Clinical Significance of Vascular Endothelial Growth Factors C and D and Chemokine Receptor CCR7 in Gastric Cancer

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Abstracts. *Background/Aim: This study was designed to investigate the clinical significance of lymphangiogenic vascular endothelial growth factors C and D, and chemokine receptor CCR7 in the lymphatic spread of gastric cancer. Patients and Methods: The expressions of VEGF-C and -D, and CCR7 were examined in 82 gastric tumors showing a discrepancy between the degree of lymphatic invasion (Ly) and the status of lymph node metastasis (N) (Ly+N–: 72, and Ly–N+: 10 patients). Results: High expression of VEGF-C and -D, and CCR7 was present in 88%, 63% and 67% of cases, respectively. The VEGF-C expression was significantly higher in Ly+N– than Ly–N+ ($p<0.05$), but VEGF-D and CCR7 were not. CCR7 expression was a prognostic factor in the Ly+N– subgroup ($p<0.05$), but VEGF-C and -D were not. Conclusion: VEGF-C and -D and CCR7 may play critical roles in lymphatic invasion in primary tumors. CCR7 expression should provide prognostic information in node-negative gastric cancer patients showing lymphatic invasion.*

Gastric cancer is the fourth most common cancer in the world, and is the second leading cause of death after lung cancer (1, 2). The status of lymph node metastasis is recognized as one of the most important prognostic factors in both of the widely used staging systems, Union Internationale Contre le Cancer (UICC) and the Japanese Classification of Gastric Carcinoma (JCGC), along with depth of wall invasion (3, 4). Lymphatic invasion is also recognized as a significant prognostic factor, strongly associated with potential lymph node metastasis (5-8). However, we have sometimes encountered gastric cancer patients showing discrepancy between the status of lymph node metastasis and the degree of lymphatic invasion (9). In fact, lymphatic spread consists of a highly complex series of relationships between tumor cells and surrounding lymphatic tissues. Therefore, lymphatic invasion of tumor cells does not necessarily indicate lymph node metastasis.

Recently several studies have demonstrated that various molecules, such as vascular endothelial growth factors (VEGFs), chemokines and their receptors (CCRs), are involved in the lymphatic metastatic process. VEGF-C and -D have been identified as major regulators of the development of lymphatic vessels (lymphangiogenesis) (10-14), and consequently correlate with lymphatic invasion and lymph node metastasis (10). Chemokine receptor 7 (CCR7), which is essential for migration of lymphocytes to lymph nodes, is also thought to play an important role in the implantation of tumor cells to metastatic sites.

In the present study, we investigated the expression of VEGF-C, -D, and CCR7 in particular gastric cancer cases showing lymphatic invasion without lymph node metastasis, and compared the results with those of cases without lymphatic invasion but showing marked lymph node metastasis in order to elucidate the detailed roles and clinical significance of these molecules in gastric cancer.

Patients and Methods

Patients and tissue samples. Between 1997 and 2006, a total of 1,020 patients with gastric cancer underwent surgical resection at Kyoto Prefectural University of Medicine Hospital. Tumor specimens as well as resected lymph nodes were obtained at the time of surgery and immediately fixed in 10% neutral-buffered formalin and embedded in paraffin after fixation. The macroscopic and microscopic classification of gastric tumors was based on the JCGC. The degree of lymphatic invasion was classified into four grades according to the JCGC: ly0 (no lymphatic invasion), ly1 (minimal lymphatic invasion), ly2 (moderated lymphatic invasion), ly3 (marked lymphatic invasion) (4). Lymphatic invasion and lymph

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node metastasis were determined by routine hematoxylin and eosin staining on the resected specimen, and immunostaining using D2-40 and cytokeratin as needed.

Of these patients, there were 82 showing a discrepancy between the degree of lymphatic invasion and the status of lymph node metastasis: 72 patients showing lymphatic invasion without lymph node metastasis (Ly+N– group) and 10 patients showing no lymphatic invasion with marked lymph node metastasis (more than seven positive nodes) (Ly–N+ group). We focused on these 82 cases for further immunostaining analyses. All patients provided written informed consent to participate in all procedures associated with the study in accordance with our institutional guidelines.

Immunohistochemical staining and evaluation. The resected primary tumors were immunostained using polyclonal antibody against VEGF-C, monoclonal antibody against VEGF-D, and monoclonal antibody against CCR7. The slides were deparaffinized with xylene and rehydrated with a graded series of ethanol. Antigen retrieval was carried out at 98˚C in citric acid buffer (10 mmol/l, pH 6.0) for 40 minutes. Endogenous peroxidase was blocked by immersing the slides in methanol containing 3% hydrogen peroxide for 30 minutes. After washing, non-specific binding was blocked at room temperature for 60 minutes with 1% bovine serum albumin in phosphate-buffered saline. The sections were incubated at room temperature for 2 hours with anti-VEGF-C (Invitrogen, CA, USA) polyclonal antibody, anti-VEGF-D monoclonal antibody (R and D systems, CA, USA) and were incubated overnight at 4˚C with anti-CCR7 (BD Pharmingen, CA, USA) with 1:100 dilution. After washing, the sections for VEGF-C and -D staining were treated with CSA II kit (Dako, CA, USA), and the sections for CCR7 staining were treated with EnVison kit (Dako) according to the manufacturer's instructions. The immunoproducts were examined by treating slides with diaminobenzidine tetrahydrochloride followed by counterstaining with hematoxylin. Positive controls for VEGF-C and -D comprised lymph nodes from patients with advanced gastric cancer and histologically evident metastasis. Positive controls for CCR7 comprised human spleen tissues (11). Negative controls were performed in all cases by omitting the first antibody.

Two independent investigators (K. D. and K. W.), who were blinded to the clinicopathological data of the patients, evaluated immunoreaction of VEGF-C and -D and CCR7. The presence of immunoreactivity in over 10% of the cancer cells was defined as high expression (12, 13). Expressions of were evaluated by high-power (×200) microscopy in 10 fields that each contained 100 cells.

Statistical analysis. The χ^2 test was used for univariate comparisons. Survival rates were calculated by the Kaplan-Meier method, with the date of gastrectomy as the starting point. Differences in survival were examined by log-rank test. The significance of differences was accepted at $p<0.05$.

Results

Clinicopathologic findings. The mean patient age was 66 (range 36-88) years, and the male: female ratio was 2.3:1. The median tumor size was 36 mm (range 8-118 mm). Other clinicopathological factors are summarized in Table I. The 72 patients with lymphatic invasion were classified into three groups according to the JCGC: 38 patients as ly1, 24 patients as ly2 and 10 patients as ly3.

Table I. *Clinicopathological features of gastric cancer patients with Ly+N– and Ly–N+ tumors.*

	Ly+N– n=72	Ly–N+ n=10
Gender		
Male	50	9
Female	22	1
Age (years)		
<65	33	5
≥65	39	5
Tumor size (mm)		
<40	43	7
≥40	29	3
Location		
Upper	21	1
Middle	28	5
Lower	23	4
Lymphatic invasion		
Ly0	0	10
Ly1	38	0
Ly2	24	0
Ly3	10	0
Lymph node metastasis		
Positive	0	10
Negative	72	0
Histological type		
Differentiated	36	2
Undifferentiated	36	8
T Classification		
T1T2	38	8
T3T4	34	2

Expression of VEGF-C and -D, and CCR7 and clinicopathological factors. VEGF-C and -D were expressed in the cytoplasm of cancer cells and CCR7 was expressed in the cell membrane and/or cytoplasm of cancer cells. High expressions of VEGF-C and -D, and CCR7 were detected in 72 (88%; Figure 1A, B) 52 (63%; Figure 1C, D), and 55 (67%; Figure 1E, F) cases, respectively.

Expression of VEGF-D and CCR7 was significantly higher in the differentiated tumor type than in the undifferentiated type (both $p<0.01$). VEGF-C also tended to be more highly expressed in the differentiated type than in the undifferentiated type, although the difference was not significant ($p=0.07$). However, the expression of none of the molecules was correlated with any other clinicopathological factor (Table II).

On comparison of Ly+N– and Ly–N+ groups, the expression of VEGF-C was significantly higher in the former than in the latter ($p<0.05$). VEGF-D also tended to be more highly expressed in Ly+N– than in Ly–N+, although the difference was not significant ($p=0.10$). There was no significant difference in the expression of CCR7.

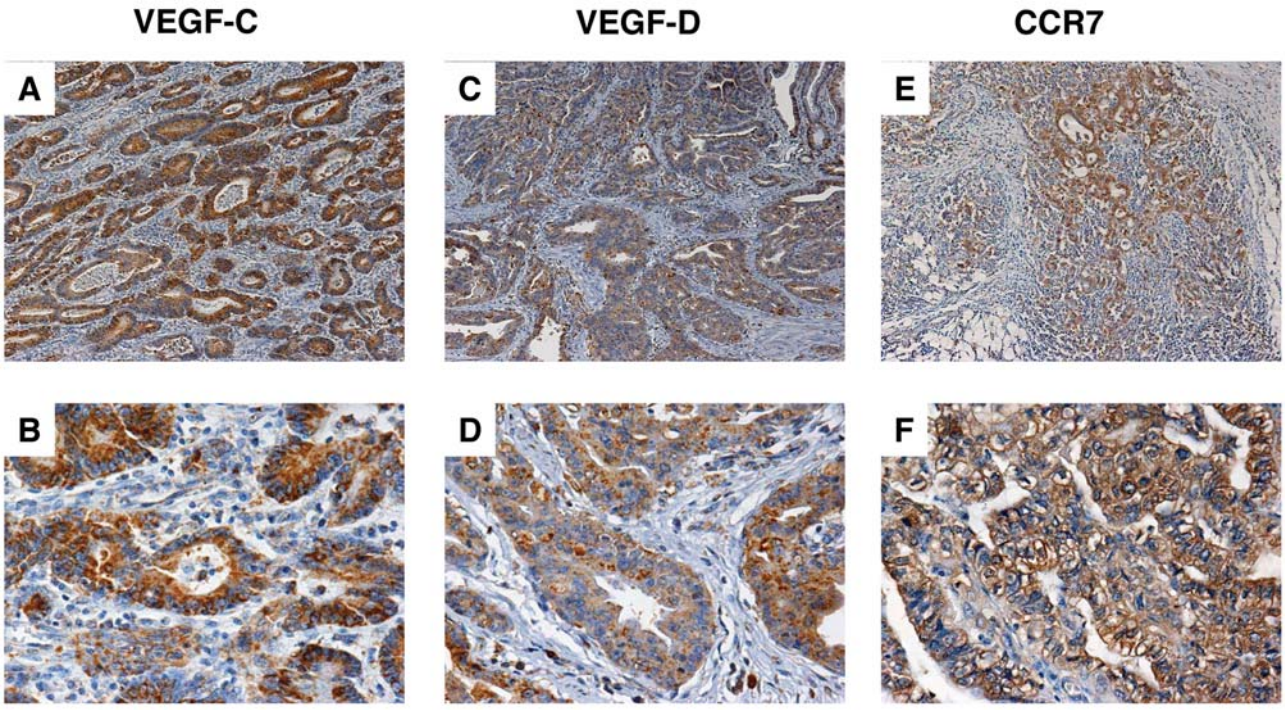

Figure 1. *Immunohistochemical staining of VEGF-C, -D and CCR7 in gastric cancer specimens. A, B: Positive VEGF-C staining: C, D: positive VEGF-D staining, E, F: positive CCR7 staining. Expression of VEGF-C and -D was observed mainly in the cytoplasm of gastric cancer cells. Expression of CCR7 was observed mainly in the membrane and/or cytoplasm. A, C, E: Original magnification ×100; B, D, F: ×400.*

Survival. Since only 10 patients were classified as Ly-N+, further prognostic analysis was performed for the 72 patients of the Ly+N- subgroup. Figure 2 shows the survival curves stratified according to the expressions of VEGF-C and -D, and CCR7. The expression of CCR7 was found to be a prognostic factor in the Ly+N- subgroup ($p<0.05$). However, there was no significant difference in survival curves when patients were divided according to the expressions of VEGF-C and -D in the present study.

Discussion

Lymphatic metastasis of tumor cells consists of a highly complex series of mechanisms as follows: i) invasion to the stromal tissues and penetration into the lymphatic vessels; ii) release of tumor cells from the primary tumor mass; iii) dissemination within the lymphatic circulating system; iv) adhesion of the tumor cells to the lymphatic endothelium in lymph nodes; v) formation of tumor deposit (14, 15). Although the details of these steps remain unknown, the step in which the tumor cells enter the lymphatic flow is the most important for lymph node metastasis. In fact, lymphatic invasion has been reported to be associated with lymph node metastasis (16, 17).

VEGF family members VEGF-C and -D are associated with the lymphatic spread of cancer cell by their interaction with VEGFR-3 expressed in the lymphatic endothelium (12, 13, 18, 19). Yonemura *et al*. reported that VEGF-C expression was closely related to lymph node metastasis, lymphatic invasion, venous invasion, and tumor infiltration in gastric cancer (18). They also reported that the prognosis of patients was significantly poorer in the group showing high VEGF-C expression than in that showing low expression, and that VEGF-C tissue status was an independent prognostic factor on multivariate analysis (18). Ishikawa *et al*. reported that the expression of VEGF-D was significantly correlated with tumor differentiation, deeper tumor invasion, lymphatic invasion, and lymph node metastasis in early gastric cancer (20). Arigami *et al*. also reported that high levels of VEGF-C and -D expression were significantly correlated with lymphatic invasion and lymph node micrometastasis (21). These findings suggest that VEGF-C and -D are likely involved in various malignant characteristics of cancer, such as multiplication, migration, and lymphangiogenesis.

However, chemokines and their receptors are also associated with various characteristics of malignant tumors. Although these were initially identified as molecules related to various normal biological functions, lymphocyte migration, angiogenesis and lymphangiogenesis, they have also recently been shown to be involved in the lymphatic spreads of various types of cancer (22-26). These

Table II. *Relationship between VEGF-C and -D and CCR7 expression and clinicopathological factors in 82 patients with Ly+N– or Ly–N+ gastric cancer.*

	VEGF-C			VEGF-D			CCR7		
	High n=72	Low n=10	*P*-value	High n=52	Low n=30	*P*-value	High n=55	Low n=27	*P*-value
Gender									
Male	52	7	0.883	38	21	0.765	38	21	0.411
Female	20	3		14	9		17	6	
Age (years)									
<65	33	5	0.804	28	10	0.073	24	14	0.483
≥65	39	5		24	20		31	13	
Tumor size (mm)									
<40	44	6	0.946	32	18	0.890	31	18	0.371
≥40	28	4		20	12		24	9	
Location									
Upper	22	0	0.061	16	6	0.520	17	5	0.137
Middle	26	7		19	14		18	15	
Lower	24	3 1		7	10		20	7	
Histological type									
Differentiated	36	2	0.075	30	8	0.006	32	6	0.002
Undifferentiated	36	8		22	22		23	21	
T classification									
T1T2	40	7	0.387	29	18	0.709	29	18	0.230
T3T4	32	3		23	12		26	9	

chemokines belongs to the small-molecule chemoattractive cytokine family, and are categorized into four groups (CXC, CC, CX3X, and C) based on the characteristic presence of four conserved *N*-terminal cysteines (27-29). Among them, CC type CCR7 is one of the most heavily researched chemokines involved in the lymphatic spread of several types of cancer.

In the present study, we examined the roles of these molecules in particular gastric cancer cases showing a discrepancy between the degree of lymphatic invasion in primary tumors and the status of lymph node metastasis. Because VEGF-C and -D have been regarded as playing critical roles in lymphangiogenesis and lymphatic metastasis, we predicted that the expression of VEGF-C and -D would be significantly higher in the Ly+N– group than in the Ly–N+ group. In contrast, we initially predicted that the expression of CCR7 would be higher in the Ly–N+ group than in the Ly+N– group because CCR7 was previously identified as an important factor in lymphocyte homing toward lymphatic tissues including lymph nodes. As expected, our results demonstrated that VEGF-C expression was significantly higher in tumor cells from the Ly+N– group than in those from the Ly–N+ group, and the expression of VEGF-D was also higher in the Ly+N– group than in the Ly–N+ group, although the difference was not significant. However, the expression of CCR7 was similar in both groups (p=0.83). These results may indicate that all of these molecules, VEGF-C and -D, and CCR7, play critical roles in lymphangiogenesis of tumors and lymphatic invasion in primary tumors. Issa *et al*. demonstrated that VEGF-C secretion and CCR7 expression by tumor cells are positively coupled to synergistically direct and enhance tumor cell invasion toward lymphatic vessels (30). However, several factors other than the molecules examined in this study are also expected to be involved in adhesion and migration of tumor cells to the lymph nodes.

Another interesting finding in the present study was that the expression of CCR7 appears to be a significant prognostic factor in Ly+N– gastric cancer. As for VEGF-C and -D, the survival of patients with positive tumors were worse than those with negative ones, although the differences were not significant. The lack of significant differences might be due to the small number of patients in the present study. Further study is needed to clarify the clinical significance of VEGF-C and -D in node-negative gastric cancer.

In conclusion, VEGF-C and -D and CCR7 appear to play critical roles in lymphatic invasion in primary tumors; factors other than these cytokines might also contribute to implantation of cancer cells in lymph nodes as a metastatic site of gastric cancer. Analyzing the expression of CCR7 should provide prognostic information in node-negative gastric cancer patients showing lymphatic invasion.

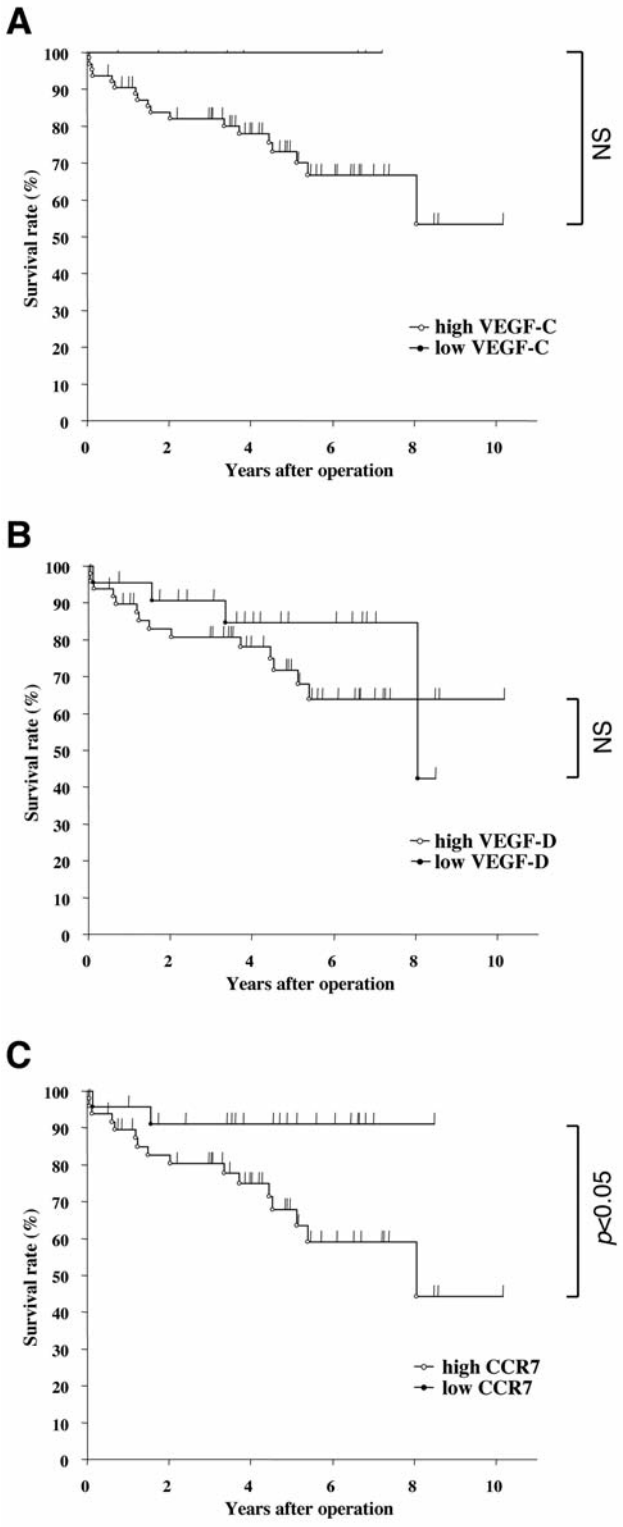

Figure 2. *Survival curve of patients with Ly+N– gastric cancer stratified according to the expression of VEGF-C and -D, and CCR7. A: VEGF-C, B: VEGF-D, C: CCR7. The patients showing high expression of CCR7 had a significantly poorer prognosis than those with low expression of CCR7 (p<0.05).*

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