

Impact of Vascular Endothelial Growth Factor-C and -D Expression in Human Pancreatic Cancer: Its Relationship to Lymph Node Metastasis

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ABSTRACT

Purpose: The aim of this study was to evaluate the expression of vascular endothelial growth factor (VEGF)-C and -D in pancreatic cancer and to reveal its relation to lymph node metastasis.

Experimental Design: Formalin-fixed, paraffin-embedded blocks were obtained from 58 patients with pancreatic head cancer. All of the patients underwent a curative resection. The total number of resected lymph nodes was 1,058. The expressions of VEGF-C and -D were evaluated by immunohistochemical staining. To evaluate the relation to lymph node metastasis, the expressions of VEGF-C and -D between the marginal and central portions in the tumor were compared. When >25% of the tumor cells showed distinct staining, the portion was judged as high expression.

Results: The two groups with high expression of VEGF-C ($P = 0.015$) and VEGF-D ($P = 0.020$) in the marginal portion had a significantly higher incidence of lymph node metastasis compared with the groups with low expression, respectively. Furthermore, the group with high expression of both VEGF-C and -D in the marginal portion had a higher incidence of lymph node metastasis compared with the group with low expression ($P = 0.007$). The 5-year survival rate of patients with high expression of both VEGF-C and -D in the marginal portion was significantly lower than that of patients with low expression of both VEGF-C and -D ($P = 0.017$).

Conclusions: VEGF-C and -D expression in tumor cells in the marginal portion of the tumor significantly associated with lymphatic metastasis and prognosis in patients with pancreatic head cancer.

INTRODUCTION

The survival of patients with adenocarcinoma of the pancreas is worse than that with any other gastrointestinal malignancy (1). The principal reasons for the poor prognosis include difficulties diagnosing pancreatic adenocarcinoma at a localized resectable stage and the propensity of the tumor for early metastasis to regional lymph nodes and the liver. The presence or absence of lymph node metastasis is one of the important prognostic factors for patients with pancreatic cancer. Therefore, the assessment of regional lymph nodes status is required to affect survival and the therapeutic method of choice.

The mechanisms by which tumor cells detach from the primary tumor, invade lymphatic vessels, and metastasize to regional lymph nodes are complex. Although studies of the lymphatic system have been overshadowed by the greater emphasis placed on the blood vascular system, recent studies have promoted the importance of the lymphatic system by the discovery of several molecules that act as markers of the lymphatic endothelium. These include molecules such as lymphatic vessel endothelial hyaluronan receptor-1, Prox-1, Podoplanin, and vascular endothelial growth factor receptor (VEGFR)-3 (2–5). In addition, vascular endothelial growth factor (VEGF)-C and -D, which are members of VEGF family, have been reported as lymphatic-specific growth factors (6, 7).

VEGF-C is the first ligand to be identified for VEGFR-3 (6). Because expression of VEGFR-3 is predominantly restricted to the lymphatic endothelium in adults (8), the major function of VEGF-C seems to be the regulation of lymphatic vessel growth. VEGF-D was first isolated as a *fos*-inducible factor from mouse skin fibroblasts. VEGF-D has a primary sequence that is similar to VEGF-C and is also a ligand for VEGFR-3 (9). Therefore, VEGF-C and -D are thought to have similar biological functions. They require proteolytic processing to generate several higher affinity forms that are better able to activate VEGFR-3. Furthermore, the fully processed forms are also able to activate VEGFR-2 (10, 11), which is predominantly expressed in the activated endothelia of blood vessels. They are thought to have the potential to induce angiogenesis as well as lymphangiogenesis. VEGF-C induces angiogenesis in the context of tissue ischemia (12) and in the cornea (13). However, VEGF-C induced specific lymphatic endothelial proliferation and hyperplasia of the lymphatic vasculature (lymphangiogenesis) in the skin of transgenic mice that overexpressed VEGF-C in keratinocytes (14). Several animal studies have shown that xenografted tumors induced lymphangiogenesis but not angiogenesis as a result of overexpression of VEGF-C and/or -D. These findings suggest that VEGF-C and -D may promote the spread of tumor cells through lymphatic channels (15–18). In fact, increased levels of VEGFR-3 have been detected in lymphatic endothelia adjacent to tumor cells that expressed

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VEGF-C and/or -D and in lymph nodes that contained carcinoma metastasis (19, 20).

The levels of VEGF-C and/or -D in primary tumors have been significantly correlated with lymph node metastasis in a variety of cancers that include the following: prostatic, ovarian, gastric, esophageal, colorectal, gallbladder, breast, and malignant melanoma (19–26). Recent studies have shown that an increased density of functional lymphatic vessels have been found in stromal tissue at the periphery of tumors, whereas the lymphatic vessels inside the tumor were flattered and, in general, without a lumen (18, 27). The invasive margin of tumors is thought to be the site where invasion of lymphatic vessels is likely to occur. Thus, the expression of VEGF-C and -D in the marginal portion of the tumors is thought to be more important than centrally.

Little is known about the expression of VEGF-C and -D in pancreatic cancer and its relevance to lymph node metastasis. The aim of this study was to evaluate the precise expression of VEGF-C and -D in pancreatic cancer and to reveal its relationship to lymph node metastasis. For this purpose, we assessed the expression considering intratumoral heterogeneity of VEGF-C and -D in invasive ductal adenocarcinoma of the pancreas and analyzed the relationship among the expression pattern, clinicopathological factors, and prognosis.

MATERIALS AND METHODS

Patients and Tumor Samples. In this study, formalin-fixed, paraffin-embedded blocks were obtained from 58 patients with invasive ductal adenocarcinoma of the pancreatic head who received surgical treatment at Kagoshima University Hospital. All of the patients underwent macroscopically curative resection by total pancreatectomy, pancreaticoduodenectomy, or pylorus-preserving pancreaticoduodenectomy with lymph node dissection. Patients included in this study had not received any preoperative chemotherapy or radiotherapy. The duration of follow-up

ranged from 6 to 152 months (median, 24 months). All of the patients were followed-up after discharge as follows: plain-film radiography every 1 to 3 months, and computerized tomography and ultrasonography every 3 to 6 months.

All of the resected primary tumors and lymph nodes were histologically examined by H&E staining according to the tumor-node-metastasis classification system (28). The study group contained 43 men and 15 women ranging in age from 42 to 78 years (median, 65.6 years). Two patients had pT1 tumors, 4 patients had pT2 tumors, 46 patients had pT3 tumors, and 6 patients had pT4 tumors. Pathologically, all of the tumors were invasive ductal adenocarcinoma (24 well differentiated, 32 moderately differentiated, and 2 poorly differentiated). The total number of resected regional lymph nodes was 1,058, and the median number per patient was 18.2. Of 58 patients, 35 were histologically node-positive, and 114 lymph nodes had metastatic tumor cells.

Immunohistochemical Staining and Evaluation. Primary tumors and histologically positive lymph nodes were immunostained by using antibodies against VEGF-C and -D. Three $\mu\text{mol/L}$ -thick sections were cut from paraffin blocks. They were deparaffinized in xylene and rehydrated with a series of graded EtOH. Endogenous peroxidase activity of the specimens was blocked by immersing the slides in absolute methanol solution containing 0.3% hydrogen peroxide for 30 minutes at room temperature. After washing three times with PBS for 5 minutes each, the sections were treated with 1% BSA for 30 minutes to block nonspecific reactions at room temperature. After being autoclaved at 120°C for antigen retrieval in 10 mmol/L citrate buffer solution (pH 6.0), the sections were incubated overnight at 4°C with anti-VEGF-C goat polyclonal antibody (Santa Cruz Laboratory, Santa Cruz, CA; ref. 21) and anti-VEGF-D rabbit polyclonal antibody (Santa Cruz Laboratory, Santa Cruz, CA; ref. 23) diluted 1:200 in PBS, respectively. After three washes in PBS for 5 minutes each, the

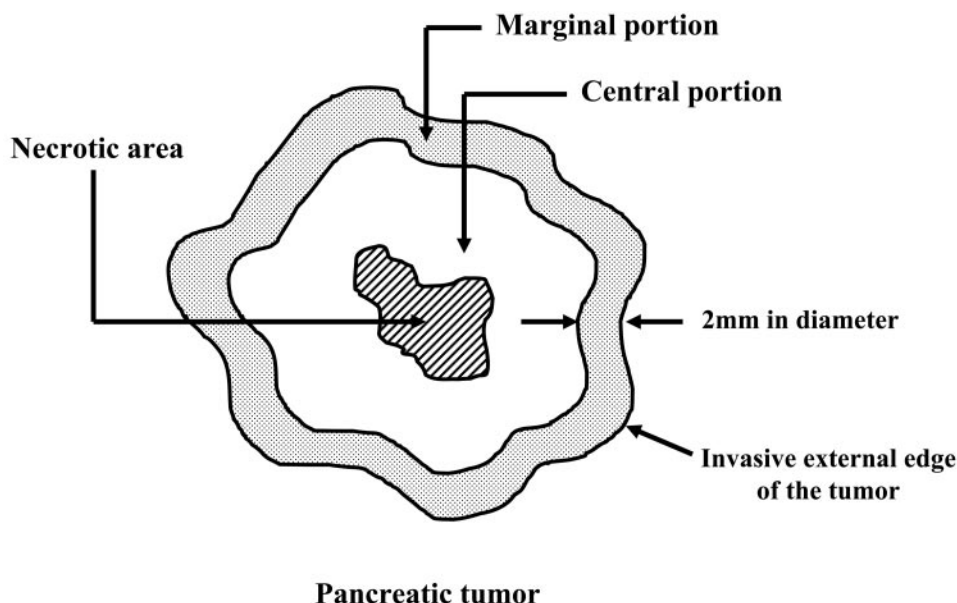


Fig. 1 Schematic illustration of the division of primary tumors. We divided primary tumors into two portions, which were marginal and central portions. The marginal portion was within 2 mm in diameter of the invasive external edge of the tumor, whereas the central portion was with the exception of the marginal portion and necrotic area. We evaluated each expression of VEGF-C and -D in the two portions of the primary tumor separately.

sections were incubated with biotinylated secondary antibodies for 30 minutes at room temperature. Then after three washes in PBS for 5 minutes each, the sections were incubated with avidin-biotin-peroxidase complex (Vectastain Elite ABC kit, Vector Laboratory, Burlingame, CA) diluted with PBS for 30 minutes at room temperature (29). After three 5-minute washes with PBS, the sections were visualized with 0.5 mg/mL diaminobenzidine tetrahydrochloride in PBS containing 0.03% H₂O₂ and counter-stained with hematoxylin before mounting. Negative control studies were done with normal goat serum or normal rabbit serum instead of the anti-VEGF-C primary antibody or anti-VEGF-D primary antibody, respectively, in each staining procedure. No significant staining was observed in the negative control sections.

Primary tumors were divided into two portions, which were marginal and central portions. The marginal portion was within 2 mm in diameter of the invasive external edge of the tumor (Fig. 1), whereas the central portion was with the exception of the marginal portion and necrotic area. To evaluate immunohistochemical staining, 10 fields were selected of the two portions,

respectively, and expression in 1,000 tumor cells (100 cells/fields) was evaluated with high-power ($\times 200$) microscopy. Because we evaluated the expression level of VEGF-C and -D in two portions of each primary tumor, 116 portions were examined in total. The immunohistochemical expression of VEGF-C and -D was defined as positive if distinct staining of the cytoplasm was observed in at least 1% of tumor cells. Each portion of the tumors was judged as high expression when $>25\%$ of tumor cells showed distinct staining. Two investigators (Hiroshi Kurahara and Sonshin Takao), who had no prior knowledge of the clinicopathological findings, assessed the expression of VEGF-C and -D.

Statistical Analysis. All of the statistical calculations were carried out with StatView statistical software version 5.5 (SAS Institute, Cary, NC). The significance of the relation between the expression of VEGF-C and -D and clinicopathological factors was estimated with the χ^2 test. Survival rates were calculated with the Kaplan-Meier method, and significant differences in survival were determined by log-rank test. A $P < 0.05$ was considered statistically significant.

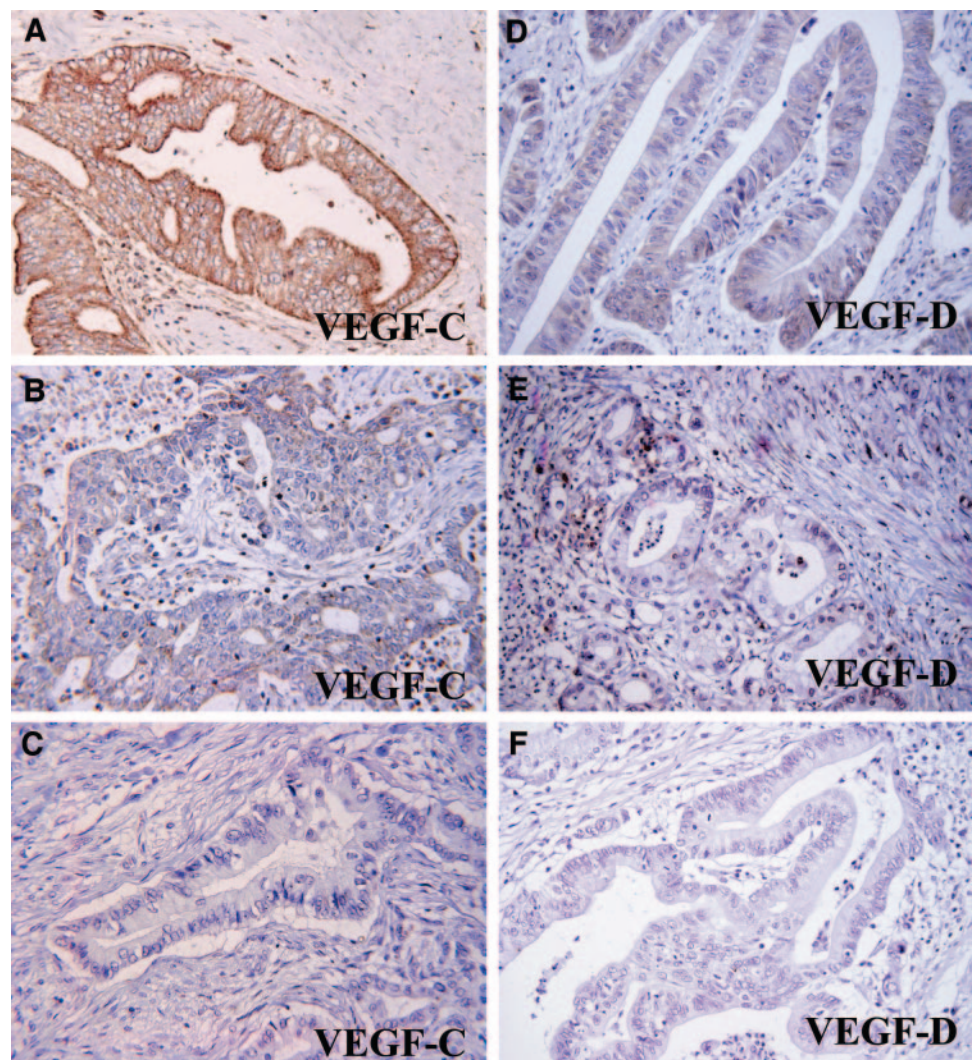


Fig. 2 Immunohistochemical expression of VEGF-C and -D in invasive ductal adenocarcinoma of the pancreatic head. VEGF-C and -D expressions were detected in the cytoplasm of tumor cells; original magnification, $\times 400$. **A.** Almost all of the tumor cells expressed VEGF-C. **B.** Very few tumor cells expressed VEGF-C. **C.** No tumor cell expressed VEGF-C. **D.** Almost all of the tumor cells expressed VEGF-D. **D.** Very few tumor cells expressed VEGF-D. **E.** No tumor cell expressed VEGF-D.

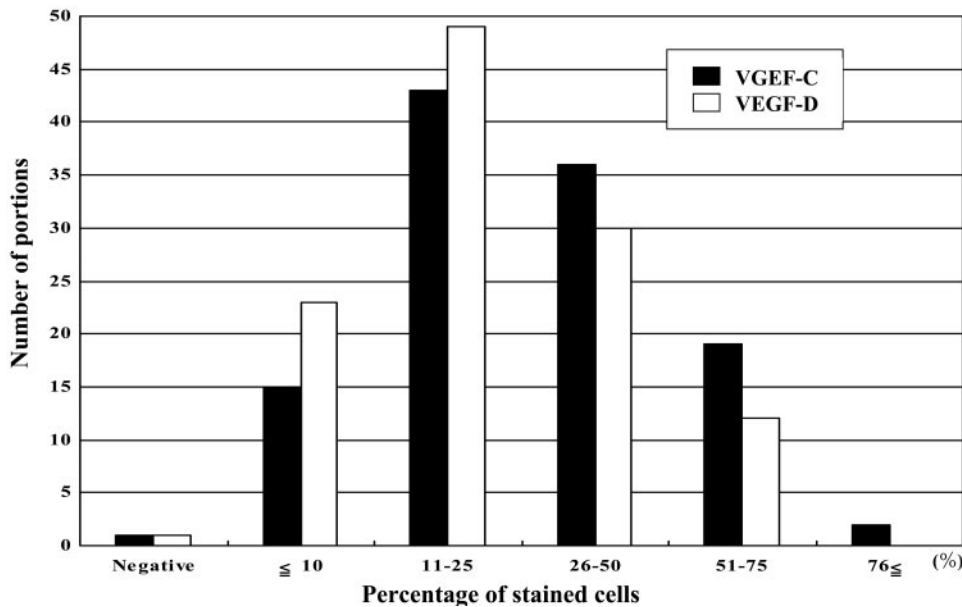


Fig. 3 Distribution of the percentage of cells stained by immunohistochemistry with antibodies for VEGF-C and -D in invasive ductal adenocarcinoma of the pancreatic head samples obtained from 58 patients. Because we evaluated immunohistochemical reactivity in two portions in each primary tumor, 116 portions were evaluated in total. The expression levels of VEGF-C and -D in the 116 portions were classified into six groups including marginal and central portions based on the percentage of immunoreactive cells.

RESULTS

Expression of VEGF-C and -D in Pancreatic Cancer and Resected Regional Lymph Nodes. Immunohistological localizations of VEGF-C and -D were cytoplasmic. VEGF-C and -D were expressed in almost all of the cases except for one. In these tumors with positive expression, the number of immunoreactive cells ranged from a very few to almost all of the tumor cells (Fig. 2). The expression levels in the 116 portions of the 58 primary tumors were classified into six groups based on the percentage of immunoreactive tumor cells (Fig. 3). VEGF-C and -D expression were heterogeneous even in the same tumors. The expression levels of VEGF-C and -D in the marginal portions were both significantly higher than those in the central portions (Table 1). Besides, VEGF-C and -D were often remarkably up-regulated at the invasive external margin of primary tumors (data not shown).

On the other hand, 35 patients with histologically positive lymph nodes were subjected to examination of the expression of VEGF-C and -D of tumor cells in metastatic lymph nodes (Fig. 4). The number of lymph nodes with high expression of VEGF-C and -D were 104 (91.2%) and 83 (72.8%) of 114 histologically positive lymph nodes, respectively.

Correlation between Vascular Endothelial Growth Factor-C and -D Expression and Clinicopathological Factors. Patients were divided into two groups according to the expression levels of VEGF-C (Table 2) and VEGF-D (Table 3)

in the two portions of the primary tumors. The correlation with clinicopathological factors was analyzed. The group with a high expression of VEGF-C in the marginal portion of the tumor had a significantly higher incidence of lymphatic invasion ($P = 0.028$) and lymph node metastasis ($P = 0.015$). The group with a high expression of VEGF-D in the marginal portion had a significantly higher incidence of lymph node metastasis ($P = 0.020$). On the other hand, there was no significant association between the expression level of VEGF-C or -D in the central portion of the tumor and clinicopathological factors.

As shown in Table 4, there was a significant association between the expression level of VEGF-C and -D in the marginal portions of the tumors ($P = 0.002$). All of the cases were then divided among the following three groups according to the expression patterns of VEGF-C and -D in the marginal portion of the tumors: both VEGF-C and -D high expression (High group), both VEGF-C and -D negative or low expression (Low group), and a group with either VEGF-C or -D high expression (Intermediate group). As shown in Table 5, in this combined analysis, the High group had a significantly higher incidence of lymph node metastasis compared with the Low group ($P = 0.007$). On the other hand, there were no significant associations between expression levels of VEGF-C or -D and venous invasion or liver metastasis.

Table 1 Comparison of VEGF-C and VEGF-D expression in marginal and central portions in invasive ductal adenocarcinoma of pancreatic head

Portion	VEGF-C expression (%)		P	VEGF-D expression (%)		P
	Negative/low	High		Negative/low	High	
Marginal	24 (41.4)	34 (58.6)	0.041	32 (55.2)	26 (44.8)	0.033
Central	35 (60.3)	23 (39.7)		43 (74.1)	15 (25.9)	

NOTE. Statistical differences in expression level of VEGF-C and -D between marginal portion and central portion were both calculated with the χ^2 test.

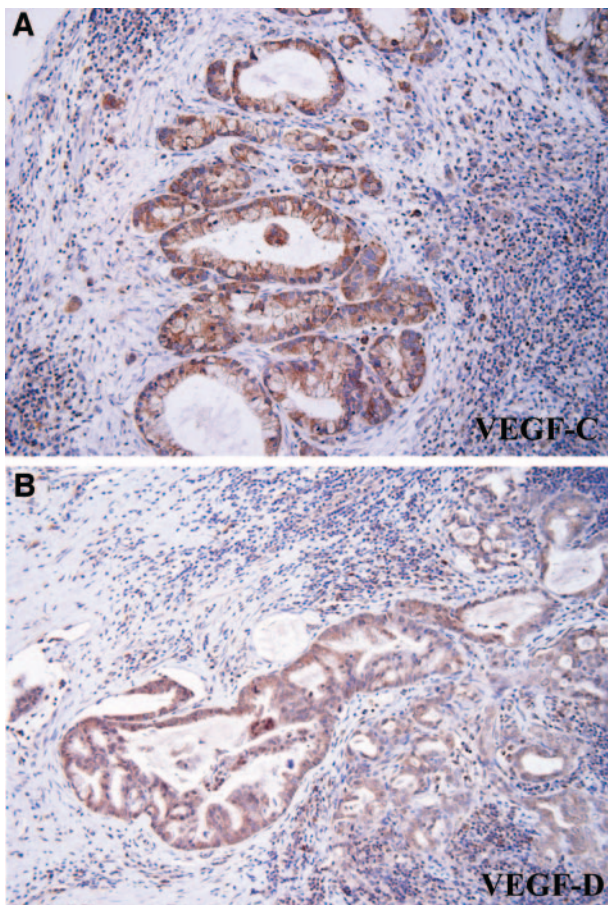


Fig. 4 Immunohistochemical expressions of VEGF-C and -D of tumor cells in metastatic lymph nodes from invasive ductal adenocarcinoma of the pancreatic head; $\times 200$. A, high level staining for VEGF-C. B, high level staining for VEGF-D.

Prognostic Impact of VEGF-C and -D Expression.

Fig. 5 shows the survival curve of patients according to their VEGF-C and -D expression levels in the marginal portions of the tumors. The 5-year survival rates of patients with high expression of VEGF-C and patients with negative or low expression of VEGF-C were 7.7% and 30.3%, respectively (Fig. 4A). Moreover, the 5-year survival rates of patients with high expression of VEGF-D and patients with negative or low expression of VEGF-D were 5.7% and 24.7%, respectively (Fig. 4B). Patients with high expression of VEGF-C and -D tended to have a poorer prognosis than patients with negative or low expression of VEGF-C and -D, respectively; however, there were no statistically significant differences. Furthermore, survival rates were evaluated according to combinations of the expression patterns of VEGF-C and -D (Fig. 4C). Patients were divided into the following three groups as above: the High group, the Intermediate group, and the Low group. The differences among the three groups were statistically significant ($P = 0.045$). The High group had a much poorer prognosis compared with the Low group ($P = 0.017$).

DISCUSSION

Lymph node metastasis is a major prognostic factor in pancreatic cancer. An understanding of metastatic mechanisms is therefore essential for development of better treatments for cancer. In the present study, our goal was to evaluate the precise expression of VEGF-C and -D considering intratumoral heterogeneity in human pancreatic cancer and to reveal its relationship to lymph node metastasis. To assess precise expression pattern considering heterogeneity of tumors, we divided primary tumors into marginal and central portions. We took into consideration of the report by Padera *et al.* (18) for the definition of tumor margin. Our definition of marginal portion in this study, which is within 2 mm in diameter of the invasive external edge of the tumor, was proper in case of human cancer, which is different from the experimental tumor (18, 27). We then examined the immunoreactivity in the two portions. The expression level in the marginal portion was significantly higher than in the central portion. VEGF-C and -D were often remarkably up-regulated at the invasive external margin of primary tumors. These findings are consistent with a study that reported that accentuation of VEGF-D was often present at the infiltrating margin of breast carcinoma (25). Jaya *et al.* (30) showed that tumor cell-host interaction can up-regulate the expression levels of VEGF-C and -D in tumors. Our results showed that VEGF-C and -D expression levels in the marginal portion had a stronger correlation with lymphatic invasion and lymph node metastasis compared with expression levels in the central portion.

In analysis of the expression of VEGF-C and -D in pancreatic cancer, the assessment differs dependent on the selected area for immunoreactive evaluation. Because the invasive margin of tumor is thought to be the most crucial site where spread of tumor cells to lymphatic vessels is likely to occur (18, 27), it is very important to know the expression of VEGF-C and/or -D produced by tumor cells near the invasive external margin. For this reason, the analyses of immunoreactivity for VEGF-C and -D in the marginal portion of the tumors signify to understand the biological behavior of pancreatic cancer.

In various malignant tumors, VEGF-C and/or -D have been reported to be significantly correlated with lymph node metastasis (19–26). However, in cases of non-small-cell lung carcinoma (31), there was no significant association between expression of VEGF-C and lymph node metastasis. In fact, low levels of VEGF-D were associated with lymph node metastasis in cases of lung adenocarcinoma (32) and head and neck squamous cell carcinoma (33). These discrepancies may result from differences in methodology as reported previously (24) and also because of intratumoral heterogeneity. In brief, VEGF-C and -D expression based on PCR analysis may not elucidate the precise tumor VEGF-C and -D levels, because the two growth factors are expressed not only in tumor cells but also in stromal components including fibroblasts (30). Furthermore, the two growth factor levels based on PCR do not exactly represent the levels of activated forms, because they require proteolytic processing to bind VEGFR-2 and VEGFR-3 with a high affinity. Even in analysis with immunohistochemistry, the result may differ dependent on the selected site for assessment.

We also found preferential expression of VEGF-C and -D in metastatic lymph nodes when compared with primary tumors.

Table 2 Correlation between clinicopathological factors and VEGF-C expression in invasive ductal adenocarcinoma of pancreatic head

Clinicopathological factors	VEGF-C expression (%) marginal portion		P	VEGF-C expression (%) central portion		P
	Negative/low	High		Negative/low	High	
Histology						
Well	9 (15.5)	15 (25.9)	0.224	14 (24.1)	10 (17.2)	0.505
Moderately	13 (22.4)	19 (32.8)		19 (32.8)	13 (22.4)	
Poorly	2 (3.4)	0 (0)		2 (3.4)	0 (0)	
Tumor size						
T1	2 (3.4)	0	0.052	2 (3.4)	0 (0)	0.561
T2	3 (5.2)	1 (1.7)		3 (5.2)	1 (1.7)	
T3	15 (25.9)	31 (53.4)		26 (44.8)	20 (34.5)	
T4	4 (6.9)	2 (3.4)		4 (6.9)	2 (3.4)	
Lymphatic invasion						
Negative	5 (8.6)	1 (1.7)	0.028	5 (8.6)	1 (1.7)	0.224
Positive	19 (32.8)	33 (56.9)		30 (51.7)	22 (37.9)	
Venous invasion						
Negative	8 (13.8)	9 (15.5)	0.572	9 (15.5)	8 (13.8)	0.458
Positive	16 (27.6)	25 (43.1)		26 (44.8)	15 (25.9)	
Lymph node metastasis						
Negative	14 (24.1)	9 (15.5)	0.015	17 (29.3)	6 (10.3)	0.087
Positive	10 (17.2)	25 (43.1)		18 (31.0)	17 (29.3)	
Liver metastasis						
Negative	19 (32.8)	21 (36.2)	0.158	27 (46.6)	13 (22.4)	0.097
Positive	5 (8.6)	13 (22.4)		8 (13.8)	10 (17.2)	

NOTE. Statistical significances of association between expression level of VEGF-C in each portion and clinicopathological factors were both calculated with the χ^2 test.

Approximately 91% and 73% of metastatic tumors in lymph nodes showed a high expression of VEGF-C and -D, respectively. These results could be ascribed to different molecular mechanisms. First, there is intratumoral heterogeneity even in the VEGF-C and/or -D low expressed tumors. VEGF-C and/or -D expressing tumor cells have a higher potential to invade

lymphatic vessels and to promote lymph node metastasis compared with nonexpressing tumor cells. In brief, VEGF-C and/or -D high-expressing tumor cells promote lymphatic metastasis by not only inducing lymphangiogenesis but also by facilitating self-entry into the lymphatic system. These findings are consistent with a recent report (16) that indicated that the tumor cells

Table 3 Correlation between clinicopathological factors and VEGF-D expression in invasive ductal adenocarcinoma of pancreatic head

Clinicopathological factors	VEGF-D expression (%) marginal portion		P	VEGF-D expression (%) central portion		P
	Negative/low	High		Negative/low	High	
Histology						
Well	13 (22.4)	11 (19.0)	0.430	16 (27.6)	8 (13.8)	0.436
Moderately	17 (29.3)	15 (25.9)		25 (43.1)	7 (12.1)	
Poorly	2 (3.4)	0 (0)		2 (3.4)	0 (0)	
Tumor size						
pT1	2 (3.4)	0	0.159	2 (3.4)	0 (0)	0.777
pT2	3 (5.2)	1 (1.7)		3 (5.2)	1 (1.7)	
pT3	22 (37.9)	24 (41.4)		33 (56.9)	13 (22.4)	
pT4	5 (8.6)	1 (1.7)		5 (8.6)	1 (1.7)	
Lymphatic invasion						
Negative	4 (6.9)	2 (3.4)	0.550	4 (6.9)	2 (3.4)	0.659
Positive	28 (48.3)	24 (41.4)		39 (67.2)	13 (22.4)	
Venous invasion						
Negative	7 (12.1)	10 (17.2)	0.168	12 (20.7)	5 (8.6)	0.691
Positive	25 (43.1)	16 (27.6)		31 (53.4)	10 (17.2)	
Lymph node metastasis						
Negative	17 (29.3)	6 (10.3)	0.020	16 (27.6)	7 (12.1)	0.519
Positive	15 (25.9)	20 (34.5)		27 (46.6)	8 (13.8)	
Liver metastasis						
Negative	20 (34.5)	20 (34.5)	0.238	29 (50.0)	11 (19.0)	0.671
Positive	12 (20.7)	6 (10.3)		14 (24.1)	4 (6.9)	

NOTE. Statistical significances of association between expression level of VEGF-D in each portion and clinicopathological factors were both calculated with the χ^2 test.

Table 4 Correlation between expression level of VEGF-C and VEGF-D in the marginal portions in invasive ductal adenocarcinoma of pancreatic head

VEGF-C expression	No. (%) of cases		<i>P</i>
	Negative/low expression of VEGF-D	High expression of VEGF-D	
Negative/low	19 (32.8)	5 (8.6)	0.002
High	13 (22.4)	21 (36.2)	

NOTE. Statistical significance of association between expression level of VEGF-C and -D in the marginal portion was calculated with the χ^2 test.

Table 5 Correlation between lymph node metastasis and expression pattern of VEGF-C and VEGF-D in the marginal portions in invasive ductal adenocarcinoma of pancreatic head

Patterns of VEGF-C and -D expressions	Node negative (n = 23)	Node positive (n = 35)	Positive rate (%)
High *	4	17	81.0†
Intermediate ‡	8	11	57.9
Low §	11	7	38.9†

* High: cases with both VEGF-C and -D high expression.

† *P* = 0.007.

‡ Intermediate: cases including those with VEGF-C high expression and VEGF-D negative or low expression and those with VEGF-C negative or low expression and VEGF-D high expression.

§ Low: cases with both VEGF-C and -D negative or low expression.

growing inside lymphatic vessels in the periphery of VEGF-C expressing tumors express more VEGF-C than the rest of the tumor. As a second possible mechanism, metastatic tumor cells expressing VEGF-C and/or VEGF-D could obtain successful growth in regional lymph nodes that they reached. The existence of an organ-specific growth promotion for tumor cells, depending on the specific protein produced by the tumor cells themselves, has been reported previously (34, 35).

Because all of the intestinal fluids in the tumor must exit from the tumor margin in the absence of functional lymphatics within the tumor (18, 27), lymphangiogenesis may depend more on the amount of VEGF-C and/or -D produced than on location of production. However, expression level of these proteins in the marginal portion had a stronger correlation with lymphatic invasion and lymph node metastasis compared with expression levels in the central portion. In addition, we revealed preferential expression of VEGF-C and -D in metastatic lymph nodes when compared with primary tumors. Taken together, these possibilities are speculated that it is important for VEGF-C and/or -D high-expressing tumor cells to exist in the marginal portion of the tumor for lymphatic metastasis because of not only lymphangiogenesis but also the high ability of the VEGF-C and/or -D high-expressing tumor cells to self-entry into the lymphatic system or to obtain successful growth in reached lymph nodes.

In the current study, we found clear and significant correlation between VEGF-C and -D expressions in the marginal portion of the tumors. This is thought to be because of the constructive similarity of the two growth factors. Combination analysis of VEGF-C and -D expression showed that tumors with

a high expression of both VEGF-C and -D had a higher incidence of lymph node metastasis compared with tumors with a high expression of either VEGF-C or -D. This indicated a strong difference in the incidence of lymph node metastasis between

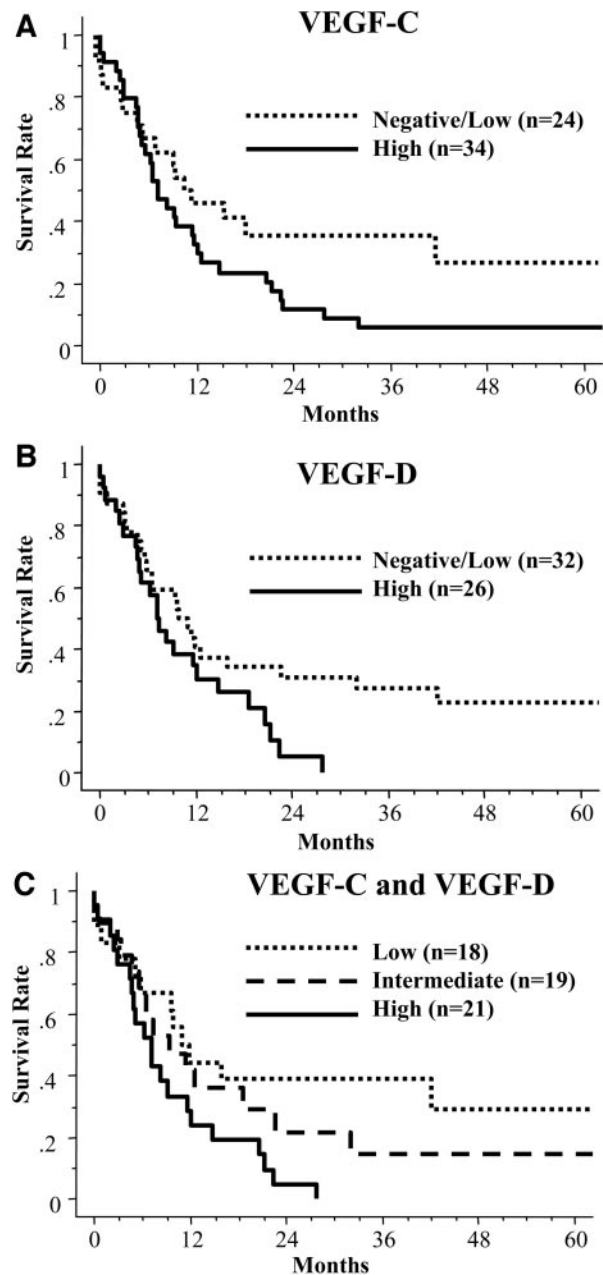


Fig. 5 Comparison of survival curves by the Kaplan-Meier method according to the expression of VEGF-C and -D in the marginal portions in patients with invasive ductal adenocarcinoma of the pancreatic head. **A**, according to VEGF-C expression, there was no significant difference between the survival curves (*P* = 0.073). **B**, according to VEGF-D expression, there was no significant difference between the survival curves (*P* = 0.055). **C**, the group of high level expression with both VEGF-C and -D showed a significant poorer prognosis compared with that of negative or low expression with both VEGF-C and -D (*P* = 0.017).

the High and Low groups. These findings revealed that tumors with a high expression of both VEGF-C and -D had a very high potential to facilitate lymph node metastasis. In this combination analysis, the postoperative prognosis of patients who had tumors with a high expression of both VEGF-C and -D were significantly poorer than the prognosis in patients who had tumors with negative or low expression of both VEGF-C and -D. These prognoses are suggested to be dependent on the incidence of lymph node metastasis according to expression patterns. In brief, the expressions of VEGF-C and/or -D not only facilitated lymphatic metastasis but also had a clear negative impact on prognosis.

In conclusion, our results showed that VEGF-C and -D produced by tumor cells in the marginal portion of the tumors significantly associated with lymphatic metastasis, and these lymphangiogenic factors were useful predictors of prognosis in cases with pancreatic head cancer. These findings may help to prospectively identify patients at increased risk for lymph node metastasis and poor outcome who should receive additional treatment, such as a molecular targeting therapy for VEGF-C/D and careful follow-up examinations after surgical treatment.

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