Clinicopathological Significance of Vascular Endothelial Growth Factor-C in Breast Carcinoma with Long-Term Follow-Up

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Expression of angiogenic and lymphangiogenic factors by tumors may influence the route of metastatic spread. The angiogenic factor vascular growth factor-C (VEGF-C) is implicated in the development of lymphatic vessels and promotion of lymphatic metastasis. The purpose of this study was to determine whether VEGF-C correlates with lymph node metastasis or prognosis. We assessed VEGF-C expression using immunohistochemistry in 123 invasive breast carcinomas with long-term follow-up. The relationship between VEGF-C expression and lymph node status and other established clinicopathological parameters was assessed. Whether VEGF-C expression plays a prognostic role in breast cancer was also investigated. VEGF-C expression was identified in 103 cases (83.7%). Positive VEGF-C was significantly correlated with lymph node metastasis ($P = 0.0131$). Survival curves determined by the Kaplan-Meier method and univariate analysis demonstrated that positive VEGF-C was associated with both disease-free survival ($P = 0.0165$) and overall survival ($P = 0.0175$). On the basis of our findings, VEGF-C plays a crucial role in lymph node metastasis and may be a significant prognostic factor for long-term survival in breast cancer.

KEY WORDS: Breast cancer, Immunohistochemistry, Metastasis, Prognosis, VEGF-C.

Mod Pathol 2003;16(4):309–314

VOL. 16, NO. 4, P. 309, 2003 Printed in the U.S.A.
Date of acceptance: January 8, 2003.
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DOI: 10.1097/01.MP.0000062858.98295.9F

The vascular endothelial growth factor (VEGF) family is a group of growth factors that regulate the growth of endothelial cells (1, 2). The well-known member VEGF-A plays essential roles in vasculogenesis and angiogenesis (3), and its crucial role in tumor angiogenesis and blood-born metastasis has been documented in a variety of cancers (4). Recently, VEGF-C, a novel VEGF member, has been found to induce not only angiogenesis but also lymphangiogenesis via VEGF receptor-2 (VEGF-R2) and VEGF receptor-3 (VEGF-R3; 5, 6). Because VEGF-R3 has been demonstrated to be expressed almost exclusively in the lymphatic endothelium and thus considered to be a major regulator of lymphangiogenesis (7, 8), VEGF-C appears to be an important lymphangiogenic factor. The correlation of VEGF-C expression with lymph node metastasis has been reported in many malignant tumors (9–19).

In breast carcinoma, tumor involvement of the axillary lymph nodes is considered to be the most important prognostic factor (20). VEGF-C was strongly correlated with lymph node metastasis in animal model (21); however, the relationship between VEGF-C expression and lymph node metastasis has not been established in clinical trials (22–24).

In this study, we analyzed the relationship between expression of VEGF-C and clinicopathological features in breast cancer with a long-term follow-up. Our studies showed that the expression of VEGF-C was strongly correlated with lymph node metastasis and patients' prognosis.

MATERIALS AND METHODS

Patients and Tumor Samples

The study included 123 women with invasive breast cancer that was diagnosed and treated in the

Osaka Police Hospital, Osaka, Japan between 1981 and 1992. The age ranged from 24 to 87 years (mean 51 years). The median follow-up period of the patients was 109 months (range, 5.5 to 230 months). All women were apparently free of distant metastasis and underwent curative surgery. Patient and tumor characteristics are shown in Table 1. The pathologic classification of each sample was confirmed by a review of hematoxylin and eosin–stained sections.

Immunohistochemistry

The primary antibody used in study was an anti–VEGF-C goat polyclonal antibody (R & D Systems, Inc, Minneapolis, MN). Paraffin sections that were 4 μm thick were deparaffinized and autoclaved for antigen retrieval. Then they were placed in a solution of absolute methanol and 3% hydrogen peroxidase for 5 minutes. They were subsequently washed in phosphate-buffered saline (PBS) and treated for 20 minutes with Protein Block Serum-free (DAKO Co, Carpinteria, CA). The sections were treated with anti-VEGF-C antibody diluted 1:400 in PBS and then were incubated at 4° C in a humidified chamber. After the overnight treatment, the slides were incubated with Histofine Simple Stain MAX PO (G; NICHIREI, Tokyo, Japan) for 60 minutes according to the manufacturer's instructions. The color was developed using diaminobenzidine with 0.01% hydrogen peroxidase. Counterstaining was done with hematoxylin. For the negative control, all reagents except for the primary antibody were used. Evaluation of VEGF-D immunoreactivity was carried out independently by two investigators who did not have clinical or laboratory knowledge of the patients. The immunostained sections were scanned by light microscopy, and all of the tumor cells were evaluated. Only cases in which ≥10% of tumor cells were immunoreactive were scored as positive according to Kinoshita *et al.* (22).

TABLE 1. The Relationship between VEGF-C Expression and Other Parameters

Factor	VEGF-C		*P* Value
	Negative	Positive	
Age			0.2189
<50	14	54	
≥51	6	49	
Histology			0.0006
IDC	14	100	
Others	6	3	
Tumor size			0.1157
≤2 cm	10	29	
>2 cm	10	69	
Lymph node metastasis			0.0131
Negative	15	44	
Positive	5	59	
ER			0.1445
Negative	5	44	
Positive	15	58	
PgR			0.1474
Negative	6	49	
Positive	14	52	
c-erbB-2			0.0575
Negative	18	69	
Positive	2	34	
p53			0.4430
Negative	15	64	
Positive	5	38	
Grade			0.4643
I and II	13	56	
III	7	47	

Covariates

Information about the patients' clinical history was obtained from the patients' medical records. The immunostaining results of estrogen receptor (ER), progesterone receptor (PgR), p53, and c-erbB-2 were obtained from our pathological data files. The size of the primary tumor was considered to be the largest tumor diameter observed after surgical excision. Lymph node status was determined by counting the number of axillary lymph nodes with histological evidence of metastatic breast carcinoma. Histological typing and nuclear grading were performed as described elsewhere (25, 26), respectively.

Statistics

Fisher's exact test was used to examine the association between different variables and clinicopathological factors. DFS (disease-free survival) curves and OS (overall survival) curves were obtained using the Kaplan-Meier method and compared using the log-rank test. A multivariate model using the Cox stepwise regression analysis was used to evaluate the statistical strength of independent association between covariates and DFS and/or OS. A *P* value of $<.05$ was considered significant. A computer program package (StatView 5.0, Abacus Concepts, Berkeley, CA) was used for all statistical testing and management of the database.

RESULTS

VEGF-C Expression in Breast Cancer Tissue

In breast cancer cells, expression of VEGF-C protein was observed in the cytoplasm. The VEGF-C staining is heterogenic. In some cases, almost all invasive cells were immunopositive for VEGF-C (Fig. 1A), whereas in others, the invasive cells were negative (Fig. 1B). According to the criteria for VEGF-C immunostaining evaluation, VEGF-C protein was positive in 83.7% (103/123) of the breast cancer patients. In addition, VEGF-C expression was often more intense in the invasive edge and/or intraductal component (Fig 1C). In contrast, very little or no staining was observed in normal mam-

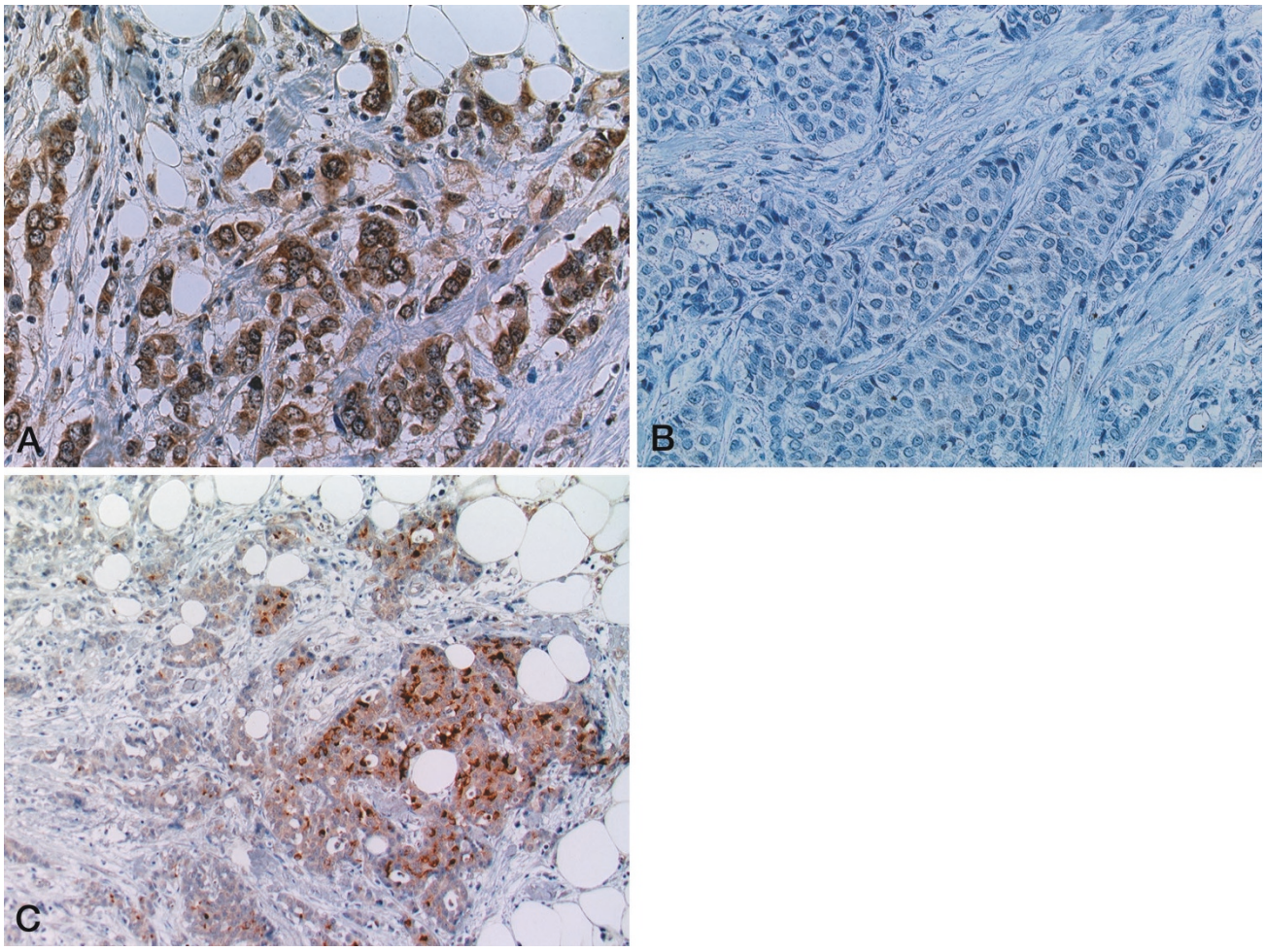

FIGURE 1. Vascular endothelial growth factor-C (VEGF-C) expression patterns in invasive breast carcinomas. **A,** almost all tumor cells show diffuse and intense immunostaining. **B,** the tumor cells are completely unreactive. **C,** VEGF-C expression was often more intense in the invasive edge.

mary cells surrounding the affected ducts, although a weak signal was seen in unaffected normal ductal epithelium occasionally.

Correlations between VEGF-C Expression and Clinicopathological Factors

The correlation between VEGF-C expression and clinicopathological factors was summarized in Table 1. VEGF-C expression was more frequently found in tumors with lymph node metastasis than in those without it ($P = .0131$). There was no significant correlation between VEGF-C expression and the age of the patients, tumor size, hormone receptor status, c-erbB-2 status, p53 status, or nuclear grading.

VEGF-C and Patients' Survival

The survival analysis was performed on all the patients and took into account the following variables: VEGF-C, patient's age, histological type, tumor size, lymph node status, hormonal status, c-erbB-2 status, p53 status, and nuclear grade. Univariate survival analysis showed that tumor size, lymph node status, ER, and VEGF-C were of significant prognostic value for DFS (Table 2; Fig. 2A); tumor size, lymph node status, PgR, and VEGF-C were of significant prognostic value for OS (Table 3; Fig. 2B). Based on multivariate cox regression analysis, only lymph node status was identified as an independent prognostic factor (DFS, $P = .004$; OS, $P = .0079$). We failed to identify VEGF-C expression as an independent prognostic factor for DFS and OS (data not shown).

DISCUSSION

In the present study, we found that VEGF-C was up-regulated in invasive breast cancer. VEGF-C expression was often more intense in the invasive edge, where release of angiogenic factors would be anticipated (27) and/or peritumoral lymphangiogenesis would be induced (28).

A previous report showed that VEGF-C mRNA expression in estrogen-dependent breast cancer cell line MCF-7 (28) is down-regulated by estrogen

TABLE 2. Univariate Analysis of Disease-Free Survival (DFS) by Various Clinicopathological Factors Factor

Factor	Number	DFS	
		Number of Recurrences (%)	P Value
Age			0.3559
<50	66	21 (31.8)	
≥51	52	20 (38.4)	
Histology			0.4879
IDC	109	39 (35.7)	
Others	9	2 (32.2)	
Tumor size			0.0048
≤2 cm	39	6 (15.3)	
>2 cm	76	33 (43.4)	
Lymph node metastasis			<0.0001
Negative	59	9 (15.2)	
Positive	59	32 (54.2)	
ER			0.0354
Negative	48	21 (43.7)	
Positive	69	19 (27.5)	
PgR			0.2210
Negative	53	20 (37.7)	
Positive	63	19 (30.1)	
c-erbB-2			0.2981
Negative	83	27 (32.5)	
Positive	35	14 (40.0)	
p53			0.7600
Negative	74	27 (36.4)	
Positive	43	13 (30.2)	
Grade			0.7587
I and II	66	23 (34.8)	
III	52	18 (34.6)	
VEGF-C			0.0165
Negative	20	2 (10.0)	
Positive	98	39 (39.7)	

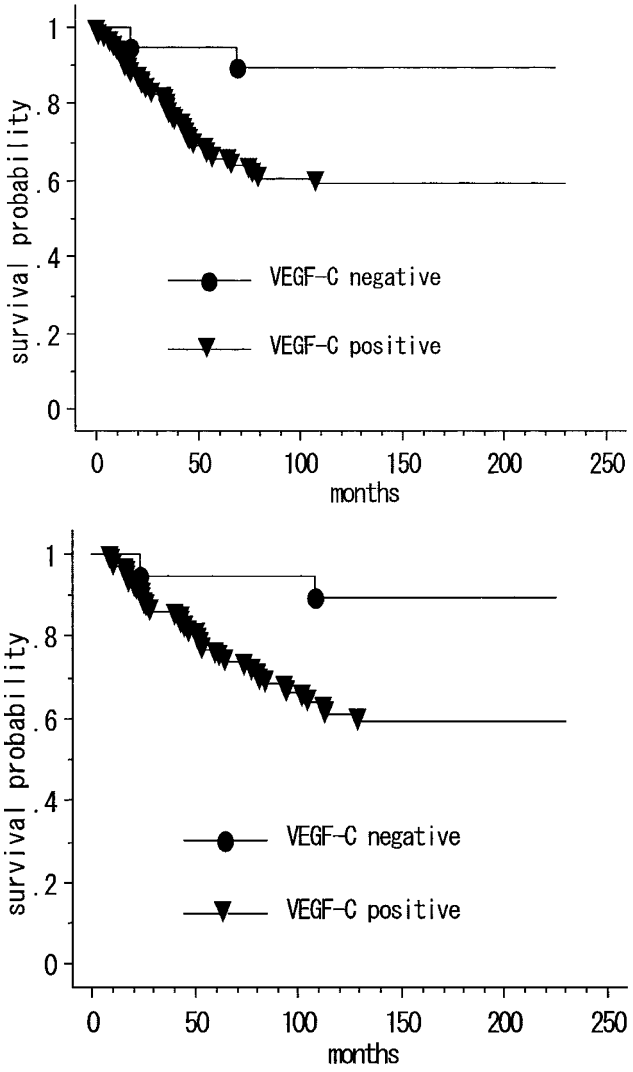

FIGURE 2. DFS (**A**) and OS (**B**) curves stratified according to vascular endothelial growth factor-C (VEGF-C) expression.

(29). However, we could not find any relationship between VEGF-C expression and ER status in our present study.

Lymph node metastasis is the oldest and most reliable prognostic indicator in breast carcinoma. A recent hypothesis is that most breast carcinomas are systemic from the onset, but the axillary lymph node status still has major prognostic implications (30–33). Therefore, it is understandable that in evaluation of the clinical stage of breast carcinoma and, consequently, therapy and outcome, emphasis continues to be placed on axillary lymph node status. Our findings showed that VEGF-C expression was associated with lymph node metastasis in breast cancer. Our results are consistent with a recent report in a Balb-cA Bom-nu mice model that VEGF-C–induced lymphangiogenesis is strongly correlated with dissemination to regional lymph nodes (29). However, Kinoshita *et al.* (22) and Gunningham *et al.* (23) did not obtain a significant relationship between VEGF-C expression and lymph node metastasis. Notably, Gunningham *et al.* (23) used RT-PCR assay for their study. As seen in our present study, VEGF-C expression could also be observed in normal breast epithelium. Therefore, RT-PCR assay could not reveal tumor VEGF-C expression correctly if microdissection was not performed. The discrepancy between our findings and those reported by Kinoshita *et al.* (22) may be due to use of different antibodies.

It is very important for practical medical purposes to clarify whether VEGF-C expression will really prove to be a prognostic indicator for breast carcinoma. In our results, survival curves determined by the Kaplan-Meier method and univariate analysis demonstrated that VEGF-C expression was associated with both DFS and OS; however, we failed to identify VEGF-C expression as an independent prognostic factor for DFS and OS. Previous study shows that VEGF-C–induced lymphangiogenesis is strongly correlated with dissemination to regional lymph nodes in animal model (29). Based on these results, VEGF-C positivity is the predominant predictor of nodal positivity. The predictive value of VEGF-C for OS and DFS was dependent on lymph node metastasis. However, Kinoshita *et al.* (22) and Yang *et al.* (24) did not obtain a significant relationship between VEGF-C expression and prog-

TABLE 3. Univariate Analysis of Overall Survival (OS) by Various Clinicopathological Factors

Factor	Number	OS	
		Number of Deaths (%)	P Value
Age			0.1414
<50	68	19 (28.0)	
≥50	55	21 (38.2)	
Histology			0.1866
IDC	114	39 (34.2)	
Others	9	1 (11.1)	
Tumor size			0.0327
≤2 cm	39	7 (18.0)	
>2 cm	79	29 (36.3)	
Lymph node metastasis			0.0002
Negative	59	11 (18.7)	
Positive	64	29 (45.3)	
ER			0.1997
Negative	49	18 (36.7)	
Positive	73	21 (28.7)	
PgR			0.0313
Negative	55	22 (40.0)	
Positive	66	16 (24.2)	
c-erbB-2			0.1108
Negative	87	25 (28.7)	
Positive	36	15 (41.6)	
p53			0.6106
Negative	79	27 (34.1)	
Positive	43	12 (27.9)	
Grade			0.8258
I and II	69	24 (34.7)	
III	54	16 (29.7)	
VEGF-C			0.0175
Negative	20	2 (10.0)	
Positive	103	38 (36.8)	

nosis. The discrepancy between our findings and those reports may be due to use of different antibodies.

In conclusion, VEGF-C expression may play a crucial role in lymph node metastasis of breast cancers. Furthermore, VEGF-C expression serves as a significant prognostic factor for long-term survival in breast cancer. It is possible that VEGF-C will become a target for anti-angiogenic therapy for breast cancer.

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Book Review

Pai GS, Lewandowski RC Jr., Borgaonkar DS: Handbook of Chromosomal Syndromes, 376 pp, New York, Wiley-Liss, 2002 ($99.95).

Every medical student knows that the Down syndrome is linked to trisomy of chromosome 21. Few of us do, however, realize that it took 93 years for this fact to become established—from 1866 when the Down syndrome was first described until 1959 when the trisomy 21 was discovered. Since then hundreds of cytogenetic abnormalities have been discovered, and chromosomal analysis has become a routine test in evaluating many childhood diseases.

In this book the authors present the most important chromosomal developmental syndromes. The disorders are listed by the affected chromosome number from 1 to 22 and the sex chromosomes. The clinical features of each syndrome are briefly outlined and illustrated with black and white photographs. The text is sketchy but to the point and informative. Key references also are included.

This book could be used for quick reference by neonatologists, genetic counselors, and general pediatricians. It also deserves to be included in hospital and departmental libraries.

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