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Expressions of Vascular Endothelial Growth Factor (VEGF)-D and VEGF Receptor-3 in Colorectal Cancer: Relationship to Lymph Node Metastasis

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Angiogenic factors play a major role in tumor growth and metastasis. Vascular endothelial growth factor (VEGF)-D is a ligand for VEGF receptor-3 (VEGFR-3/Flt-4), which mainly expressed on the lymphatic endothelium. Recent experimental studies have shown that VEGF-D induces tumor lymphangiogenesis and promote metastatic spread of tumor cells via lymphatic vessels. However, the contribution of VEGF-D to lymph node metastasis in human colorectal cancer is less understood. We therefore examined VEGF-D and VEGFR-3 expression in patients with colorectal cancer. Sections of formalin-fixed and paraffin-embedded specimens from 76 colorectal cancers were immunohistochemically stained for VEGF-D and VEGFR-3. Staining for VEGF-D was positive in the cytoplasm of tumor cells in 43 of 76 examined tumors (56.6%). Staining for VEGFR-3 was positive in endothelial cells in 38 (50.0%) tumors. Univariate analysis showed that both VEGF-D and VEGFR-3 expressions correlated significantly with lymph node metastasis, histological type and depth of tumor invasion. However, logistic regression analysis indicated that VEGF-D expression, but not that of VEGFR-3, was an independent predictor for lymph node metastasis. Our data suggest that VEGF-D plays an important role in lymph node metastasis in colorectal cancer.

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Key Words: vascular endothelial growth factor (VEGF)-D, lymph node metastasis, colorectal cancer, lymphangiogenesis

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Introduction

The capacity of tumor cells to induce angiogenesis and lymphangiogenesis may regulate the probability of hematogenous or lymphatic metastasis. Lymph-angiogenesis is controlled, in part, by members of the vascular endothelial growth factor (VEGF) family, i.e., VEGF-C, and VEGF-D, and their receptor on lymphatic endothelium, VEGFR-3[1,2]. VEGF-C was initially identified as a ligand for VEGFR-3[3]. Because expression of VEGFR-3 is mainly restricted to the lymphatic endothelium, the major function of VEGF-C appears to be the regulation of lymphatic vessel growth[4,5]. VEGF-C has been shown to regulate the growth of lymphatic vessels in various experimental models[1,6].

VEGF-D is the most recently discovered member of the VEGF family. It was isolated as a *fos*-inducible factor from mouse skin fibroblasts and the mature form shares about 60% amino acid sequence identity with VEGF-C[2,7]. In addition to their sequence identity, VEGF-C and VEGF-D are thought to have similar biological functions because they can bond to common receptors. These secreted growth factors are synthesized as propeptides that are activated by proteolysis to form high-affinity ligands that activate VEGFR-3 and stimulate lymphangiogenesis[8,9]. In experimental mouse tumor model, it is reported that VEGF-C and VEGF-D can induce tumor lymphangiogenesis and direct metastasis to the lymphatic vessels and lymph nodes[10-12]. In human, recent studies indicated that VEGF-D is upregulated in malignant melanoma[13], inflammatory breast carcinoma[14], and colorectal cancer[15], suggesting that VEGF-D expression is associated with lymph node metastasis in various human malignancies.

In the present study, we examined the expression of VEGF-D and VEGFR-3 in colorectal cancer and investigated their relationship to lymph node metastasis, one of the most important prognostic factors in colorectal cancer.

Material and Methods

Patients

Seventy-six tissue samples were obtained from patients with colorectal cancer who had been operated between 1997 and 1998 at the Division of Surgical Oncology, Department of Translational Medical Science, Nagasaki University Graduate School of Biomedical Sciences. The group consisted of 52 males and 24 females, with a mean age at the time of surgery of 62.5±12.0 years (± SD, range: 29-88 years). None had received chemotherapy or radiotherapy before surgery. Forty tumors were localized in the colon and 36 tumors were in the rectum. Seventy-three patients underwent curative operation. The criteria of the American Joint Committee on Cancer Classification and stage grouping was used to classify tumors[16]. The 76 patients included 1 patient with stage 0, 12 with stage I, 30 with stage II, 22 with stage III, and 11 with stage IV cancer. Eight tumors were classified as 'well-differentiated adenocarcinoma', 61 tumors as 'moderately-differentiated adenocarcinoma', 5 tumors as 'poorly-differentiated adenocarcinoma', and 2 tumors as 'mucinous carcinoma'. A written informed consent was obtained from each patient for use in the present study.

Immunohistochemistry for VEGF-D and VEGFR-3

The mouse monoclonal anti-VEGF-D antibody purchased from R&D systems, Inc (Minneapolis, MN) was used for staining at a concentration of 10 μg/ml. The rabbit polyclonal anti-VEGFR-3 antibody purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) was used at 2 μg/ml. Immunohistochemical staining was performed as follows. Serial sections (3-4-μm thick) from formalin-fixed, paraffin-embedded tissue blocks were deparaffinized and then heated in a microwave oven in 10 mM citrate buffer, pH 6.0, for 10 min. The sections were then treated in methanol containing 3% hydrogen peroxide for 10 min at room temperature. They were subsequently washed in distilled water, rinsed for phosphate buffered saline (PBS), and treated with nonspecific staining blocking reagent containing 0.25% casein (DAKO, Glostrup, Denmark) for 10 min at room temperature. The slides were then incubated overnight at 4°C in a humidified chamber with anti-VEGF-D or anti-VEGFR-3 antibody diluted in PBS with 1% bovine serum albumin (BSA). After washing the specimens with PBS, the slides were incubated with biotinylated anti-mouse or anti-rabbit IgG for 30 min at room temperature. After three washes in PBS, the sections were incubated with streptavidin-peroxidase reagent for 30 min at room temperature. VEGF-D antigen and VEGFR-3 antigen were developed by incubating the slides in diaminobenzidine (DAKO) solution containing 0.06 mM diaminobenzidine and 2 mM hydrogen peroxide in 0.05% PBS (pH 7.6) for 5 min. Sections were counterstained with hematoxylin, dehydrated with alcohol and xylene, and mounted in a routine fashion. Negative controls were performed in all cases by omitting the first antibody.

Assessment of immunoreactivity

VEGF-D expression in the tumors was measured by assessing the percentage of positively stained cells. Tumors were classified into three categories according to the extent of staining: (+), over 20% of tumor cells were stained; (±), less than 20% of tumor cells were stained, and (−), completely negative. (−) and (±) were classified as negative for VEGF-D, (+) was classified as positive for VEGF-D. For VEGFR-3 expression in endothelial cells, we scored the tumors as positive for VEGFR-3 when VEGFR-3 positive vessels were found at the tumor periphery.

Statistical analysis

Patients were divided into two groups based on the median age (62 years). The statistical significance among VEGF-D, VEGFR-3 expressions, and clinicopathological features was evaluated using chi-square test. Backward stepwise logistic regression model was used for multivariate adjustments for all covariates simultaneously. All statistical analyses described in this study were conducted using STATISTICA™ software (StatSoft, Inc. Tulsa, OK).

Results

VEGF-D expression in colorectal cancer

VEGF-D was detected in the cytoplasm of tumor cells, and no expression was observed in the nucleus (Figure 1). Expression of VEGF-D tended to be localized to the deep layer of the tumor. In specimens of normal colonic mucosa, no VEGF-D expression was observed. Among the 76 tumors, 43 (56.6%) were positive for VEGF-D expression. The relationship between VEGF-D expression and clinicopathological features is summarized in Table 1. VEGF-D expression was significantly associated with histological type and depth of invasion ($p=0.03$ and <0.01, respectively). There was no correlation between VEGF-D expression and

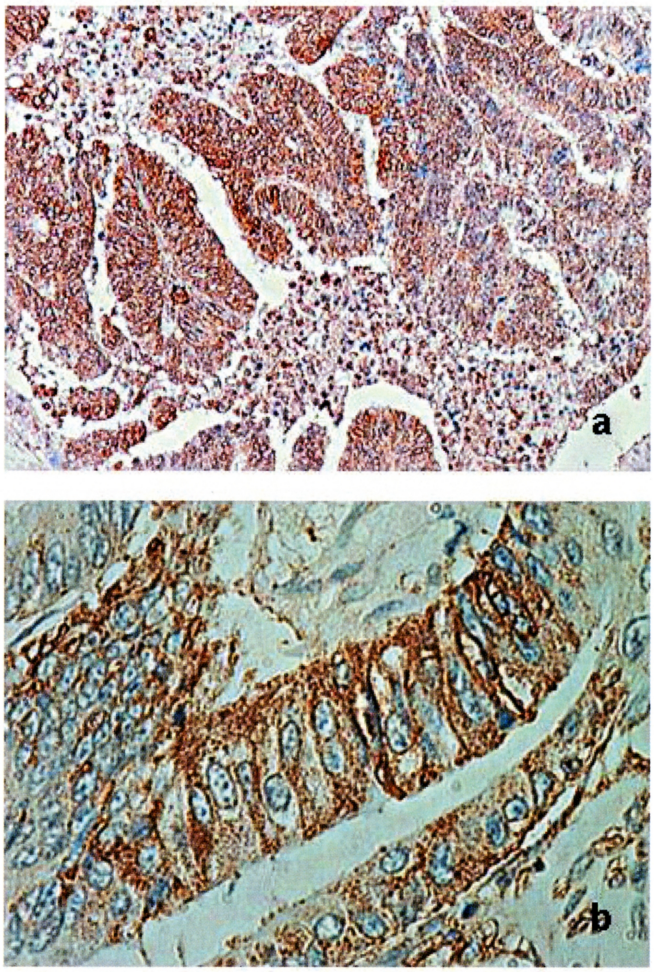

Figure 1. Immunohistochemical staining for VEGF-D in colon cancer.

Note the positive immunoreactivity for VEGF-D predominantly in the cytoplasm of tumor cells. Magnification: (a), x100; (b), x400.

gender, lymphatic involvement, venous involvement, or liver metastasis. The positive expression of VEGF-D was significantly higher in tumors with lymph node metastasis than in those without lymph node metastasis (Table 1). Multivariate analysis revealed that VEGF-D expression in tumor cells is an independent factor influencing lymph node metastasis (Table 2).

VEGFR-3 expression in colorectal cancer

VEGFR-3 was detected in a subset of vessels, which were typically thin-walled and devoid of red blood cells (Figure 2). Thirty-eight of 76 tumors (50%) were positive for VEGFR-3 expression in endothelial cells. Although VEGFR-3 was detected in the cytoplasm of tumor cells with a staining pattern similar to that of VEGF-D (49 of 76 tumors, 64.4%), in this study we evaluated VEGFR-3 only in endothelial cells. Like

Table 1. VEGF-D and VEGFR-3 expressions, and clinicopathological features.

	VEGF-D expression			VEGFR-3 expression		
	Negative	Positive	*P* value	Negative	Positive	*P* value
Age			0.04			0.65
<62 years	19	15		17	19	
≧62 years	14	28		21	19	
Gender			0.77			0.32
Male	22	30		28	24	
Female	11	13		10	14	
Histological type			0.03			<0.01
well	5	3		8	0	
moderate	28	33		28	33	
poor, mucinous	0	7		2	5	
Depth of invasion			<0.01			0.02
Tis, T1, T2	13	3		12	4	
T3, T4	20	40		26	34	
Lymphatic involvement			0.38			0.38
negative	8	7		9	6	
positive	25	36		29	32	
Venous involvement			0.86			0.64
negative	17	23		19	21	
positive	16	20		19	17	
Lymph node metastasis			<0.01			0.02
negative	27	17		27	17	
positive	6	26		11	21	
Liver metastasis			0.38			0.77
negative	28	33		31	30	
positive	5	10		7	8	

well, well-differentiated adenocarcinoma; moderate, moderately-differentiated adenocarcinomas; poor, poorly-differentiated adenocarcinoma; mucinous, mucinous carcinoma.

Table 2. Logistic regression analysis of VEGF-D with respect to lymph node metastasis.

	Odds ratio (95% CI)	*P* value
Histological type		
well	1	
moderate	1.31 (0.09 – 19.08)	0.84
poor, mucinous	8.81 (0.23 – 338.58)	0.23
Depth of invasion		
Tis, T1, T2	1	
T3, T4	1.90 (0.31 – 11.71)	0.48
Venous involvement		
negative	1	
positive	0.64 (0.18 – 2.28)	0.48
Lymphatic involvement		
negative	1	
positive	24.31 (1.80 – 328.34)	0.015
VEGF-D expression		
negative	1	
positive	5.31 (1.56 –18.09)	<0.01

CI, confidence interval. Other abbreviations as in Table 1.

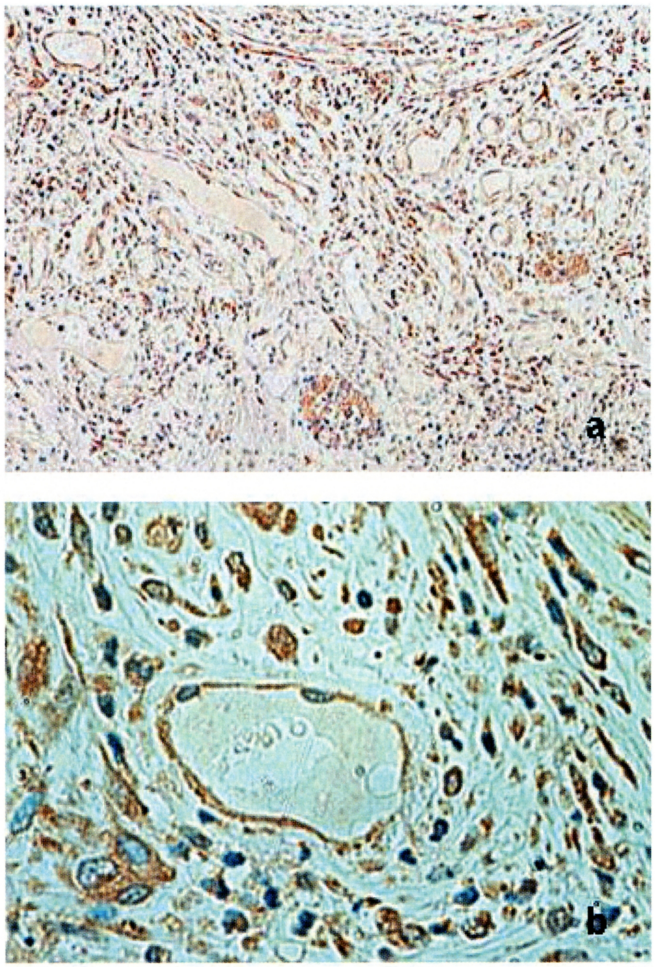

Figure 2. Immunohistochemical staining for VEGFR-3 in colon cancer.
Note the positive immunoreactivity for VEGFR-3 in the endothelium of vessels. Magnification: (a), x100; (b), x400.

VEGF-D, VEGFR-3 expression was significantly associated with histological type and depth of invasion ($p < 0.01$ and $p < 0.02$, respectively). VEGFR-3 expression was significantly higher in tumors with lymph node metastasis than in those without lymph node metastasis (Table 1). However, multivariate analysis revealed that VEGFR-3 expression was not an independent factor influencing lymph node metastasis (Table 3). VEGFR-3 expression in endothelial cells was significantly associated with VEGF-D expression in tumor cells (Table 4).

Discussion

The VEGF family consists of VEGF-A, -B, -C, -D, and -E as well as placenta growth factor[17]. The founding member, VEGF-A, plays essential roles in vasculogenesis and angiogenesis[18]. Although its crucial role in tumor angiogenesis and hematogenous metastasis has been documented in a variety of cancers[19], little is known about the physiological and pathological roles of other family members. Recent experimental studies with VEGF-C and VEGF-D have shown that they can induce tumor lymphangiogenesis. However, few studies assessed the relevance of VEGF-D to lymph node metastasis in human malignancies. VEGF-D activates VEGFR-2 (KDR/Flk-1) and VEGFR-3[2,20], both of which are essential for vascular development[17,21]. VEGFR-2 is expressed on vascular endothelial cells[22] and VEGFR-3 is mainly expressed in the endothelium of lymphatic vessels[5]. A recent study indicated that VEGF-D can stimulate both angiogenesis and lymphangiogenesis[12]. However, another study showed that VEGF-D binds only to VEGFR-3 and induces only lymphangiogenesis in mouse tumor models[6].

In the present study, VEGF-D was highly expressed

Table 3. Logistic regression analysis of VEGFR-3 with respect to lymph node metastasis.

	Odds ratio (95% CI)	*P* value
Histological type		
well	1	
moderate	0.89 (0.07 – 11.83)	0.92
poor, mucinous	9.28 (0.26 – 335.62)	0.22
Depth of invasion		
Tis, T1, T2	1	
T3, T4	2.37 (0.41 – 13.39)	0.32
Venous involvement		
negative	1	
positive	0.64 (0.19 – 2.20)	0.48
Lymphatic involvement		
negative	1	
positive	22.01 (1.67 – 290.05)	0.02
VEGFR-3 expression		
negative	1	
positive	2.26 (0.70 – 7.34)	0.16

Abbreviations as in Tables 1 and 2.

Table 4. Relationship between VEGF-D expression and VEGFR-3 expression.

VEGF-D expression	VEGFR-3 expression		
	Negative	Positive	*P* value
Negative	25	8	<0.01
Positive	13	30	

in primary tumors of colorectal cancer with lymph node metastasis. On the other hand, the expression of VEGF-D was not related to hematogenous metastasis, such as liver metastasis or venous involvement. Recent studies demonstrated that VEGF-D is proteolytically processed in a manner similar to VEGF-C[8,9]. It exists in numerous forms, as some molecules are completely processed, whereas others are partially processed or remain unprocessed[9]. There is evidence indicating that the unprocessed or partially processed VEGF-Ds are capable of binding VEGFR-3, albeit with low affinity, but fully proteolytic cleavage is necessary to generate the mature form that binds VEGFR-2 as well. These data suggest the possibility that the angiogenic versus lymphangiogenic responses to VEGF-D may depend on, at least in part, the degree of proteolytic processing of this growth factor in different tumor environment, and that in colorectal cancer VEGF-D does not act as an angiogenic factor, but plays a role in the development of lymphatic vessels thereby promoting only lymphatic metastasis. Although the protease responsible for the processing is yet to be determined, we speculate that in colorectal cancer the protease is not expressed enough to generate fully processed VEGF-D.

Our results also showed a significant correlation between expression of VEGF-D and depth of tumor invasion. That VEGF-D tended to be expressed in the deep layers of tumors suggests the increased potential of lymphangiogenesis following growth of such tumors, or reflects the malignant potential of tumor invasion.

Several studies have examined the relationship between VEGF-D and human malignancies[13,14,23,24]. Although recent studies have examined the expression of VEGF-D in colorectal cancer, the role of this factor in lymph node metastasis is still controversial. White et al.[15] studied 84 patients with colorectal cancer and used univariate analysis to conclude that the expression of VEGF-D correlated with lymph node metastasis. On the other hand, George et al.[25] reported that there was no correlation between VEGF-D expression and lymph node metastasis in 70 patients with colorectal cancer. Our results were consistent with the former study. To our knowledge, the present study is the first report to indicate that VEGF-D expression is an independent predictor of lymph node metastasis in colorectal cancer using multivariate analysis.

In the same population of patients, we examined the expression of VEGFR-3. VEGFR-3 expression was significantly associated with tumor histological type and with depth of invasion. Furthermore, VEGFR-3 expression was significantly associated with VEGF-D expression, suggesting that the co-expression of VEGF-D and VEGFR-3 may play an important role in tumor lymphangiogenesis. However, multivariate analysis revealed that VEGFR-3 expression was not an independent factor influencing lymph node metastasis. In addition, VEGFR-3 expression was also detected in tumor cells (data not shown). Similar pattern of VEGFR-3 in colorectal cancer was also reported[26]. Although the significance of such expression is still unclear, it is possible that VEGFR-3 plays a role in tumor growth in an autocrine manner. Further studies should be performed to elucidate the role of VEGFR-3 on tumor cells.

In conclusion, we have demonstrated in the present study a close relationship between VEGF-D expression and lymph node metastasis in colorectal cancer. Our study suggests that the detection of VEGF-D protein in the primary tumor may represent a potential risk of lymph node metastasis in colorectal cancer.

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