Prognostic Significance of Platelet-derived Growth Factor-BB Expression in Human Esophageal Squamous Cell Carcinomas

SOHEI MATSUMOTO[1], YUKISHIGE YAMADA[2], MICHIHIRO NARIKIYO[1], MASATO UENO[1], HIDETOSHI TAMAKI[1], KATSUHIKO MIKI[1], KOHEI WAKATSUKI[1], KOJI ENOMOTO[1], TOMOYO YOKOTANI[1] and YOSHIYUKI NAKAJIMA[1]

[1]*Department of Surgery, Nara Medical University School of Medicine, Nara;*
[2]*Kokuho Central Hospital, Nara, Japan*

Abstract. *Background: Cancer metastases are commonly found in the lymphatic system and tumor lymphangiogenesis requires the interplay of several growth factors. The expression of platelet-derived growth factor (PDGF)-BB and vascular endothelial growth factor (VEGF)-C in esophageal cancer was investigated to define their clinicopathological significance. Materials and Methods: Using immunohistochemistry, the expression of PDGF-BB and VEGF-C was examined, along with lymphatic vessel density (LVD) in 53 patients with esophageal cancer. Results: PDGF-BB and VEGF-C expression was positive in 31 cases (58.5%) and 38 cases (71.7%), respectively, and expression correlated with lymph node metastasis and lymphatic invasion. Furthermore, PDGF-BB expression correlated with the depth of tumor invasion and the size of the tumor, and PDGF-BB-positive patients had a significantly poorer prognosis than PDGF-BB-negative patients. The LVD in positive PDGF-BB or VEGF-C tumors was higher than in negative tumors. Conclusion: PDGF-BB may play a pivotal role in lymphangiogenesis and tumor growth in esophageal cancer.*

Esophageal squamous cell carcinoma has a poor prognosis despite advances in perioperative care and multimodal treatment. Poor survival is due to early lymph node metastasis, which is propagated by a rich lymphovascular network and lack of serosal lining. The lymphatic drainage system of the esophagus, which is well developed in the submucosal layer, forms a complex interconnecting network that extends longitudinally. Deep penetration of the esophageal wall increases tumor entry into the lymphatics, increasing the potential for lymph node metastasis (1). In Japan, extended lymph node dissection, which is called 3-field lymph node dissection, is advocated as a standard surgical procedure since dissection of metastatic nodes may improve survival and lead to a potential cure (2).

The platelet-derived growth factor (PDGF) family includes at least four structurally related members, PDGF-AA, -BB, -CC and -DD, that can form both homodimers and heterodimers (3, 4). Members of the PDGF family bind to tyrosine kinase receptors encoded by two genes, PDGF-α and PDGF-β. Both genetic studies and recent work have demonstrated that members of the PDGF family, especially PDGF-BB, are important lymphangiogenic factors (5). PDGF-BB expression has been found in breast cancer tissues (6) and the PDGF family may contribute to tumor lymphangiogenesis and lymphatic metastasis (7, 8).

Vascular endothelial growth factor (VEGF) is a major inducer of angiogenesis and vessel permeability (9, 10). To date, the following members of this family have been characterized: VEGF-A, VEGF-B, VEGF-C, VEGF-D and VEGF-E (11). VEGF-C is a ligand for the VEGF receptor (VEGFR)-3, a tyrosine kinase receptor that is expressed predominantly in lymphatic endothelial cells (12). Breast cancer has been shown to express VEGF-C and show lymph node metastasis (13).

The recently developed monoclonal antibody D2-40, which reacts with the oncofetal membrane antigen M2A identified in ovarian cancer cell lines and germ cell tumors, was reported to be a selective marker for lymphatic endothelium and has been shown to be useful in identifying the presence of lymphatic invasion of various malignant neoplasms (14).

Using immunohistochemical techniques, the expression of PDGF-BB and VEGF-C was examined, and the number of lymphatic vessels stained by D2-40 antibody in human esophageal cancer was determined; the correlation between these parameters and various clinicopathological features was investigated.

Correspondence to: Sohei Matsumoto, Department of Surgery, Nara Medical University School of Medicine, 840 Shijo-cho, Kashihara-city, Nara, 634-8522, Japan. Fax: +81 74422 6866, e-mail: msohei@naramed-u.ac.jp

Key Words: Esophageal cancer, platelet-derived growth factor, PDGF-BB, vascular endothelial growth factor, VEGF-C.

Materials and Methods

Patients and tissue samples. Tissue samples were obtained from 53 patients undergoing esophagectomy with lymph node dissection for esophageal cancer at the Department of Surgery, Nara Medical University Hospital between January 1998 and December 2002. None of the patients had previously received chemotherapy or radiotherapy. Patients who had died within 30 days after operaion were excluded from this study. Formalin-fixed, embedded tissue samples were used for immunohistochemistry. Tumor stage and histological classification were determined according to the TNM classification (15).

Immunohistochemistry. Primary antibodies used in this study were anti-PDGF-BB (Abcam, UK; 1:100 dilution), anti-VEGF-C (Zymed Laboratories Inc, USA; 1:100 dilution) and anti-D2-40 (Dako Cytomation Inc, USA; 1:50 dilution). Immunohistochemical staining was performed according to the following immunoperoxidase technique. Formalin-fixed paraffin-embedded 4 μm-thick sections of tumor tissues were cut onto coated slides. After deparaffinization, antigen retrieval was performed in an autoclave at 100˚C for 5 min. Endogenous peroxidase activity was blocked using 0.03% hydrogen peroxide containing sodium azide for 10 min. Tissue sections were then incubated with the PDGF-BB and VEGF-C antibody overnight at 4˚C. After three washes in tris-buffered saline (TBS), the sections were incubated for 30 min with biotinylated goat anti-rabbit immunoglobulin G, washed three times with TBS, and incubated with avidin-biotinated peroxidase complex for 30 min. D2-40 staining was performed using the Dako Envision system (Dako Cytomation Inc; USA). After deparaffinization, slides were washed with phosphate-buffered saline (PBS) and sections were incubated with peroxidase blocking reagent (0.03% hydrogen peroxide containing sodium azide) for 10 min. After three washes in PBS, the sections were incubated for 1 h at 37˚C with anti-D2-40 antibody, washed three times with PBS and incubated with the peroxidase complex for 1 h at 37˚C. All sections were incubated with 0.01% H_2O_2 and 0.05% diaminobenzidine tetrahydrochloride and counterstained with hematoxylin. Immunohistochemical staining was evaluated by three independent observers who were blinded to the patient's clinicopathological status. Positive staining was defined as the presence of PDGF-BB and VEGF-C immunoreactivity in at least 30% of the cancer cells.

Statistical analysis. The χ^2 exact test or Mann-Whitney *U*-test was used to assess the association between PDGF-BB, VEGF-C expression, lymphatic vessel density (LVD), and clinicopathological features. The Kaplan-Meier method was used to analyze survival rates and the significance of differences was determined by the log-rank test. For all tests, a *p*-value of <0.05 was considered statistically significant.

Evaluation of vessel density. Lymphatic vessels were stained with anti-D2-40 antibody and LVD was determined in peritumoral tissue on two fields of high density (magnification x200).

Results

PDGF-BB and VEGF-C expression in human esophageal cancer. In the esophagus, PDGF-BB and VEGF-C expression was mainly present in the cytoplasm of cancer cells (Figure 1A, B). The relationship between PDGF-BB, VEGF-C expression and clinicopathological features is shown in Table I. Among 53 patients with esophageal cancer, PDGF-BB and VEGF-C expression was positive in 31 cases (58.5%) and 38 cases (71.7%), respectively. PDGF-BB expression was significantly associated with the depth of tumor invasion. Only 11 of 26 patients (42.3%) whose tumor depth was from Tis to T2 showed PDGF-BB-positive staining, but 20 of 27 patients (74.1%) with tumor depth from T3 to T4 were PDGF-BB-positive. PDGF-BB expression was significantly associated with the presence of lymph node metastasis and lymphatic invasion. Overall survival of patients with tumors positive for PDGF-BB was significantly worse than for those with PDGF-BB-negative tumors (Figure 2A).

VEGF-C expression was significantly associated with lymphatic invasion and lymph node metastasis, but there was no significant association between VEGF-C expression and other clinicopathological features. Patients with VEGF-C- positive tumors exhibited a tendency for lower survival rates, but this did not reach statistical significance (Figure 2B).

Immunohistochemical staining for D2-40. D2-40 staining was used to investigate the number of lymphatic vessels within peritumoral tissue. D2-40 was strictly present in the lymphatic endothelial cells and peritumoral lymphatics were dilated (Figure 1C). The average peritumoral LVD was 8.3 (range 1 to 22) on 2 fields at x200 magnification. The LVD in PDGF-BB-positive tumors (average 9.6/field) was significantly higher than in negative tumors (average 6.3/field) (Figure 3A). Similarly, the LVD in VEGF-C-positive tumors (average 9.1/field) was significantly higher than in negative tumors (average 6.1/field) (Figure 3B).

Discussion

Metastatic tumor spread through blood or lymphatic vessels occurs in most forms of human cancer, with regional lymph node metastasis often being the most important prognostic factor for carcinoma patients. Lymphatic metastases are the consequence of a complex metastatic process that includes: (i) dissemination of malignant cells from a primary tumor to the lymphatics; (ii) transport of tumor cells *via* the lymphatics to local lymph nodes; (iii) settling of tumor cells in the lymph nodes; (iv) growth of metastatic tumors in the lymph nodes. Each of these steps is critical in facilitating clinical detection of lymph node metastases in cancer patients. Recent work in tumor lymphangiogenesis research has been focused on the VEGF family, especially VEGF-C and its receptor VEGFR-3. VEGF-C can regulate physiological and pathological blood vessel growth *in vivo* (16). In addition, VEGF-C has been shown to regulate the growth of lymphatic vessels in various experimental models

(17). In a breast cancer model, VEGF-C promoted enhanced spreading of tumor cells to regional lymph nodes, and the degree of tumor lymphangiogenesis correlated with lymph node metastases (18).

PDGF is a potent mitogen for cells of mesenchymal origin (19), but overexpression of PDGF and PDGF receptors has been reported in various human tumors, including gastric, pancreatic, lung and ovarian carcinomas (20). PDGF promotes cell growth, which is mediated by activation of the phosphoinositol-3-kinase (PI3K)/Akt signaling pathway by the binding of PDGF to its receptor, which in turn leads to phosphorylation of the serine/threonine kinase (21). Another study demonstrated that PDGF-BB can induce lymphangiogenesis and lymphatic metastasis by a VEGFR-3-independent mechanism. PDGF-BB was observed to induce lymphangiogenesis in the mouse cornea *in vivo*. Interestingly, the lymphangiogenesis induced by PDGF-BB could not be restricted by inhibitors blocking interaction of VEGF-C with VEGFR-3, suggesting that, in this model, PDGF-BB exerts its effect *via* an independent pathway that may involve PDGF receptors on lymphatic vessels (5). In this report, PDGF-BB significantly induced lymphangiogenesis compared to PDGF-AA and -AB; therefore our study focused on PDGF-BB.

In this study, we observed that PDGF-BB and VEGF-C expression using immunohistochemistry was significantly associated with lymph node status and lymphatic invasion. In addition, we confirmed that PDGF-BB expression was significantly associated with tumor depth of invasion and tumor size. We determined that that PDGF-BB-positive esophageal cancer patients had a significantly poorer prognosis than PDGF-BB-negative patients. Many studies have indicated that VEGF-C levels in primary tumors correlate with lymph node metastasis in thyroid (22), prostate (23), gastric (24, 25), colorectal (26) and lung carcinomas (27), and our study suggested that expression of VEGF-C correlated with lymphatic metastasis in esophageal carcinoma. Although VEGF-C has been reported as a potential prognostic factor in cervical (28) and ovarian carcinomas (29), it was not a prognostic factor in this study or in our previous work on VEGF-C expression in gastric cancer (30).

In this study, we also investigated the number of lymphatic vessels in esophageal cancer tissues using D2-40 immunostaining. Within the tumor, lymphatic vessels were disordered, uncountable and considered to be nonfunctional. Another study indicated that cancer cells compress intratumour vessels (31). Within the peritumoral tissue, however, the lumen of lymphatics was maintained and some vessels contained cancer cells; therefore, we counted the number of lymphatic vessels in these regions. We observed that peritumoral LVD in PDGF-BB- or VEGF-C-positive tumors was significantly higher than in the negative tumors. Our result showed that LVD did not influence prognosis in survival analysis, but high LVD corresponded to recurrence of lymph node metastasis in esophageal cancer (data not shown).

Table I. *Relationship between clinicopathological findings and PDGF-BB or VEGF-C expression.*

	DGF-BB			VEGF-C		
	positive	negative	*p*-value	positive	negative	*p*-value
Age (years)						
<65	16	11	0.9079	18	9	0.4073
≥65	15	11		20	6	
Gender						
Male	23	17	0.7974	29	11	0.8202
Female	8	5		9	4	
Depth of invasion						
Tis, T1, T2	11	15	0.0190	18	8	0.6956
T3, T4	20	7		20	7	
Tumor size						
<40 mm	13	16	0.0265	18	11	0.0871
≥40 mm	18	6		20	4	
Lymph node status						
Positive	24	9	0.0069	27	6	0.0357
Negative	7	13		11	9	
Lymphatic invasion						
Positive	25	12	0.0414	30	7	0.0211
Negative	6	10		8	8	
Blood vessel invasion						
Positive	11	3	0.0755	12	2	0.1767
Negative	20	19		26	13	
TNM stage						
0	0	1	0.0074	0	1	0.0377
I	2	7		6	4	
II	6	8		8	5	
III	12	2		9	5	
IV	11	4		15	0	

p-value determined using the χ^2 exact test.

Conclusion

We have shown for the first time that PDGF-BB is a prognostic marker for human esophageal cancer. We found that PDGF-BB expression in human esophageal cancer was significantly associated with the depth of primary tumor, tumor size, lymph node metastasis and TNM stage. Furthermore, our data suggest that PDGF-BB may play a critical role, not only in esophageal cancer lymphangiogenesis, but also in cancer progression. Thus, development of an antagonist for PDGF-BB may be an important approach for control of tumor growth and metastasis of esophageal cancer.

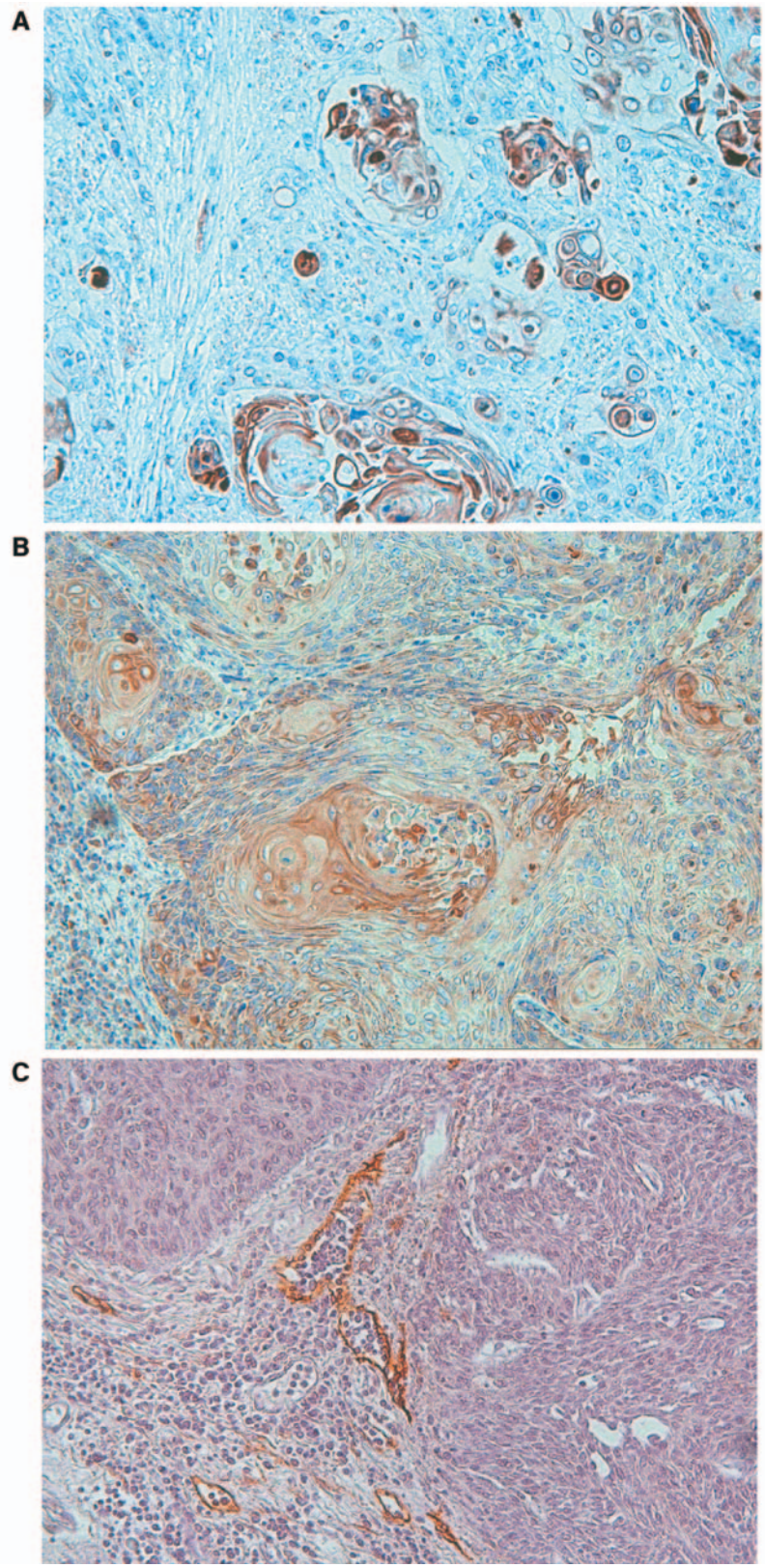

Figure 1. *Immunohistochemical staining of human esophageal cancer tissue for PDGF-BB (A) and VEGF-C (B). (Original magnification, x200) Immunohistochemical staining of lymphatic vessels for D2-40 (C). (Original magnification, x200).*

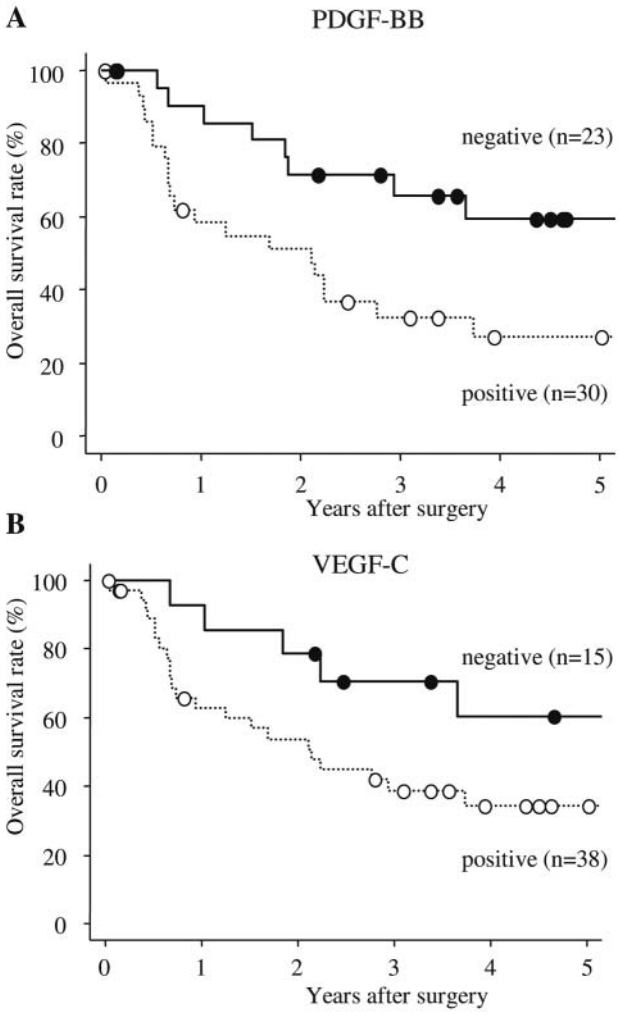

Figure 2. *A) Overall survival of 53 patients with esophageal cancer in relation to tumor PDGF-BB status. PDGF-BB-positive patients had a poorer prognosis than PDGF-BB negative patients (p=0.0314). B) Overall survival of 53 patients with esophageal cancer in relation to tumor VEGF-C status. Patients with VEGF-C positive tumors exhibited a tendency for lower survival rates, but this did not reach statistical significance (p=0.0934). The p-value was determined using the log-rank test.*

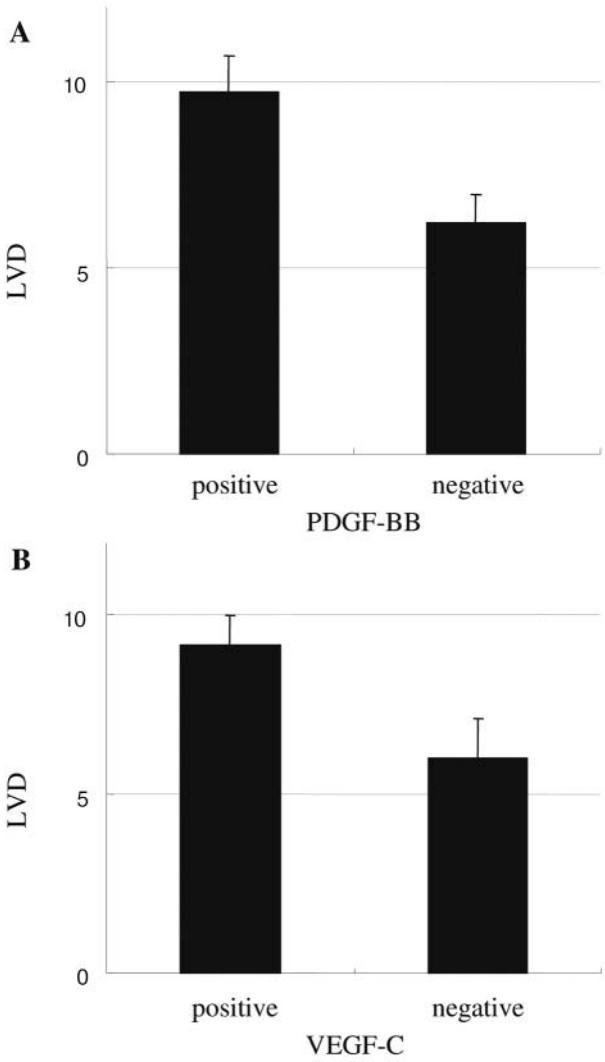

Figure 3. *A) The LVD in positive PDGF-BB tumors was significantly higher than in negative tumors (p=0.0124). B) The LVD in positive VEGF-C tumors was significantly higher than in negative tumors (p=0.0410). The p-value was determined by the Mann-Whitney U-test.*

References

1 Igaki H, Kato H, Tachimori Y, Sato H, Daiko H and Nakanishi Y: Prognostic evaluation for squamous cell carcinomas of the lower thoracic esophagus treated with three-field lymph node dissection. Eur J Cardiothorac Surg *19*: 887-893, 2001.

2 Igaki H, Tachimori Y and Kato H: Improved survival for patients with upper and/or middle mediastinal lymph node metastasis of squamous cell carcinoma of the lower thoracic esophagus treated with 3-field dissection. Ann Surg *239*: 483-490, 2004.

3 Kazlauskas A: A new member of an old family. Nat Cell Biol *2*: E78-79, 2000.

4 Heldin CH and Westermark B: Mechanism of action and *in vivo* role of platelet-derived growth factor. Physiol Rev *79*: 1283-1316, 1999.

5 Cao R, Bjorndahl MA, Religa P, Clasper S, Garvin S, Galter D, Meister B, Ikomi F, Tritsaris K, Dissing S, Ohhashi T, Jackson DG and Cao Y: PDGF-BB induces intratumoral lymphangiogenesis and promotes lymphatic metastasis. Cancer Cell *6*: 333-345, 2004.

6 Coltrera MD, Wang J, Porter PL and Gown AM: Expression of platelet-derived growth factor B-chain and the platelet-derived growth factor receptor beta subunit in human breast tissue and breast carcinoma. Cancer Res *55*: 2703-2708, 1995.

7 Achen MG, McColl BK and Stacker SA: Focus on lymphangiogenesis in tumor metastasis. Cancer Cell *7*: 121-127, 2005.

8 Cao Y: Opinion: emerging mechanisms of tumour lymphangiogenesis and lymphatic metastasis. Nat Rev Cancer *5*: 735-743, 2005.
9 Berse B, Brown LF, Van de Water L, Dvorak HF and Senger DR: Vascular permeability factor (vascular endothelial growth factor) gene is expressed differentially in normal tissues, macrophages, and tumors. Mol Biol Cell *3*: 211-220, 1992.
10 Ferrara N: Molecular and biological properties of vascular endothelial growth factor. J Mol Med *77*: 527-543, 1999.
11 Veikkola T and Alitalo K: VEGFs, receptors and angiogenesis. Semin Cancer Biol *9*: 211-220, 1999.
12 Lymboussaki A, Partanen TA, Olofsson B, Thomas-Crusells J, Fletcher CD, de Waal RM, Kaipainen A and Alitalo K: Expression of the vascular endothelial growth factor C receptor VEGFR-3 in lymphatic endothelium of the skin and in vascular tumors. Am J Pathol *153*: 395-403, 1998.
13 Kinoshita J, Kitamura K, Kabashima A, Saeki H, Tanaka S and Sugimachi K: Clinical significance of vascular endothelial growth factor-C (VEGF-C) in breast cancer. Breast Cancer Res Treat *66*: 159-164, 2001.
14 Dumoff KL, Chu C, Xu X, Pasha T, Zhang PJ and Acs G: Low D2-40 immunoreactivity correlates with lymphatic invasion and nodal metastasis in early-stage squamous cell carcinoma of the uterine cervix. Mod Pathol *18*: 97-104, 2005.
15 Sobin LH and Wittekind C: TNM classification of malignant tumours, 6th ed. New York: John Wiley & Sons Inc, 2002.
16 Cao Y, Linden P, Farnebo J, Cao R, Eriksson A, Kumar V, Qi JH, Claesson-Welsh L and Alitalo K: Vascular endothelial growth factor C induces angiogenesis *in vivo*. Proc Natl Acad Sci USA *95*: 14389-14394, 1998.
17 Witzenbichler B, Asahara T, Murohara T, Silver M, Spyridopoulos I, Magner M, Principe N, Kearney M, Hu JS and Isner JM: Vascular endothelial growth factor-C (VEGF-C/VEGF-2) promotes angiogenesis in the setting of tissue ischemia. Am J Pathol *153*: 381-394, 1998.
18 Skobe M, Hawighorst T, Jackson DG, Prevo R, Janes L, Velasco P, Riccardi L, Alitalo K, Claffey K and Detmar M: Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. Nat Med *7*: 192-198, 2001.
19 Kim R, Emi M, Arihiro K, Tanabe K, Uchida Y and Toge T: Chemosensitization by STI571 targeting the platelet-derived growth factor/platelet-derived growth factor receptor-signaling pathway in the tumor progression and angiogenesis of gastric carcinoma. Cancer *103*: 1800-1809, 2005.
20 George D: Platelet-derived growth factor receptors: a therapeutic target in solid tumors. Semin Oncol *28*: 27-33, 2001.
21 Zhang SX, Gozal D, Sachleben LR, Jr., Rane M, Klein JB and Gozal E: Hypoxia induces an autocrine-paracrine survival pathway *via* platelet-derived growth factor (PDGF)-B/PDGF-beta receptor/phosphatidylinositol 3-kinase/Akt signaling in RN46A neuronal cells. Faseb J *17*: 1709-1711, 2003.
22 Bunone G, Vigneri P, Mariani L, Buto S, Collini P, Pilotti S, Pierotti MA and Bongarzone I: Expression of angiogenesis stimulators and inhibitors in human thyroid tumors and correlation with clinical pathological features. Am J Pathol *155*: 1967-1976, 1999.
23 Tsurusaki T, Kanda S, Sakai H, Kanetake H, Saito Y, Alitalo K and Koji T: Vascular endothelial growth factor-C expression in human prostatic carcinoma and its relationship to lymph node metastasis. Br J Cancer *80*: 309-313, 1999.
24 Yonemura Y, Endo Y, Fujita H, Fushida S, Ninomiya I, Bandou E, Taniguchi K, Miwa K, Ohoyama S, Sugiyama K and Sasaki T: Role of vascular endothelial growth factor C expression in the development of lymph node metastasis in gastric cancer. Clin Cancer Res *5*: 1823-1829, 1999.
25 Ichikura T, Tomimatsu S, Ohkura E and Mochizuki H: Prognostic significance of the expression of vascular endothelial growth factor (VEGF) and VEGF-C in gastric carcinoma. J Surg Oncol *78*: 132-137, 2001.
26 Akagi K, Ikeda Y, Miyazaki M, Abe T, Kinoshita J, Maehara Y and Sugimachi K: Vascular endothelial growth factor-C (VEGF-C) expression in human colorectal cancer tissues. Br J Cancer *83*: 887-891, 2000.
27 Kajita T, Ohta Y, Kimura K, Tamura M, Tanaka Y, Tsunezuka Y, Oda M, Sasaki T and Watanabe G: The expression of vascular endothelial growth factor C and its receptors in non-small cell lung cancer. Br J Cancer *85*: 255-260, 2001.
28 Ueda M, Terai Y, Yamashita Y, Kumagai K, Ueki K, Yamaguchi H, Akise D, Hung YC and Ueki M: Correlation between vascular endothelial growth factor-C expression and invasion phenotype in cervical carcinomas. Int J Cancer *98*: 335-343, 2002.
29 Nishida N, Yano H, Komai K, Nishida T, Kamura T and Kojiro M: Vascular endothelial growth factor C and vascular endothelial growth factor receptor 2 are related closely to the prognosis of patients with ovarian carcinoma. Cancer *101*: 1364-1374, 2004.
30 Hachisuka T, Narikiyo M, Yamada Y, Ishikawa H, Ueno M, Uchida H, Yoriki R, Ohigashi Y, Miki K, Tamaki H, Mizuno T and Nakajima Y: High lymphatic vessel density correlates with overexpression of VEGF-C in gastric cancer. Oncol Rep *13*: 733-737, 2005.
31 Padera TP, Stoll BR, Tooredman JB, Capen D, di Tomaso E and Jain RK: Pathology: cancer cells compress intratumour vessels. Nature *427*: 695, 2004.

Received February 26, 2007
Revised May 14, 2007
Accepted May 17, 2007