VEGF-D in Association With VEGFR-3 Promotes Nodal Metastasis in Human Invasive Lobular Breast Cancer

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Key Words: Breast; Lymphangiogenesis; Growth factor; Breast cancer; Metastasis; Immunohistochemistry

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Abstract

We assessed the expression of vascular endothelial growth factors (VEGF-C and VEGF-D) in breast cancer cells and the density of lymph vessels and VEGF receptor-3 (VEGFR-3)–positive vessels in and around the tumor in invasive lobular breast cancer.

We found significant correlation between peritumoral lymph vessel density and presence of lymph node metastases \((P = .001)\) and the number of metastatic lymph nodes \((P < .001)\). A significant correlation was detected between tumor cell VEGF-D expression and lymph node status \((P = .001)\) and density of lymphatic vessel endothelial receptor (LYVE)-1–positive vessels \((P = .035)\). VEGFR-3+/VEGF-D+ and VEGFR-3+/VEGF-C+ tumors had a significantly higher number of metastatic lymph nodes than tumors with other staining patterns \((P < .001)\). Tumors positive for neither VEGF-D nor VEGFR-3 had a lower density of LYVE-1+ vessels than tumors with other staining patterns \((P = .033)\).

Our results indicate that peritumoral lymph vessel density is associated with lymph node metastases in invasive lobular breast cancer and that invasive lobular cancer producing VEGF-D, surrounded by VEGFR-3+ vessels, has a significantly higher peritumoral lymph vessel density and a higher number of metastatic lymph nodes.

In breast cancer, the most significant prognostic factor is the status of the axillary lymph nodes. Therefore, the presence of metastases in the lymph nodes influences the choice of adjuvant therapy after surgery. Tumor size, grade, lymphatic or vascular invasion, tumor palpability, and patient age are the most commonly reported independent factors to predict axillary metastasis. Recent evidence suggests that tumor lymphangiogenesis, the growth of tumor-associated lymphatic vessels, promotes lymphatic metastatic spread. This may mean that the ability of the tumor to spread through the lymph vessels to regional lymph nodes would be predicted also by measuring lymphangiogenesis.

It is now possible to measure lymph vessel density in human cancer specimens by using recently discovered lymphatic specific markers such as lymphatic vessel endothelial receptor (LYVE)-1. Furthermore, the molecular regulation of lymphangiogenesis has been clarified in numerous studies, and tumor growth factors and their receptors can be assessed. However, most of the evidence of the molecular regulation of lymphangiogenesis is based on animal in vivo and in vitro studies. These studies advocate that lymphangiogenesis is promoted by binding of vascular endothelial growth factors VEGF-C and VEGF-D to their receptor VEGFR-3. Basically, this model states that binding of VEGF-C and/or VEGF-D produced by tumor cells to lymph vessels expressing VEGFR-3 induces lymphangiogenesis. In turn, a dense lymphatic network promotes dissemination of tumor cells to regional lymph nodes. Therefore, it has been postulated that the process of tumor lymphangiogenesis promotes metastatic spread to regional lymph nodes.

VEGF-C and VEGF-D are members of the vascular endothelial growth factor family. They are secreted as polypeptides,
both binding to the tyrosine kinase receptor, VEGFR-3. The molecular structure of VEGF-C is 30% identical to that of VEGF. VEGF-C is stepwise proteolytically processed with increasing binding affinity to VEGFR-3.12 VEGF-D is more recently discovered and is 61% identical to VEGF-C. There are also several forms of VEGF-D generated by proteolytic processing with increasing binding affinity to VEGFR-3.13

In embryos, VEGFR-3 is expressed in lymph and blood vessel endothelial cells. In adults, it is confined to the lymphatic endothelium.14 However, in pathologic conditions, such as wound healing and tumor angiogenesis, VEGFR-3 expression is also found in blood vessel endothelial cells.15

In breast cancer, several clinical studies have reported a significant association between expression of VEGF-C and lymph node metastases or survival,16-18 but others failed to show this correlation.19,20 The same controversial results are reported for VEGF-D expression and lymph node metastases and survival.21,22 Direct evidence of lymphangiogenesis in human tumors mediated by VEGF-C and/or VEGF-D remains uncertain.

The previous studies on breast cancer have focused mainly on patients with invasive ductal carcinoma, the most common histologic type of breast cancer. Therefore, the aim of this study was to investigate the role of lymphangiogenesis in lymphatic dissemination in invasive lobular breast cancer by examining peritumoral and intratumoral lymph vessel density and the expression of VEGF-C, VEGF-D, and VEGFR-3 in tumors.

Material and Methods

Patients

Between May 2000 and October 2002, 634 consecutive patients with breast cancer underwent surgery at the Breast Surgery Unit, Helsinki University Central Hospital, Helsinki, Finland. Of the cases, 160 were typed as invasive lobular breast cancer according to a histopathologic review of the tumor specimens. Owing to restrictive availability of the breast tissue samples and technical difficulties during preparation or staining, the total study population consisted of 95 invasive lobular breast cancer samples. The project plan was approved by the Helsinki University Hospital Ethical Committee, and the study was performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from each patient.

Axillary Staging

Axillary staging was performed using sentinel node biopsy. Preoperative lymphoscintigraphy was performed the day before surgery, 4 hours after intratumoral injection of 80 to 100 MBq of technetium Tc 99m–labeled human albumin colloid in a volume of 0.2 mL. At least 5 minutes before the injection 1 mL of Patent Blue dye was injected intratumorally. Sentinel nodes were harvested using a handheld γ probe and seeking the blue-stained lymphatic vessels and nodes. The sentinel nodes were examined histologically using serial sectioning and immunohistochemical analysis, as described in detail in a previous report.23 Axillary clearance was performed when the sentinel nodes in the axilla were involved.

Immunohistochemical Analysis

We cut 4-µm sections from paraffin-embedded blocks, deparaffinized them in xylene, and rehydrated them in a series of graded alcohols. The sections were pretreated in a microwave oven and peroxidase was activity blocked with 1.6% hydrogen peroxide in methanol for 30 minutes. Nonspecific binding sites were blocked with normal serum, and the antibody was applied overnight at 4°C. The next day, detection was performed with the DAKO EnVision kit (DAKO, Carpinteria, CA) or Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Stainings were developed using 3-amino-9-ethylcarbazole (red), counterstained with hematoxylin, and mounted. Every series contained a positive and a negative control.

LYVE-1 Staining

For staining with the anti–LYVE-1 antibody (a kind gift from David G. Jackson, PhD), the sections were pretreated in a microwave oven in 10 mmol/L of citrate buffer, pH 6.0, at 600 W for 20 minutes to retrieve tissue antigen. Endogenous peroxidase activity was blocked with 1.6% hydrogen peroxide in methanol for 30 minutes. Nonspecific binding sites were blocked with normal goat serum in phosphate-buffered saline (PBS) for 30 minutes. Primary polyclonal antibody for LYVE-1 (1.4 µg/mL affinity-purified LYVE-1 immunoglobulin), diluted 1:300, was applied overnight. The detection was performed using the DAKO EnVision kit.

VEGFR-3 Staining

For staining for VEGFR-3, the 9D9 antibody (a kind gift from Professor Kari Alitalo) was used. The sections were pretreated in a microwave oven in 10 mmol/L of citrate buffer, pH 6.0, at 600 W for 20 minutes to retrieve tissue antigen. Nonspecific binding sites were blocked with TNB blocking buffer (TSA Biotin System, NEN Life Science, Boston, MA). The primary polyclonal antibody for VEGFR-3, diluted 1:200, was applied overnight. The next day, biotinylated goat antimouse immunoglobulins were applied as the second antibody. Immunoreactions were developed using the tyramide amplification system (NEN Life Science), followed by the application of streptavidin-horseradish peroxidase complex for 30 minutes.

CD34 Staining

To distinguish between lymph and blood vessels, the sections were also immunostained for CD34. For staining of CD34 receptor–positive vessels, the anti-CD34 antibody from Professor Kari Alitalo was used. The sections were pretreated in a microwave oven in 10 mmol/L of citrate buffer, pH 6.0, at 600 W for 30 minutes to retrieve tissue antigen. Nonspecific binding sites were blocked with normal goat serum in phosphate-buffered saline (PBS) for 30 minutes. Primary polyclonal antibody for CD34 (1.4 µg/mL affinity-purified CD34 immunoglobulin), diluted 1:300, was applied overnight. The detection was performed using the DAKO EnVision kit.
Becton Dickinson Biosciences, San Jose, CA, was used. The sections were pretreated in a microwave oven in tris(hydroxymethyl)aminomethane (Tris) hydrochloride, pH 8.5, at 600 W for 20 minutes to retrieve tissue antigen, and endogenous peroxidase activity was blocked with 1.6% hydrogen peroxide in methanol for 30 minutes. Nonspecific binding sites were blocked with normal horse serum in PBS for 30 minutes, and primary monoclonal CD34 antibody was applied overnight. The detection was performed using the Vectastain Elite ABC kit.

**VEGF-C Staining**

An anti–VEGF-C antibody from Zymed Laboratories, South San Francisco, CA, was used for tumor cell VEGF-C staining. The sections were pretreated with Tris hydrochloride, pH 8.5, in a microwave oven at 600 W for 20 minutes. Endogenous peroxidase activity was blocked with 1.6% hydrogen peroxide in methanol for 30 minutes. Nonspecific binding sites were blocked with normal goat serum in PBS for 30 minutes. The VEGF-C antibody diluted 1:500 was applied overnight and detected with the Vectastain Elite ABC kit.

**VEGF-D Staining**

For VEGF-D staining, an anti–VEGF-D antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used. The sections were pretreated in a microwave oven in Tris hydrochloride, pH 8.5, at 600 W for 20 minutes to retrieve tissue antigen. Endogenous peroxidase activity was blocked with 1.6% hydrogen peroxide in methanol for 30 minutes. Normal rabbit serum to block nonspecific binding sites was used. The VEGF-D antibody, diluted 1:1,000, was applied overnight, and detection was performed with the Vectastain Elite ABC kit.

**Scoring of Slides**

Slides were scored for LYVE-1, VEGFR-3, and CD34 vessel density according to the method described by Weidner et al. Briefly, tumor sections were scanned at low power (×40 and ×100), and 3 areas containing the maximum number of distinctive lymphatic vessels were selected. A 25-point eyepiece-mounted Chalkley Point Array graticule (Graticules, Tonbridge, England) was used at ×250 magnification and oriented over the selected area so that the maximum number of points coincided with positively stained vessel endothelium. The mean of the 3 Chalkley counts was used for statistical analysis.

For scoring of VEGF-C and VEGF-D staining, the percentage of strongly stained tumor cells was assessed. Plain percentages as continuous values were used for statistical analysis. When combining the results of the stainings, the median was used as the cutoff point.

**Statistical Analyses**

The association between Chalkley vessel count for LYVE-1+, VEGFR-3+, and CD34+ vessels and clinicopathologic factors was analyzed with the Mann-Whitney U test and the Kruskal-Wallis test. These same tests were also used to analyze the relation between the percentage of tumor cells positive for VEGF-C and VEGF-D and clinicopathologic factors. The Spearman rank correlation coefficients were used to evaluate the association between continuous variables, i.e., staining of VEGFR-3, VEGF-C, and VEGF-D and vessels positive for LYVE-1 and CD34. Stainings were divided into a positive and a negative group according to their median. Three groups of combined staining patterns for VEGF-C, VEGF-D, and VEGF-3 were correlated with the LYVE-1 Chalkley counts and number of metastatic lymph nodes using the Kruskal-Wallis test. All tests were 2-sided, and P values less than .05 were considered significant. All statistical analyses were performed using SPSS 10.0 for Windows (SPSS, Chicago, IL).

**Results**

**Vessel Densities in Lobular Breast Cancer**

Practically all LYVE-1+ vessels were located at the tumor periphery. Intratumoral LYVE-1+ vessels were rarely present. Vessels positive for LYVE-1 were small, without muscle layer or RBCs in their lumen, morphologically matching lymph vessels. No tumor cells, stroma, or other types of cells expressed LYVE-1 [Image 11].

We found a significant correlation between peritumoral lymph vessel density and the presence of lymph node metastases (P = .001; Mann-Whitney U test). Lymph vessel density also correlated with the number of metastatic lymph nodes (P < .001; Spearman rank test). The mean Chalkley count for LYVE-1+ vessels was 3.7 (SD, 1.6; range, 0-7.3). There was no correlation between lymph vessel density and patient age, tumor size, tumor grade, and tumor palpability [Table 1].

CD34+ blood vessels were located at the tumor edge and intratumorally. The mean CD34 Chalkley count was 5.2 (SD, 3.1; range, 0.7-12). A high Chalkley count of CD34+ vessels correlated with lymph node status (P < .001; Mann-Whitney U test) and with the number of positive lymph nodes (P < .001; Spearman rank test). We did not find a correlation between CD34 vessel density and other clinicopathologic factors (Table 1).

Vessels expressing VEGFR-3 were located in areas with dense staining for CD34 and for LYVE-1. The mean VEGFR-3 Chalkley count was 0.9 (SD, 1.6; range, 0-5.3). We did not observe intratumoral VEGFR-3+ vessels. We found a positive correlation between VEGFR-3 staining and LYVE-1 (P = .021) and CD34 (P = .007) [Table 2].

**Expression of VEGF-C and VEGF-D in Lobular Breast Cancer**

VEGF-C+ cells were almost exclusively tumor cells; only occasionally were positively stained macrophages...
The mean VEGF-C expression was 18.2% (SD, 19.6%; range, 0%-80%). We did not find a correlation between VEGF-C expression and any clinicopathologic factor. There was not a correlation between VEGF-C expression and LYVE-1 or CD-34 vessel stainings.

Like VEGF-C staining, VEGF-D was almost exclusively expressed in tumor cells.

**Table 1**

<table>
<thead>
<tr>
<th>Factor</th>
<th>LYVE-1</th>
<th>P</th>
<th>CD34</th>
<th>P</th>
<th>VEGFR-3</th>
<th>P</th>
<th>VEGF-C</th>
<th>P</th>
<th>VEGF-D</th>
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<td>.915†</td>
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<td>7.0 (3.5)</td>
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<td>2.3 (2.0)</td>
<td>&lt;.001†</td>
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LYVE, lymphatic vessel endothelial receptor; VEGF, vascular endothelial growth factor.

* Spearman rank test.

**Table 2**

<table>
<thead>
<tr>
<th>VEGFR-3</th>
<th>VEGF-C</th>
<th>VEGF-D</th>
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<td>LYVE-1</td>
<td>.021†</td>
<td>.360†</td>
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<tr>
<td>CD34</td>
<td>.007†</td>
<td>.108†</td>
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LYVE, lymphatic vessel endothelial receptor; VEGF, vascular endothelial growth factor.

* Spearman rank test.
Sometimes macrophages positive for VEGF-D were observed. The mean VEGF-D expression was 35.9% (SD, 29.8%; range, 0%-100%). A significant correlation was detected between tumor cell VEGF-D expression and lymph node status ($P = .001$; Mann-Whitney $U$ test) (Table 1). VEGF-D expression also correlated with the density of LYVE-1+ vessels ($P = .035$; Spearman rank test) (Table 2). No association was found between VEGF-D and CD34.

**Low VEGFR-3 Density With Low VEGF-C/VEGF-D Expression Predicts Node-Negative Disease**

We classified the tumors as positive or negative for VEGF-C, VEGF-D, and VEGFR-3 using the medians as cutoff points. For example, tumors with 25% or more of the cells with VEGF-D+ staining were considered VEGF-D+ and tumors with less than 25% of the cells with positive VEGF-D staining as VEGF-D−. Six categories of tumors with different staining patterns were created: (1) VEGF-D−/VEGFR-3−, (2) VEGF-D+/VEGFR-3− or VEGF-D−/VEGFR-3+, (3) VEGF-D+/VEGFR-3+, (4) VEGF-C−/VEGFR-3−, (5) VEGF-C+/VEGFR-3− or VEGF-C−/VEGFR-3+, and (6) VEGF-C+/VEGFR-3+.

Tumors that were neither VEGF-D+ nor VEGFR-3+ had a lower density of LYVE-1+ vessels compared with the tumors with other staining patterns ($P = .033$). Tumors positive for VEGFR-3 and VEGF-D or VEGF-C had a significantly higher number of metastatic lymph nodes than tumors with other staining patterns ($P < .001$) Table 3.

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![Image 2](Anatomic Pathology/Original Article) Immunohistochemical staining of human lobular breast cancer samples. **A** and **B**, Vascular endothelial growth factor (VEGF)-C+ tumor cells. **B** is a higher magnification of the framed area in **A** (**A**, ×300; **B**, ×800). **C** and **D**, VEGF-D+ tumor cells. **D** is a higher magnification of the framed area in **C** (**C**, ×100; **D**, ×400).
Only 1 case with tumors that were VEGF-D– and VEGFR-3– was lymph node positive, with a single metastatic node.

**Discussion**

Our data indicate that a high density of peritumoral lymph vessels is associated with lymph node metastases in invasive lobular carcinoma. This finding is in concordance with the results of earlier clinical studies, suggesting that a dense network of lymph vessels around the tumor increases the chance of spread of tumor cells through lymph vessels to regional lymph nodes.

Based on series of animal experimental studies, reviewed, among others, by Saharinen and Petrova,11 the theory of lymphangiogenesis stimulated by tumor growth factors has been developed. According to this theory, VEGF-D, produced by tumor cells, binds to its receptor VEGFR-3 present on lymph vessel endothelial cells surrounding the tumor, thus activating the growth of new lymph vessels. This is the first study with human breast cancer samples showing a significant relationship between VEGF-D and peritumoral lymph vessel density, suggesting the actual process of lymphangiogenesis. Furthermore, we found that tumor cell expression of VEGF-D correlates with lymph node status. To our knowledge, VEGF-C and VEGF-D expression has not been studied in patient material simultaneously with LYVE-1, VEGFR-3, and CD34 immunostaining.

Lymphangiogenesis is not just production of VEGF-D by the tumor cells. To promote lymphangiogenesis, VEGF-D needs also to bind to its receptor, VEGFR-3, available on lymph vessel endothelium. We found a correlation between high peritumoral lymph vessel density and simultaneous VEGF-D and VEGFR-3 positivity. This finding provides further evidence of lymphangiogenesis around the tumor, mediated by production of VEGF-D in the presence of VEGFR-3+ lymph vessels. This same expression pattern correlated even more significantly with the number of metastatic lymph nodes. Previous studies of breast carcinoma have not found a correlation between the density of LYVE-1+ vessels and lymph node status.26 However, a high density of LYVE-1+ vessels predicted lymph node metastases and worse survival in a more recent study.25 The vast majority of the patients in these earlier studies had invasive ductal carcinoma, the most common histologic type of breast cancer.

Our study population consisted entirely of patients with invasive lobular carcinoma. It is the second most common histologic type of breast cancer, with steadily increasing incidence rates.27 Invasive lobular carcinoma is distinctively different morphologically, with a diffuse growth pattern with single file and skip lesions. Also, the patterns of metastatic spread and local recurrence differ from those encountered in connection with invasive ductal carcinoma.28-30 Invasive lobular carcinoma metastasizes to lymph nodes in a scattered manner, lacking severe cytologic atypia. Metastatic cells tend to fill the sinuses, mimicking sinus histiocytosis.30 The use of immunohistochemical analysis enhances the detection of nodal metastases in invasive lobular carcinoma.31 The metastases of invasive lobular carcinoma are more readily detected when using sentinel node biopsy instead of axillary clearance in axillary staging owing to the enhanced histopathologic assessment of sentinel lymph node metastases by applying serial sectioning and immunohistochemical analysis.32 The differences in the study populations and in the methods of axillary staging may explain the discordant findings regarding the association between peritumoral lymph vessel density and lymph node status in the present and the earlier studies.

Our results showed also a correlation between vessels positive for VEGF-3 and those positive for LYVE-1, as well as those expressing CD34, indicating that VEGF-3 is expressed in lymph and blood vessel endothelium in cancer growth, as found in earlier studies.33,34 Although in this study on invasive lobular breast cancer, VEGF-D had a prominent role in lymphatic dissemination, VEGF-C did not correlate with any of the examined clinicopathologic

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Relation Between Staining Pattern and Peritumoral Lymph Vessel Density and Number of Lymph Node Metastases*</th>
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<tbody>
<tr>
<td></td>
<td>No. of Cases</td>
</tr>
<tr>
<td>VEGF-D– and VEGFR-3–</td>
<td>24</td>
</tr>
<tr>
<td>VEGF-D+ and VEGFR-3– or VEGF-D– and VEGFR-3+</td>
<td>22</td>
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<td>VEGF-D+ and VEGFR-3+</td>
<td>11</td>
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<tr>
<td>VEGF-C– and VEGFR-3–</td>
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<tr>
<td>VEGF-C+ and VEGFR-3– or VEGF-C– and VEGFR-3+</td>
<td>25</td>
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<td>VEGF-C+ and VEGFR-3+</td>
<td>11</td>
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</table>

LYVE, lymphatic vessel endothelial receptor; VEGF, vascular endothelial growth factor.

* Data for LYVE-1 vessel density and number of metastatic lymph nodes are expressed as mean (range). P values were calculated using the Kruskal-Wallis test.
factors or with any vessel staining. Therefore, the role of VEGF-C in the dissemination of invasive lobular breast cancer seems limited.

Peritumoral LYVE-1+ vessel density is associated with lymph node metastases in invasive lobular breast cancer. It is more exciting that our results indicate that invasive lobular cancers producing VEGF-D, surrounded by VEGFR-3+ vessels, show a significant correlation with peritumoral lymph vessel density and lymph node status.

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