Flt-4-Positive Vessel Density Correlates with Vascular Endothelial Growth Factor-D Expression, Nodal Status, and Prognosis in Breast Cancer

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ABSTRACT

Purpose: Metastasis to the regional lymph nodes through the lymphatic vessels is a common step in the progression of cancer and an important prognostic factor in many types of cancer. Recent evidence suggests that tumor lymphangiogenesis promotes lymphatic metastasis, and that the presence of Flt-4 on tumor blood and lymphatic vessels may play an important role in mediating lymphangiogenic factor-induced neovascularization. We assessed flt-4-positive vessel density (FVD) in breast cancer, and examined whether FVD associates with lymph node metastasis, VEGF-D expression, or prognosis.

Experimental Design: One hundred three invasive breast carcinomas with long-term follow-up were included in our study. Flt-4 was assessed using immunohistochemistry, then we analyzed the relationship between FVD and lymph node status, as well as VEGF-D expression and other established clinicopathological parameters. The relationship between FVD and prognosis was also investigated.

Results: Mean FVD of “hot spot” was 29.3 ± 22.5 for each case. FVD was correlated significantly with lymph node metastasis (P < 0.0001), VEGF-D expression (P = 0.0019), tumor size (P = 0.0015), estrogen receptor (P = 0.0211), progesterone receptor (P = 0.0462), and c-erbB-2 (P = 0.0326). Survival curves determined by the Kaplan-Meier method and univariate analysis demonstrated that high FVD was associated with both worse disease-free survival (P = 0.0035) and overall survival (P = 0.0336).

Conclusions: Increased FVD was correlated with lymph node metastasis and VEGF-D expression. High FVD may be a significant unfavorable prognostic factor for long-term survival in breast cancer. It is possible that Flt-4 becomes a target for antiangiogenic therapy to breast cancer.

INTRODUCTION

Metastasis to the regional lymph nodes via the lymphatic vessels is a common step in the progression of cancer, an important prognostic factor in many types of cancer, and the basis for surgical and radiation treatment of local lymph nodes. Recent evidence suggests that tumor lymphangiogenesis, the growth of tumor-associated lymphatic vessels, promotes lymphatic metastasis (1–4). However, the absence of specific markers for lymphatics in cancer has made the identification of tumor lymphatics difficult. The discovery recently of VEGFR-3 (also known as Flt-4), a receptor for lymphangiogenic VEGF-C and VEGF-D, changed the landscape for lymphatic studies. Flt-4 is specifically activated by VEGF-C and VEGF-D, and has been associated with the development and maintenance of lymphatic vessels (5, 6). Flt-4 has been demonstrated to be expressed almost exclusively in the lymphatic endothelium and is, thus, considered to be a major regulator of lymphangiogenesis in normal tissue (7, 8). However, Flt-4 expression on blood vessels has been documented in different types of malignant tumors and granulation tissue (9–11). Recent data suggest that Flt-4 expression may be a new microvascular progression marker, and that the presence of Flt-4 on tumor blood and lymphatic vessels may play an important role in mediating lymphangiogenic factor-induced neovascularization (12).

To evaluate whether Flt-4-positive vessels may affect tumor metastasis and prognosis in breast cancer, we assessed FVD, and investigated the relationship between FVD and lymph node status, as well as VEGF-D expression. Furthermore, we examined whether FVD has any value or relevance with respect to predicting the disease outcome.

MATERIALS AND METHODS

Patients and Tumor Samples. The study included 103 women with invasive breast cancer, diagnosed and treated in the Osaka Police Hospital, Osaka, Japan, between 1981 and 1991. All of these cases had no family history of breast cancer or malignancy in first-degree relatives as judged by questioning at the time of admission for surgery. The patients had received
mastectomy with axillary lymph node dissection. All of the women were apparently free of distant metastasis. All of the cases received postoperative adjuvant therapy consisting of combination chemotherapy and hormone treatment. Twenty-four patients only received chemotherapy, and 6 patients only received tamoxifen. Only 2 of them had postoperative radiotherapy to the breast. Patient and tumor characteristics are shown in Table 1.

**Immunohistochemistry.** Paraffin sections 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 min. They were subsequently washed in PBS and treated for 20 min with Protein Block Serum-free (DAKO Co., Carpinteria, CA). The primary antibody used in this study was an anti-human VEGFR-3 (Flt-4) goat antibody (R & D Systems, Inc., Minneapolis, MN). The sections were treated with anti-Flt-4 antibody diluted 1:200 with PBS and incubated at 4°C in humidified chamber. After the overnight treatment, to avoid the nonspecific biotin reaction, we used Histofine Simple Stain MAX PO (Nichirei, Tokyo, Japan) as a second antibody for 60 min by the manufacturer’s instructions. The antigen localization was visualized using 3,3′-diaminobenzidine tetrachloride/nickel-cobalt kit (Zymed Laboratories, Inc., San Francisco, CA) to make determination of FVD easily by enhancing the color development. Counterstaining was done with nuclear fast red stain solution. For the negative control, all of the reagents except for the primary antibody were used.

**FVD Assessment.** Determination of FVD was performed according to Weidner et al. (13). The immunostained sections were scanned by light-microscopy using a low magnification (×4), and the areas of tissue with the greatest number of distinctly highlighted flt-4-positive vessels (hot spot) were selected. FVD was then determined by counting all of the immunostained vessels at a total magnification of ×200 from five areas for each case. The mean number of Flt-4-positive vessels in each case was evaluated. The scoring and count were performed by three investigators blindly without clinical knowledge of the patients.

**Covariates.** Information about the clinical history of the patients was obtained from the patient medical records. The immunostaining results of VEGF-D, ER, PgR, and c-erbB-2 were obtained from our pathological data files (14). The size of the primary tumor was obtained from the surgical specimen. 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 done according to the WHO classification (15) and the Protocol of the Japan National Surgical Adjuvant Study of Breast Cancer Pathology Section (16), respectively.

**Statistics.** Mann-Whitney test was used to examine the association of FVD with age, histological type, nodal status, ER, PgR, c-erbB-2, p53, and VEGF-D immunoreactivity. Correlation of FVD with tumor size and histological grade was investigated by the Kruskal-Wallis test. OS curves and DFS curves were obtained using 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 < 0.05 was considered significant. A computer program 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 103 patients was 51 years (range, 24–87 years). Fifty-two percent of the patients were <50 years (n = 53), and 51% (n = 52) of the patients had lymph node metastasis at the time of surgery. Median follow-up time for the 103 subjects was 116 months (range, 10–230 months). Forty-one subjects had relapsed by the time of last follow-up. Thirty-one patients died of breast carcinoma.

**Flt-4 Expression in Breast Cancer Tissue.** Flt-4-positive vessels were present in all of the invasive breast carcinoma. All of the stained vessels were typically thin-walled. Consistent with the previous report (10), Flt-4 was restricted to the endothelium not only at peritumoral lesion but also at intratumoral lesion, indicating Flt-4-positive vessels did not appear to form any specific distribution pattern. Occasionally, invasion of the carcinoma cells into the Flt-4-positive vessels could be observed (Fig. 1). In normal breast tissue, Flt-4-positive vessels were also present in the interductal stroma. These vessels did not form any specific pattern but were scattered throughout the stroma. In
intraductal carcinoma, Flt-4-positive vessels formed an arch-like pattern (“necklace pattern”) around the affected ducts. FVD was determined independently by three observers. The mean FVD of all of the samples was 29.3 ± 22.5 per case.

Flt-4 Expression Is Correlated with VEGF-D Expression and Lymph Node Metastasis. As we described previously (14), the expression of VEGF-D protein was observed as diffuse cytoplasmic staining in breast cancer cells. With the cutoff of 10%, VEGF-D expression was positive in 81% (84 of 103) of the breast cancer patients. As shown in Table 1, FVD was correlated with VEGF-D expression (P = 0.0019) and lymph node metastasis (P < 0.0001).

Correlations between FVD and Other Clinicopathological Factors. As shown in Table 1, FVD was significantly associated with tumor size (P = 0.0015), ER (P = 0.0211), PgR (P = 0.0462), and c-erbB-2 (P = 0.0326). There was no significant correlation between FVD and the age of the patients, p53 status, or nuclear grading.

FVD Is Correlated with Patient Survival. To examine the correlation between FVD and patient prognosis, the patients were divided into two groups, those with < (low FVD) and >30 (high FVD) Flt-4-positive vessels per case. The survival analysis was performed on 103 patients and took into account the following variables: FVD, patient age, histological type, tumor size, lymph node status, hormonal status, c-erbB-2, p53, and nuclear grade. Univariate survival analysis showed that tumor size, lymph node status, c-erbB-2, and FVD were of significant prognostic value for DFS (Table 2; Fig. 2A); tumor size, lymph node status, and FVD were of significant prognostic value for OS (Table 3; Fig. 2B). On the basis of multivariate Cox regression analysis, only lymph node status was identified as an independent prognostic factor (DFS, P < 0.0001; OS, P = 0.0022). We failed to identify FVD as an independent prognostic factor for DFS or OS (data not shown).

DISCUSSION
Tumor metastasis may depend on the capacity of tumor cells to induce angiogenesis and/or lymphangiogenesis. VEGF-D, which is a potent angiogenic factor in vivo and stimulates endothelial cell proliferation and migration (6, 17), has been demonstrated involvement in promoting tumor angiogenesis and lymphangiogenesis (3). It has also shown to be required for the growth and establishment of lymphatic vessels within tumors (6). It is widely accepted that invasion of tumor cells into blood or lymphatic vessels is one of the critical steps for the establishment of metastasis (18). VEGF-D has been the focus of recent debate with regard to their potential role in promoting tumor angiogenesis.
and lymphangiogenesis. In humans, VEGF-D activates VEGFR-2 (KDR/Flk-1) and VEGFR-3 (Flt-4) receptor tyrosine kinases (6, 19), both of which are essential for vascular development (20, 21). Flt-4 differs from VEGFR-2 by being expressed predominantly in lymphatic endothelial cells in adult normal tissues (7). However, Flt-4 as a specific marker for lymphatic endothelium has been called into question by reports that the receptor is also expressed to a variable extent by endothelial blood vessels in number of cancers and granulation tissue (9–11). Flt-4 expression may be a new microvascular progression marker, and the presence of Flt-4 on tumor blood and lymphatic vessels may play an important role in mediating lymphangiogenic factor-induced neovascularization (12). In this study, we found that FVD was associated with VEGF-D expression, which is the first to demonstrate that there is significant correlation between VEGF-D expression and high FVD in human breast cancer tissue. Our results suggested that VEGF-D may play an important role in neovascularization labeled by Flt-4. Our findings are consistent with a recent report in SCID/NOD mice model that VEGF-D is capable of activating Flt-4. In their animal model, expression of VEGF-D in tumor cells leads to spread of the tumor to lymph nodes.

Flt-4 expression is up-regulated in breast cancer and a number of other tumor types (8, 10, 22). In this study, we found that FVD was correlated with lymph node status. However, Jacquemier et al. (23) and Gunningham et al. (24) have not obtained a significant relationship between Flt-4 expression profile and lymph node metastasis. Notably, Gunningham et al. (24) have used reverse transcription-PCR assay for their study. As described in our study, Flt-4-positive vessels could also be noted in normal breast tissue. Therefore, reverse transcription-PCR assay could not reveal Flt-4 expression in tumors correctly if microdissection was not performed. The discrepancy between our findings and that reported by Jacquemier et al. (23) may be because of cases selection bias.

It is very important for practical purpose to examine whether FVD has any value or relevance with respect to predicting the disease course in breast carcinoma. In our results, survival curves determined by Kaplan-Meier method and log-rank test demonstrated that high FVD was associated with both DFS and OS. Therefore, antiangiogenic therapy using antagonistic strategies to block Flt-4 signaling may be an important tool for breast cancer treatment in the future. A recent report showed that administration of anti-Flt-4 blocking antibodies inhibited lymph node metastasis (25), which may become a novel potential strategy for breast cancer treatment.

In conclusion, increased Flt-4 expression is associated significantly with lymph node metastasis and VEGF-D expression, and

Table 3 Univariate analysis of OS by various clinicopathological factors

<table>
<thead>
<tr>
<th>Factor</th>
<th>No.</th>
<th>No. (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age ≤50</td>
<td>53</td>
<td>14 (22.4)</td>
<td>0.2490</td>
</tr>
<tr>
<td>Age &gt;51</td>
<td>50</td>
<td>17 (34.0)</td>
<td>0.2341</td>
</tr>
<tr>
<td>Histology IDC</td>
<td>94</td>
<td>30 (21.9)</td>
<td>0.0150</td>
</tr>
<tr>
<td>Histology Others</td>
<td>9</td>
<td>1 (11.1)</td>
<td></td>
</tr>
<tr>
<td>Tumor size ≤2 cm</td>
<td>34</td>
<td>5 (14.7)</td>
<td></td>
</tr>
<tr>
<td>Tumor size &gt;2 cm</td>
<td>69</td>
<td>26 (30.1)</td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis Negative</td>
<td>51</td>
<td>8 (15.6)</td>
<td>0.0003</td>
</tr>
<tr>
<td>Lymph node metastasis Positive</td>
<td>52</td>
<td>23 (44.2)</td>
<td></td>
</tr>
<tr>
<td>ER Negative</td>
<td>39</td>
<td>12 (30.7)</td>
<td>0.6426</td>
</tr>
<tr>
<td>ER Positive</td>
<td>64</td>
<td>19 (29.6)</td>
<td></td>
</tr>
<tr>
<td>p53R Negative</td>
<td>42</td>
<td>15 (35.7)</td>
<td>0.2157</td>
</tr>
<tr>
<td>p53R Positive</td>
<td>61</td>
<td>16 (26.2)</td>
<td></td>
</tr>
<tr>
<td>c-erbB-2 Negative</td>
<td>76</td>
<td>21 (27.6)</td>
<td>0.3302</td>
</tr>
<tr>
<td>c-erbB-2 Positive</td>
<td>27</td>
<td>10 (37.0)</td>
<td></td>
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<tr>
<td>Grade I and II</td>
<td>62</td>
<td>21 (33.8)</td>
<td>0.5069</td>
</tr>
<tr>
<td>Grade III</td>
<td>41</td>
<td>10 (24.3)</td>
<td></td>
</tr>
<tr>
<td>FVD Low</td>
<td>63</td>
<td>14 (22.2)</td>
<td>0.0336</td>
</tr>
<tr>
<td>FVD High</td>
<td>40</td>
<td>17 (42.5)</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2 Association of FVD with patients prognosis in breast cancer (Kaplan-Meier method and log-rank test). High FVD is significantly related to recurrence (A, P = 0.0035) and death (B, P = 0.0336).
may play a important role for lymph node metastasis in breast cancer. Furthermore, FVD may serve as a significant prognostic factor for long-term survival in breast cancer. It is possible that Flt-4 becomes a target for antiangiogenic therapy to breast cancer.

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