Prognostic Significance of Vascular Endothelial Growth Factor D in Breast Carcinoma with Long-Term Follow-Up

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ABSTRACT

Purpose: Expression of angiogenic and lymphangiogenic factors by tumors may influence the route of metastatic spread. The angiogenic factor vascular endothelial growth D (VEGF-D) is implicated in the development of lymphatic vessels and promotion of lymphatic metastases. The purpose of this study is to determine whether VEGF-D correlates with lymph node metastasis or prognosis.

Experimental Design: We assessed VEGF-D expression using immunohistochemistry in 105 invasive breast carcinomas with long-term follow-up. The relationship among VEGF-D expression, lymph node status, and other established clinicopathological parameters was assessed. Whether VEGF-D expression plays prognostic role in breast cancer was also investigated.

Results: VEGF-D expression was identified in 86 cases (81.9%). Positive VEGF-D was significantly correlated with lymph node metastasis (P = 0.0238) and high c-erbB-2 expression (P = 0.0211). Survival curves determined by the Kaplan-Meier method and univariate analysis demonstrated that positive VEGF-D was associated with both disease-free survival (P = 0.0023) and overall survival (P = 0.0222). In multivariate analysis using the Cox regression model, positive emerged as an independent indicator for disease-free survival (P = 0.0452).

Conclusions: VEGF-D expression is associated with lymph node metastasis and may be a novel prognostic factor in breast cancer. VEGF-D may be useful in the treatment of breast cancer as a decision-making biomarker for aggressive treatment after operation.

INTRODUCTION

Breast cancer is one of the most common malignancies among women, and its cumulative risk by age 85 is 1 in 8 women in the United States and 1 in 40 women in Japan (1). Some patients at an early stage of breast carcinoma can be cured by surgery, but ~50% of patients die of carcinoma recurrence, even when they have undergone curative surgery and adjuvant chemotherapy (2). Recurrence probably arises from the growth of occult micrometastasis that have already been established by the time of surgery. Breast cancer could metastasize via the blood stream or the lymphatic vasculature, but the mechanisms that determine the route of metastatic spread are largely unknown.

Tumor metastasis may depend on the capacity of tumor cells to induce angiogenesis and/or lymphangiogenesis, and VEGF-D has been demonstrated to be involved in promoting tumor angiogenesis and lymphangiogenesis (3). VEGF-D was first isolated from a differential display screening of murine genes expressed in fibroblasts from normal mice but not expressed in fibroblasts from mice carrying a targeted inactivation of the c-fos gene (4). The identified protein was first denoted c-fos-induced growth factor, but was later renamed as VEGF-D. VEGF-D consists of a central receptor-binding domain, called the VHD, and NH2- and COOH-terminal propeptides, which are proteolytically cleaved to generate a mature form consisting only of the VHD (5). The mature VHD binds both VEGFR-2 and VEGFR-3 with much greater affinity than the unprocessed form. Therefore, proteolytic processing is important for activating (5). The COOH-terminal propeptide of VEGF-D is cysteine-rich; the location of many of the cysteine residues resembles the spacing of the repeat units found in the Balbiani ring 3 protein, a protein synthesized in the salivary glands of the midge Chironomus tentans and which is thought to be involved in the process of forming larval silk (6, 7). VEGF-D may play a role in embryonic development and tumor biology, as it is angiogenic (5) and lymphangiogenic (3).

To date, only few clinicopathological studies on VEGF-D expression in malignant tumors have been reported (8, 9). VEGF-D has been demonstrated to be an independent prognostic marker for survival in colorectal carcinoma (10). In this study, we examine the expression of VEGF-D in breast carcinoma and investigate the relationship between VEGF-D expression and lymph node status, as well as other established clinicopathological parameters. Furthermore, we would also like to examine whether the immunohistochemical detection of VEGF-D has any value or relevance with respect to predicting the disease course.
the slides were washed in 10 mM PBS following by Protein
anol solution containing 3% hydrogen peroxide for 5 min, and
endogenous peroxidase activity was blocked in absolute meth-
xylene and rehydrated through a graded alcohol series. Then,
Paraffin sections on silane-coated slides were dewaxed with
which contained representative histology of the breast cancer.

Table 1 The relationship between VEGF-D expression and other
parameters

<table>
<thead>
<tr>
<th>Factors</th>
<th>VEGF-D</th>
<th>P</th>
</tr>
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<tbody>
<tr>
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<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 50</td>
<td>12 (22%)</td>
<td>42 (78%)</td>
</tr>
<tr>
<td>≥ 50</td>
<td>7 (14%)</td>
<td>44 (86%)</td>
</tr>
<tr>
<td>Histology</td>
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<td></td>
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<tr>
<td>IDC</td>
<td>14 (15%)</td>
<td>82 (85%)</td>
</tr>
<tr>
<td>Others</td>
<td>5 (56%)</td>
<td>4 (44%)</td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 2 cm</td>
<td>9 (24%)</td>
<td>28 (76%)</td>
</tr>
<tr>
<td>&gt; 2 cm</td>
<td>10 (15%)</td>
<td>58 (85%)</td>
</tr>
<tr>
<td>Nodal metastasis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>14 (27%)</td>
<td>38 (73%)</td>
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<tr>
<td>Positive</td>
<td>5 (9%)</td>
<td>48 (91%)</td>
</tr>
<tr>
<td>Grade</td>
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<td></td>
</tr>
<tr>
<td>I and II</td>
<td>14 (22%)</td>
<td>49 (78%)</td>
</tr>
<tr>
<td>III</td>
<td>5 (12%)</td>
<td>37 (88%)</td>
</tr>
<tr>
<td>ER</td>
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<tr>
<td>Negative</td>
<td>6 (14%)</td>
<td>36 (86%)</td>
</tr>
<tr>
<td>Positive</td>
<td>13 (21%)</td>
<td>49 (79%)</td>
</tr>
<tr>
<td>PgR</td>
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<td></td>
</tr>
<tr>
<td>Negative</td>
<td>6 (14%)</td>
<td>38 (79%)</td>
</tr>
<tr>
<td>Positive</td>
<td>13 (22%)</td>
<td>47 (78%)</td>
</tr>
<tr>
<td>P53</td>
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<tr>
<td>Negative</td>
<td>13 (20%)</td>
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</tr>
<tr>
<td>Positive</td>
<td>6 (16%)</td>
<td>31 (84%)</td>
</tr>
<tr>
<td>c-erbB-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>18 (23%)</td>
<td>59 (77%)</td>
</tr>
<tr>
<td>Positive</td>
<td>1 (4%)</td>
<td>27 (96%)</td>
</tr>
</tbody>
</table>

PATIENTS AND METHODS

Patients and Samples. Paraffin-embedded tissue was
obtained from 105 patients who underwent surgery in Osaka
Police Hospital, Osaka, Japan between 1981 and 1991. All had
histological evidence of invasive breast cancer, and none had a
family history in first-degree relatives as judged by questioning
at the time of admission for surgery. Patient and tumor charac-
teristics are shown in Table 1. All of the patients had received
mastectomy with axillary lymph node dissection. Seventy-three
(70%) received postoperative adjuvant therapy consisting of
combination chemotherapy and tamoxifen treatment. Twenty-
seven patients only received chemotherapy, 3 patients only
received tamoxifen, and 2 patients have not received any adju-
vant treatment. Only 2 patients had postoperative radiotherapy.

Immunohistochemical Studies. For the immunohisto-
chemical study, 4-μm sections were cut from paraffin blocks,
which contained representative histology of the breast cancer.
Paraffin sections on silane-coated slides were dewaxed with
xylene and rehydrated through a graded alcohol series. Then,
endogenous peroxidase activity was blocked in absolute meth-
anol solution containing 3% hydrogen peroxide for 5 min, and
the slides were washed in 10 mM PBS following by Protein
Block Serum-free (DAKO, Carpinteria, CA) treatment for 20
min. The slides were reacted with an anti-VEGF-D monoclonal
antibody (R & D Systems, Inc., Minneapolis, MN) with a
dilution of 1:1000 overnight at 4°C in a humidified chamber.
After the overnight treatment, to avoid the nonspecific biotin
reaction, we used Histofine Simple Stain MAX (NICHIREI,
Tokyo, Japan) as second antibody for 60 min according to the
manufacturer’s instructions. The immunoreactions were visual-
ized with diaminobenzidine as chromogen. Counterstaining was
performed using hematoxylin. For the negative control, all of
the reagents except for the primary antibody were used.

Evaluation of VEGF-D immunoreactivity was carried out
independently by three investigators without clinical or labora-
tory knowledge of the patients. Cytoplasmic labeling of tumor
cells was classified as either negative (if no staining or positive
staining was present in <10% of tumor cells) or positive (if
≥10% of tumor cells stained positively).

Covariates. Information about the patient clinical history
was obtained from the patient medical records. The immuno-
staining results of ER, PgR, p53, and c-erbB-2 were obtained
from our pathological data files. Age at diagnosis was consid-
ered as the age of the patient. The size of the primary tumor was
the largest tumor diameter observed after surgical excision. Lymph
node status was determined with histological evidence of
metastatic breast carcinoma. Histological typing and nuclear
grading were carried out according to the WHO classification
(11) and Protocol of the Japan National Surgical Adjuvant Study
of Breast Cancer Pathology Section (12), respectively.

Statistical Analysis. Descriptive statistics comparing
VEGF-D expression with conventional markers of tumor ag-
gressiveness were analyzed by standard χ² tests or Fisher’s
exact test. Estimates of DFS and OS were calculated by the
Kaplan-Meier product-limit method and the differences as-
essed by the log rank test. Probabilities of DFS and OS were
calculated from the date of breast carcinoma diagnosis to either
the date at which relapse from breast carcinoma was clinically
identified or the date of last contact. Multivariate survival anal-
ysis using the Cox proportional hazard regression model was
carried out to assess the independent contribution of each vari-
able to DFS and OS. All of the Ps were two-tailed, and the 0.05
level was considered statistically significant. A computer pro-
gram package (StatView 5.0; Abacus Concepts, Berkeley, CA)
was used for all of the statistical testing and management of the
database.

RESULTS

Demographics and Clinical Data. The median age at
diagnosis for the 105 subjects was 51 years (range, 24–87
years). Fifty-one percent of the patients were <50 years
(54), and 51% (n = 53) of the patients had lymph node metas-
tases at the time of surgery. Median follow-up time for the 105
subjects was 114 months (range, 10–229 months). Forty-four
subjects had relapsed by the time of last follow-up. Thirty-three
patients died of breast carcinoma.

VEGF-D Expression is Up-regulated in Breast Carci-
noma. Immunohistochemical localization of VEGF-D protein
was cytoplasmic. Tumor VEGF-D expression was heterogene-
ous, and frequently up-regulated at the infiltrating tumor edge
and intraductal component. Immunoreactivity was completely
absent in some tumors, whereas in others the number of immu-
noreactive cells ranged from very few to almost all of the tumor
cells (Fig. 1). According to the criteria for VEGF-D immuno-
histochemical evaluation, VEGF-D-positive staining was de-
tected in 86 cases (81.9%). Occasionally, normal mammary
Immunohistochemical analysis for VEGF-D in breast cancer. A, most tumor cells showed diffuse cytoplasmic staining for VEGF-D. B, the tumor cells showed immunonegativity to VEGF-D. C, the immunonegativity was up-regulated at the infiltrating tumor edge.
cells and stromal components including fibroblasts were also positive.

**Associations between VEGF-D and Conventional Prognostic Factors.** We compared the VEGF-D expression with the clinicopathological profiles and immunostaining of the other biological markers of the 105 patients with breast cancer. The profile included age, histological typing, primary tumor size, nodal involvement, and nuclear grading. Biological markers, of which the importance has been well established in sporadic breast cancers, include ER and PgR for hormone dependency, and p53 and c-erbB-2 for biological prognostic indicators. As shown in Table 1, positive VEGF-D expression was significantly associated with lymph node metastasis \((P = 0.0238)\) and c-erbB-2 overexpression. We found no significant association between the VEGF-D expression and the other parameters.

**VEGF-D in Univariate and Multivariate Analysis of Survival.** The survival analysis was performed on 105 patients and took into account the following variables: VEGF-D, p53, ER, PgR, c-erbB-2, histological type, nuclear grade, tumor size, lymph node status, and patient age. As shown in Table 2, univariate analysis focusing on DFS revealed axillary lymph node status \((P = 0.0001)\), log rank test), tumor size \((P = 0.0008)\), log rank test), VEGF-D \((P = 0.0023)\), log rank test), and c-erbB-2 \((P = 0.0198)\), log rank test) to be significant prognostic factors. There was a statistically significant difference in both DFS and OS between patients with tumors showing positive VEGF-D immunoreactivity and those whose tumors did not (Fig. 2). To exclude the possibility that treatment regimen influence the prognostic analysis for VEGF-D, we also analyzed the imbalance of surgical and adjuvant treatment in the VEGF-D-negative and -positive cohorts, but no significant difference could be found (data not shown).

Multivariate Cox regression analysis of the lymph node status, tumor size, VEGF-D expression, and c-erbB-2 expression focusing on DFS identified VEGF-D as an independent statistically prognostic factor (Table 3). The odds ratio for VEGF-D is 4.392. The risk of patients with VEGF-D-positive relapse within a specific time was 4 times as high than the risk of patients (to relapse within the same time course) with VEGF-D-negativity. On the basis of Multivariate Cox regression analysis, we failed to identify VEGF-D immunostaining as an independent prognostic factor for OS.

**DISCUSSION**

The dissemination of malignant cells from the primary tumor to local tissue or to distant organs via the lymphatic or blood stream is an important characteristic of cancer progression (13). Understanding the metastatic mechanisms is central to developing treatments for cancer. VEGF-D has been the focus...
of recent debate with regard to their potential role in promoting tumor angiogenesis and lymphangiogenesis.

In this study, we found that VEGF-D was up-regulated in both invasive elements and intraductal component of breast tumors. Accentuation of VEGF-D was often present at the infiltrating tumor margin, in which release of angiogenic factors would be anticipated (14). These findings support that VEGF-D, secreted by tumor cells, binds target endothelial cells to regulate tumor angiogenesis. VEGF-D is a tumor angiogenesis factor, probably because of its ability to activate VEGFR-2 (15), but is also inducing the formation of lymphatics, presumably through the lymphatic receptor VEGFR-3, although activation of VEGFR-3-VEGFR-2 heterodimers cannot be excluded. Previous studies have suggested (4, 16) that VEGF-D plays some roles in cancer progression. At present, little is known about whether factors such as hypoxia, growth factors, cytokines, and hormones regulate expression of VEGF-D. In our present study, VEGF-D expression is not related to ER or PgR expression, which suggests that VEGF-D is not regulated by estrogen or progesterone.

Our results also suggest that VEGF-D may play a role in lymphangiogenesis, because we found that positive VEGF-D was correlated with lymph node metastasis. Our findings are consistent with a recent report in animal models (3). However, there is a discrepancy between our findings and those reported in lung adenocarcinoma, which showed that expression of VEGF-D was inversely correlated with metastatic spread to lymph nodes (9). Notably, Niki et al. (9) have assessed VEGF-D expression on clinical samples based on reverse-transcription PCR analysis. As seen in our present study, VEGF-D may also be observed in normal epithelium and/or stromal cells. Therefore, VEGF-D expression based on reverse-transcription PCR analysis may be not able to reveal the real tumor VEGF-D levels if microdissection was not used. Immunohistochemical analysis may provide a more accurate evaluation for VEGF-D expression in solid tumors. In addition, as mentioned by Stacker et al. (3), analysis of VEGF-D in tumors based on RNA levels might be misleading, as VEGF-D must be proteolytically processed to bind VEGFR-2 and VEGFR-3 with high affinity. Expression of the gene encoding VEGF-D in tumors in the absence of processing may lead to accumulation of biologically inactive VEGF-D in the tumor (3).

Lymph node metastasis is the oldest and most reliable prognostic indicator in breast carcinoma. A more recent hypothesis is that most breast carcinomas are systemic from the onset, but the axillary lymph nodes status still has major prognostic implications (17–20). 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. On the basis of our present study, VEGF-D expression may be a novel predictive factor for axillary lymph node metastasis.

It is very important for practical medical purpose to clarify whether VEGF-D expression will really prove to be a prognostic indicator for breast cancer. Notably, survival curves determined by the Kaplan-Meier method and univariate analysis demonstrated that positive VEGF-D was associated with both DFS and OS. Furthermore, multivariate analysis using the Cox proportional hazard model demonstrated that positive VEGF-D expression was still related to poor DFS after consideration of other prognostic factors. Thus, VEGF-D expression appears to be a reliable prognostic biomarker. Although adjuvant chemotherapy and hormonal therapy improve survival of radically resected breast cancer, ~42% (44 cases) of all patients eventually relapsed in our present population, and resistance to anticancer agents is thought to be responsible for chemotherapy failures in breast cancer. Notably, 42 of the 44 relapsed cases (95%) showed positive VEGF-D expression. Antiangiogenic therapies

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Results of multivariate Cox regression analysis for DFS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variable</td>
<td>Odds ratio (95% CI)</td>
</tr>
<tr>
<td>Lymph node status</td>
<td></td>
</tr>
<tr>
<td>Negative (referent)</td>
<td>1.0</td>
</tr>
<tr>
<td>Positive</td>
<td>7.197 (2.889–17.926)</td>
</tr>
<tr>
<td>VEGF-D</td>
<td></td>
</tr>
<tr>
<td>Negative (referent)</td>
<td>1.0</td>
</tr>
<tr>
<td>Positive</td>
<td>4.392 (1.032–18.692)</td>
</tr>
</tbody>
</table>

Fig. 2 Association of VEGF-D expression with patient prognosis in breast cancer (Kaplan-Meier method and log rank test). Positive VEGF-D is significantly related to recurrence (A, P = 0.0023) and death (B, P = 0.0222).
using antagonistic strategies to block signaling by the VEGF-D may be important in treatment of these patients.

In conclusion, VEGF-D expression may play a crucial role for lymph node metastasis of breast cancers. Our data identifying VEGF-D expression in tumors of patients with a poor survival prognosis provides, to our knowledge, a first analysis of this VEGF as prognostic factor in patients with breast cancer. Prospective studies in a larger population should be carried out to demonstrate our present findings. It is still interesting to speculate that the VEGF-D expression may be useful in the treatment of breast cancer as a decision-making biomarker for aggressive treatment after operation.

REFERENCES

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