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Role of Vascular Endothelial Growth Factor-C and -D mRNA in Breast Cancer

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ABSTRACT

Vascular endothelial growth factor (VEGF)-C and VEGF-D belong to the VEGF family, and are thought to be involved in lymphangiogenesis and angiogenesis. At present, this is the only known system that can induce lymphatic vessel growth in the body. However, the roles of VEGF-C and VEGF-D in breast cancer tissue have not been clarified. In the present study, we measured the mRNA expression of VEGF-C and VEGF-D in the breast cancer tissue of 109 patients by real-time polymerase chain reaction (RT-PCR). Between non-infiltrating breast cancer (n=6) and infiltrating breast cancer (n=103), there were no significant differences in the mRNA expression of VEGF-C and VEGF-D. In infiltrating cancer, the expression of HER2 exhibited a positive correlation to VEGF-C and VEGF-D mRNA expression (p=0.027, p=0.048). However, mRNA expression of VEGF-C and VEGF-D did not exhibit any significant correlation to lymphatic vessel invasion or lymph node metastasis. Among patients without lymph node metastasis, the mRNA expression of VEGF-C and VEGF-D for patients with lymphatic vessel invasion was significantly higher than that for patients without lymphatic vessel invasion (p=0.001, p=0.050). The results suggest that, in breast cancer, VEGF-C and VEGF-D are involved in lymphatic vessel invasion prior to lymph node metastasis, and their expression decreases after lymph node metastasis occurs.

Key words: *Breast Cancer, VEGF-C, VEGF-D*

In many malignant tumors, the role of vascular endothelial growth factor (VEGF)-A, a member of the VEGF family, has been investigated, and VEGF-A is known to be involved in angiogenesis[4, 6]. VEGF-C and VEGF-D possess lymphangiogenic and angiogenic activities, and their receptors are VEGF receptor-2 and VEGF receptor-3. Because they bind more strongly to VEGF receptor-3., they are believed to possess stronger lymphangiogenic activities[1, 12, 16, 23]. In gastric cancer[3, 33], colon cancer[7], prostate cancer[28], breast cancer[25], and thyroid cancer[5], the intratumoral expression of VEGF-C has been reported to correlate to lymph node metastasis. In addition, in breast cancer, it has been reported that VEGF-C correlates to the lymphatic vessel invasion of breast cancer cells[19]. As the gene sequences of VEGF-C and VEGF-D[2, 29] are highly homologous, they apparently have similar activities, but in lung cancer, the balance between VEGF-C and VEGF-D is important[21]. Studies have documented the up-regulation of VEGF-C and the down-regulation of VEGF-D in head and neck squamous cell carcinoma and colon cancer[8, 13, 22].

Reports on VEGF-D are scarce, but Stacker et al reported that VEGF-D aids the spread of cancer cells in lymph ducts[27]. Some studies have found that VEGF-D correlates to lymph node metasta-

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sis in gastric cancer[11], colon cancer[24] and ovarian cancer[31]. Several studies have also documented how the expression of VEGF-D is down-regulated in malignant tumors[13, 21, 22]. Only Kurebayashi[17], Nakamura[20], Yang[30] and Koyama[15] have reported VEGF-D in breast cancer. These studies found that the expression of VEGF-C and VEGF-D was closely involved with lymph node metastasis and lymphatic vessel invasion. The significance of VEGF-C and VEGF-D expression in breast cancer has not been clarified. The present study measured the mRNA expression of VEGF-C and VEGF-D in breast cancer tissue by real-time polymerase chain reaction (RT-PCR) and investigated the relationship between VEGF-C/VEGF-D expression and various clinicopathological factors.

MATERIALS AND METHODS

The subjects were 109 patients who underwent resective surgery at the National Hospital Organization Kure Medical Center. Of the 109 patients, 60 underwent mastectomy and 49 underwent partial resection. In all cases, axillary lymph node dissection was performed. With each excised tissue, a piece of the tumor was cut out, frozen in liquid nitrogen and stored at -80°C. The patients ranged in age from 29 to 86 years, with an average of 55.8 years. Tumor diameters ranged from 0.7 to 15 cm, with an average of 2.7±2.0 cm. Pathological tests were conducted to determine axillary lymph node metastasis. Clinical staging and TMN classification followed the UICC system.

RNA extraction and real-time monitoring PCR

Using the RNeasy kit (Qiagen, Valencia, CA), the total RNA of each sample was extracted according to the manual, and the purity of total RNA was measured by spectrophotometry at 260/280 nm. Total RNA (1 μg) in each sample was then used to prepare cDNA by reverse transcription using the following primers:
VEGF-C(5'-GTCGCGACAAACACCTTCTTTAAACC -3' and 5'-GGCATCTGCAGATGTGATTATTCC-3'), VEGF-D (5'-GACTCTCGCTCAGCATCCATCGG-3' and 5'-CCACGCACGTTTCTCTAGGGCTGC-3'), and GAPDH (5'-CGACAGTCAGCCGCATCTT-3' and 5'-GCTCAGACACCATGGGGAAG-3').

Each PCR product (1 μl) was subjected to ligation, placed in LB medium and incubated at 37°C for 18 hr. With the resulting colonies, the Plasmid Mini Purification Kit (QIAGEN) was used to prepare plasmids containing the VEGF-C, VEGF-D and GAPDH genes as inserts. Standards with known copy numbers were prepared by successive dilutions to draw a calibration curve using the Smart Cycler® (Takara). Using this calibration curve, copy numbers of the VEGF-C and VEGF-D genes in the cDNA of each sample were determined. Relative to GAPDH (internal standard), the copy number of each gene was used to determine its mRNA expression.

Estrogen receptor (ER), progesterone receptor (PR) and HER2 were subjected to immunohistochemical staining. ER was immunohistochemically stained using anti-human ER α mouse monoclonal antibody (mAb) (DAKO, Glostrup, Denmark) and PR using anti-human PgR mAb (DAKO, Glostrup, Denmark). The expression of HER2 was assessed using the DAKO Hercep Test in four grades (0, 1+, 2+ and 3+), and 2+ and 3+ were considered significant. Because the present study was largely performed in 2001, genetic recombination did not undergo any review process by the hospital's ethics review board.

STATISTICAL ANALYSIS

The data was not recognized as normal distribution and therefore non-parametoric tests were used. The relationships between the expression of VEGF-C, VEGF-D and clinicopathological factors were evaluated by the Mann-Whitney U-test. Calculation was performed using the computer program Stat View (Abacus Concepts,Berkeley,CA). The results were considered significant if $p < 0.05$.

RESULTS

1) Expression of VEGF-C and VEGF-D in non-infiltrating and infiltrating cancers

No significant differences were seen in the expression of VEGF-C or VEGF-D between non-infiltrating and infiltrating cancers (Fig. 1). However, expression of VEGF-C tended to be higher than that of VEGF-D. In addition, expression of VEGF-C in infiltrating cancer tended to be higher than that in non-infiltrating cancer.

2) Expression of VEGF-C and VEGF-D in infiltrating cancer and correlations to various clinicopathological factors

No significant differences were seen in the expression of VEGF-C and VEGF-D with respect to T factor, macroscopic tumor diameter, or pathological stage. In terms of n factor, expression of VEGF-C and VEGF-D was higher for patients without lymph node metastasis, and in terms of lymphatic vessel invasion, the expression of VEGF-C and VEGF-D was higher, albeit not significantly, for patients with lymphatic vessel invasion. No significant differences were seen in the expression of VEGF-C and VEGF-D between patients with three or fewer lymph node metastases and those with four or more lymph node metastases, but the tendency was that the greater the number of metastases, the lower the expression (p=0.6993, p=0.1030) (Table 1).

HER-2 expression was investigated immunohistochemically, and its relationship to VEGF-

Table 1. Expression of VEGF-C and –D in Infiltrating Breast Cancer

	n	VEGF-C/GAPDH	P-value	VEGF-D/GAPDH	P-value
Age	<49 (n=34)	0.13±0.07	0.565	0.12±0.07	0.207
	50≧ (n=69)	0.15±0.08		0.07±0.06	
T status	T1 (n=50)	0.12±0.05	n.s	0.08±0.03	n.s
	T2 (n=36)	0.16±0.04		0.09±0.04	
	T3 (n=6)	0.05±0.02		0.19±0.18	
	T4 (n=11)	0.30±0.22		0.02±0.01	
Tumor size	~1cm (n=3)	0.07±0.04	n.s	0.10±0.08	n.s
	1~2cm (n=47)	0.11±0.09		0.08±0.03	
	2~3cm (n=29)	0.14±0.04		0.04±0.02	
	3~4cm (n=11)	0.18±0.12		0.12±0.08	
	4~ 5cm (n=5)	0.14±0.07		0.17±0.13	
	5~cm (n=8)	0.20±0.15		0.12±0.10	
n status	Negative(n=56)	0.16±0.05	0.332	0.09±0.03	0.908
	Positive (n=47)	0.12±0.02		0.08±0.03	
	n number =1~3 (n=28)	0.14±0.04	n.s	0.10±0.03	n.s
	>4 (n=19)	0.07±0.01		0.06±0.02	
lymphatic invasion	Negative(n=62)	0.09±0.02	0.069	0.05±0.02	0.210
	Positive (n=41)	0.17±0.04		0.10±0.03	
p-Stage	I (n=36)	0.12±0.05	n.s	0.05±0.03	n.s
	II (n=47)	0.16±0.10		0.09±0.03	
	III (n=15)	0.07±0.03		0.07±0.05	
	IV (n=5)	0.31±0.25		0.23±0.22	
ER,PgR status	ER-/+ or PgR-/+ (n=56)	0.12±0.03	0.244	0.06±0.04	0.259
	ER- and PgR- (n=45)	0.17±0.10		0.11±0.04	
HER-2 status	Negative(n=67)	0.12±0.07	0.03	0.03±0.02	0.05
	Positive (n=36)	0.28±0.08		0.14±0.07	

Means were shown with standard deviations(SD).
Significant differences were analyzed with Mann-Whitney U Test.
n.s : not signifificant

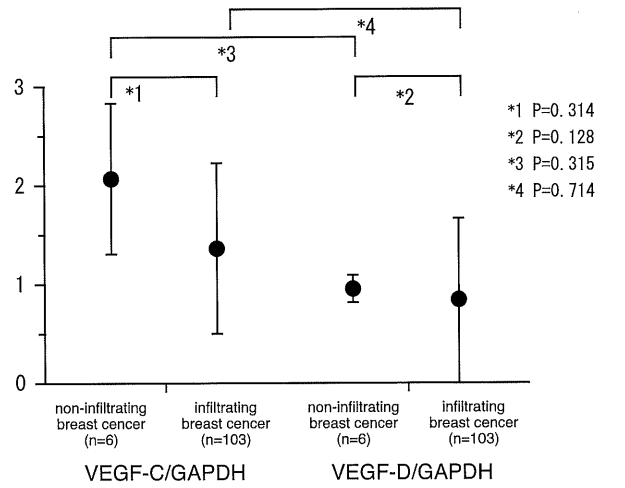

Fig. 1. Relation between Expression of VEGF-C and VEGF-D in Breast Cancer and Histology

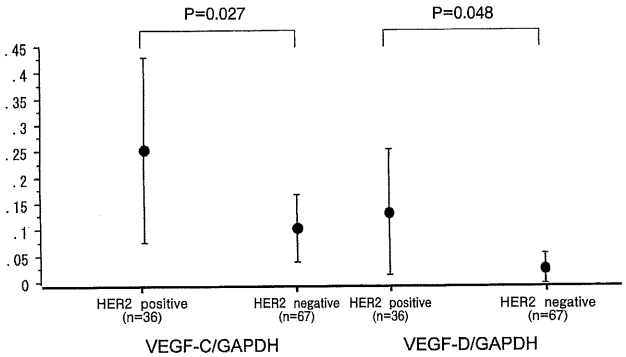

Fig. 2. Relation between Expression of VEGF-C and VEGF-D in Infiltrating Breast Cancer and HER-2 status

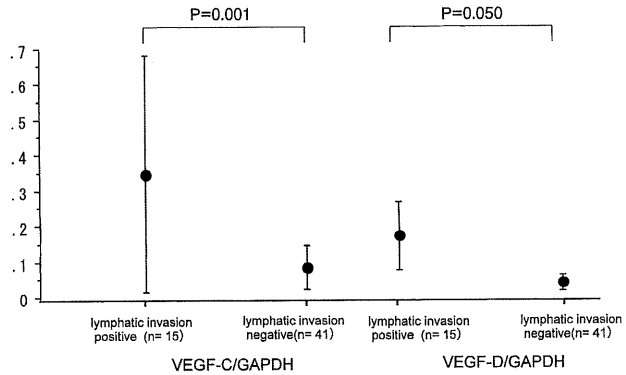

Fig. 3. Relation between Expression of VEGF-C and VEGF-D in Infiltrating Breast Cancer with no lymph node metastasis and lymphatic invasion

C and VEGF-D expressions was investigated (negative: 0 and 1+, and positive: 2+ and 3+). When compared to HER-2-negative patients, the expression of VEGF-C and VEGF-D was significantly higher for HER-2-positive patients (Fig. 2). Among 56 patients without lymph node metastasis, the expression of VEGF-C and VEGF-D for 15 patients with lymphatic vessel invasion was significantly higher than that for 41 patients without lymphatic vessel invasion (Fig. 3).

DISCUSSION

In the present study, the expression of VEGF-C and VEGF-D was compared between non-infiltrating and infiltrating cancers, but no significant differences were seen. Several studies have reported that the expression of VEGF-C and VEGF-D in cancerous tissue is lower when compared to healthy tissue in patients with lung or breast cancer[15, 21], and it is generally accepted that changes in VEGF-C and VEGF-D expression are involved in carcinogenesis. However, the present study suggests that there are no marked differences in VEGF-C and VEGF-D expression between non-infiltrating and infiltrating cancers.

In the present study, there were no significant differences in the expression of VEGF-C or VEGF-D with respect to lymph node metastasis or lymphatic vessel invasion. While some studies have

documented a positive correlation between VEGF-C expression and lymphangiogenesis[3, 7, 25, 28, 32], some studies have found no correlation between VEGF-C expression and lymph node metastasis[14]. In breast cancer, VEGF-C may be involved with lymphatic vessel invasion prior to lymph node metastasis. In addition, in esophageal cancer, VEGF-C and VEGF-D have been reported to correlate to early-stage esophageal carcinogenesis[10]. In the present study, a positive correlation was seen between lymphatic vessel invasion and VEGF-C and VEGF-D expression among patients without lymph node metastasis, thus suggesting that VEGF-C and VEGF-D are involved in lymphatic vessel invasion prior to lymph node metastasis and that their expression decreases as the number of lymph node metastases increase; in other words, the expression of VEGF-C and VEGF-D lowers as breast cancer advances. Subsequently, VEGF-C and VEGF-D cause lymphatic vessel invasion of cancer, and are involved with tumor progression as their expression decreases with lymph node metastasis. Irrespective, further investigation is needed to elucidate the regulatory mechanisms of VEGF-C and VEGF-D expressions.

HER2, the gene product of the oncogene HER2/neu, has a tyrosine kinase active region in its intracellular region, and is involved in cell growth regulation. In 20-30% of breast cancer patients, HER2/neu gene amplification is seen[26], and because these cases are sometimes resistant to hormone therapy or chemotherapy, HER2/neu gene amplification is known to be a marker for poor prognosis[9, 33-35]. In breast cancer, the expression of HER2 exhibits a positive correlation to the expression of the VEGF family, thus suggesting that HER2 may be involved in the switch mechanism of angiogenesis and lymphangiogenesis[30]. It has been reported that excessive HER2 expression induces VEGF expression in response to hypoxia and the core promoter region[18]. A close correlation between HER-2 and VEGF expressions is known. In the present study, expression of VEGF-C and VEGF-D in HER2-positive patients was significantly higher, thus supporting previous studies. Trastuzumab is a humanized monoclonal antibody developed to target the HER2 receptor.Binding with high affinity to the extracellular domain of HER2, trastuzumab inhibits the proliferation of tumor cells and has clinical activity in breast cancer that overexpresses HER2. The above findings suggest that trastuzumab may be used to suppress the expression of VEGF-C or VEGF-D. In addition, antibodies targeting VEGF-C or VEGF-D may be therapeutically useful, particularly in HER2-positive patients, and combination therapy involving trastuzumab may be effective in improving prognosis.

In the present study, the relationship of VEGF-C and VEGF-D expression to prognosis was not investigated, and this issue needs to be investigated in future long-term studies.

CONCLUSIONS

The results suggest that, in breast cancer, VEGF-C and VEGF-D are involved in lymphatic vessel invasion prior to lymph node metastasis, and after lymph node metastasis occurs, expression of VEGF-C and VEGF-D decreases. In other words, VEGF-C and VEGF-D appear to be involved with lymphatic vessel invasion in early-stage breast cancer.

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