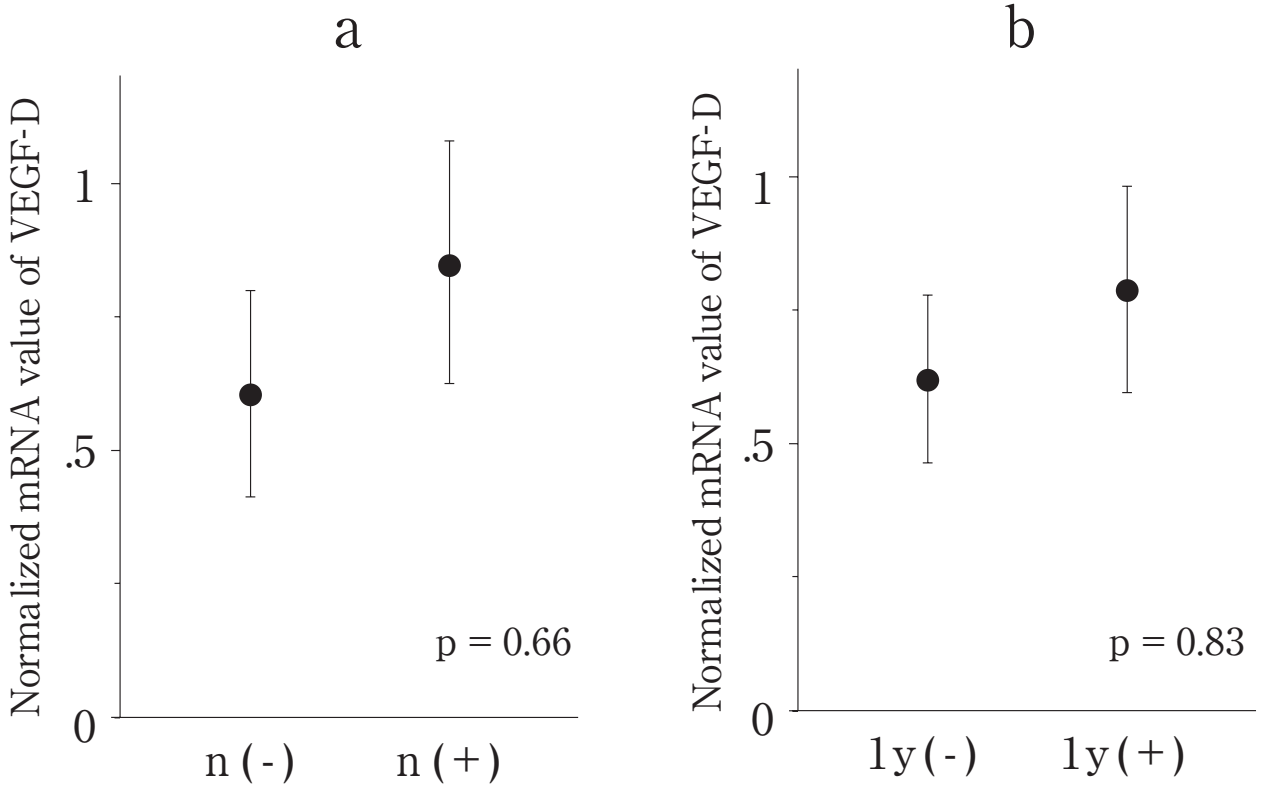

Fig. 2 The normalized mRNA value of VEGF-C determined by RTQ RT-PCR. (a), (b) No significant difference was observed between two groups (p = 0.66). n(−):,tumors without lymph node metastasis; n(+), tumors with lymph node metastasis; ly(−),tumors without lymphatic invasion; ly(+),tumors with lymphatic invasion. Bars represent the mean ± SE.

mors ranged from 0.68 to 841.9 and from 1.7 to 1241.4, respectively. This normalized mRNA value of VEGF-D ranged from 0.016 to 2.69. The normalized mRNA values were not different between the patients with lymph node metastasis and those without (p = 0.66) (Figure 2a). No difference was detected between the lymphatic invasion-positive group and negative group (0.62 ± 0.16) (p = 0.66) (Figure 2b).

Table 2 Correlation between value of VEGF-C and VEGF-D mRNA levels and the other clinocopathological factors

		VEGF-C	VEGF-D
Depth of invasion	T1, T2	0.88±0.21	0.63±0.24
	T3, T4	2.02±0.42	0.84±0.21
	p value	p=0.10	p=0.52
Histological grade	G1, G2	1.31±0.39	0.41±0.09
	G3, G4	1.85±0.44	1.05±0.26
	p value	p=0.51	p=0.081
Venous invasion	negative	0.88±0.22	0.75±0.20
	Positive	1.94±0.40	0.77±0.22
	p value	p=0.17	p=0.62

(mean±SE)

Relationships between VEGF-C and D mRNA levels and the other clinicopathological factors

The correlation of the normalized mRNA values of VEGF-C and VEGF-D with other clinicopathological parameters, such as depth of invasion, histological type and venous invasion, was also studied (Table 2). The deeper the tumor invasion, the higher the normalized mRNA values of VEGF-C tended to be; the lower the tumor differentiation, the higher the normalized mRNA values of VEGF-D tended to be. However, these differences were not statistically significant.

Prediction of lymph node metastasis by the analysis of VEGF-C mRNA level

To determine the logical cut-off point, two

cut-off points of the normalized mRNA values of VEGF-C based on those of patients without lymph node metastasis (mean + SD and mean + 2SD) were statistically searched. The normalized mRNA values above the cut-off point indicate that lymph node metastasis is likely. When the cut off point was mean + SD (1.21), the sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy were 85.2%, 75.0%, 85.2%, 75.0% and 81.8%, respectively. When mean + 2SD (1.71) was used as the cut-off point, those diagnostic indexes were 85.7%, 100%, 100%, 47.1% and 59.1% (Table 3).

Table 3 Survey of diagnostic indexes of real time PCR for diagnosis of lymph node metastasis

Diagnostic indexes	cut-off point	
	mean + SD	mean + 2SD
sensitivity	92.9% (13/14)	35.7% (5/14)
specificity	75.0% (6/8)	100% (8/8)
positive predictive value	86.6% (13/15)	100% (5/5)
negative predictive value	85.7% (6/7)	47.1% (8/17)
diagnostic accuracy	86.4% (19/22)	59.1% (13/22)

DISCUSSION

VEGF-C and VEGF-D have been reported in several human carcinomas, such as colorectal[5], gastric[6,16,17], and prostatic[10] carcinomas. However, in most studies, scientists have investigated protein expression using immunohistochemical staining, and only a few have examined expression at the transcriptional level. The clinical application of the conventional RT-PCR technique in which amplified products are analyzed by electrophoresis is limited. Since this technique is essentially qualitative in nature, exact comparison of accurate as well as relative expression levels of mRNA is not feasible. To overcome these problems in the current study, we used the RTQ RT-PCR technique to assess and quantify the expression of VEGF-C and VEGF-D simultaneously in gastric cancer tissue. RTQ RT-PCR was established as a rapid and sensitive technique for the precise quantification of mRNA in tissues and cells[18,19,20].

In this study, VEGF-C mRNA expression was up-regulated in primary tumors with lymph node metastasis compared to those without. This result was consistent with the previous reports[6,16,17] and suggests that VEGF-C may play an important role in lymphatic metastasis in gastric carcinoma. We also investigated whether VEGF-C could be a useful diagnostic marker for the diagnosis of lymph node metastasis in gastric carcinomas. We arbitrarily chose two cut-off points which were mean + SD and mean + 2SD of the normalized mRNA values of VEGF-C in the patients without lymph node metastasis. The mean + 2SD may not be an adequate cut-off point because the sensitivity would then be too low. In the case of mean + SD, the sensitivity and specificity are more acceptable. However, this is preliminary research and it is too early to conclude with certainty that VEGF-C could be a satisfying diagnostic parameter of lymph node metastasis because the number of samples was too small. To confirm the usefulness of VEGF-C as a diagnostic marker for lymph node metastasis, further studies investigating a greater number of patients are required.

Relationships between VEGF-D expression and lymph node metastasis have been reported in malignant melanoma[14], breast carcinoma[4], lung carcinoma[15]. However, the results are not consistent. In our study, VEGF-D expression did not correlate with any of the clinicopathological factors and the role of VEGF-D in progression of gastric carcinoma remains uncertain.

It is often a challenge for the surgeon to decide whether or not minimally invasive treatment such as EMR or laparoscopic surgery is indicated for early gastric carcinoma. It would be of great use to have a measurable marker that indicates the presence or absence of lymph node metastasis. Because most of the patients in the present study had advanced gastric carcinoma, to extrapolate that a similar result would obtain in patients with early gastric carcinoma may be inaccurate. Further studies will be required in order to investigate the role of VEGF-

C and other molecules in the assessment of lymph node metastasis in early gastric carcinoma.

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