Microvessels That Predict Axillary Lymph Node Metastases in Patients With Breast Cancer

S. David Nathanson, MD; Richard J. Zarbo, MD; D. Lynne Wachna, BSN; Caryle A. Spence; Tanja A. Andrzejewski; Judith Abrams, PhD

Hypothesis: The density of vasoactive endothelial growth factor receptor 3–immunostained microvessels in primary breast cancers correlates with the incidence of axillary lymph node metastasis.

Design: Breast cancer microvessel clusters ("hot spots") were sequentially immunostained for factor VIII, type IV collagen, and vasoactive endothelial growth factor receptor 3. Microvessels were counted under light microscopy at a magnification of ×200. Axillary lymph nodes were evaluated for metastases by light microscopy.

Setting: A multidisciplinary breast cancer clinic and laboratory.

Patients: Sixty patients with T2 breast cancers treated by lumpectomy (or mastectomy) and axillary lymphadenectomy.

Main Outcome Measures: Putative lymphatic microvessel density compared with axillary metastases.

Results: There were 16% (SE, 1.4%) vs 4% (SE, 0.8%) vasoactive endothelial growth factor receptor 3–immunostained microvessels (P < .001), 38% (SE, 3.9%) vs 65% (SE, 3.1%) type IV collagen–immunostained microvessels (P < .001), and 46% (SE, 4.1%) vs 31% (SE, 3.2%) unstained microvessels (P = .004) in node-positive vs node-negative patients, respectively. A fitted logistic model based on the relative percentage of putative lymphatic microvessels to blood microvessels correctly predicted that 23 (96%) of 24 patients would have a low risk and that 26 (96%) of 27 patients would have a high risk of lymph node metastases. Six (67%) of 9 patients predicted to have an intermediate risk had lymph node metastases.

Conclusion: The odds of a patient with breast cancer having axillary lymph node metastasis increased substantially as the proportion of putative lymphatic microvessels increased and the relative proportion of blood microvessels in angiogenic hot spots decreased (log likelihood = 14.6; χ² = 53.4; P < .001; area under the receiver operation characteristic curve = 0.97).


Regional lymph nodes are the most frequent site of breast cancer metastasis. Patients with breast cancer who have an increased likelihood of regional lymph node metastasis include those with large, high-grade, hormone receptor–negative primary tumors in which angiolymphatic invasion is identified. Other possible predictors of axillary lymph node metastasis include patient age, palpability and histological type of the tumor, S-phase fraction, ploidy, overexpression of certain genes or gene products (HER2, NEU, or p53) or proteins (cathepsin D, plasminogen activator, or laminin receptor), underexpression or loss of a gene (nm23), and microvessel density. However, none of these variables accurately predicts axillary metastases.

While the rationale for axillary dissection as a therapeutic procedure is debated, there is little doubt that knowledge of the tumor status of the axillary nodes is valuable prognostically and for the determination of appropriate adjuvant therapies. To obtain such information requires lymphadenectomy, since no clinical, radiological, or laboratory test has supplanted histological lymph node analysis in the diagnosis of axillary metastases. An accurate marker that predicts axillary metastasis is a desirable objective.

Suitable markers of hematogenous metastasis are those that predict metastatic propensity based on expression and/or activity of a molecule with an established role in metastasis. Other markers include those for which there is no established pathogenic association with...
PATIENTS AND METHODS

PATIENT SELECTION

The Josephine Ford Cancer Center and the Department of Surgery tumor database, Henry Ford Health System, Detroit, Mich, was used to identify patients with breast cancer who had a primary tumor resection by lumpectomy and axillary lymphadenectomy or by modified radical mastectomy between January 1991 and December 1997. The study was limited to patients with T2 breast cancer whose tumors were found to have angiogenic hot spots on initial screening with hematoxylin-eosin (HE) staining and factor VIII immunostaining. Enrollment was restricted to women in whom the diagnosis was made since 1991 because new standardization of fixation techniques and paraffin embedding of tissues for pathological evaluation did not occur until then. Of 300 women with T2 tumors, 240 were excluded for the following reasons: there were no angiogenic hot spots on initial screening with HE staining (n = 123); there was incomplete clinical and pathological information (n = 77); unavailability of the primary tumor block (n = 30); inadequate fixation of the primary tumor (n = 7); tubular or mucinous morphologic features (n = 2); and the tumor, on pathological review, was found to be non-invasive (n = 1). In the case of women with more than 1 tumor, we studied and analyzed only the earliest one.

ACQUISITION OF PRIMARY BREAST CANCER TISSUE BLOCKS

The total number of formalin-fixed, paraffin-embedded tumor blocks evaluated was 1,671, ranging from 9 to 33 for each primary tumor. Sections (5 µm thick) were cut from all the primary tumor tissue blocks and the immediate peritumoral tissues and stained with HE. All slides were examined using light microscopy at low (×40) and high (×200) power, and a standard pathological report was generated. The report identified gross pathological features, recording the size of the tumor in 3 dimensions. The largest diameter was used for the tumor classification. Microscopic morphologic features were assessed, and the grade was recorded. Estrogen and progesterone receptors were reported as positive or negative on immunostaining. Each slide was carefully evaluated for a hot spot in the primary tumor, an area where an aggregation of microvessels was clearly visible. Those blocks in which hot spots were clearly identifiable were stained and evaluated to identify lymphatic vessels and blood vessels.

The bivalved regional lymph nodes were sectioned, stained with HE, and examined for metastases, and the number of lymph nodes with metastatic breast cancer was recorded.

IMMUNOHISTOCHEMISTRY

Immunostaining was performed using a modification of the technique described by Valtola et al. Briefly, 5-µm-thick sections were cut from formalin-fixed, paraffin-embedded tissue blocks of primary breast cancers and mounted on slides (ProbeOn Plus Slides; Fisher Biotech, Pittsburgh, Pa) and stood on end for 48 hours to dry. Slides were baked in an incubator (Thermolyne Rosi 1000; Fisher Biotech) for 1 hour at 55°C. Sections were deparaffinized in 100% xylene (3 changes for 5 minutes each) and rehydrated through 100% ethanol (3 changes for 5 minutes; twice at 95% for 5 minutes and once at 70% for 4 minutes) and tap water (twice for 5 minutes). Slides were microwaved in a 5-minute target retrieval solution (Dako, Carpathia, Calif) twice for 5 minutes each and cooled for 5 to 8 minutes. Sections were incubated in 3% hydrogen peroxide (Diamond Drug, Westhaven, Conn) for 15 minutes and washed in phosphate-buffered saline (PBS) (Fisher Scientific, Pittsburgh) for 5 minutes. The immunoperoxidase counterstaining procedure was accomplished with a staining kit according to manufacturers' instructions (Vectorstain Elite ABC kit; Vector Laboratories, Burlingame, Calif). The slides were incubated for 20 minutes in blocking serum diluted with PBS. The blocking serum was shaken gently from the slides. Immunostaining was carried out with antibody at the appropriate dilution and time of incubation. Optimal antibody dilutions and the time and temperature of incubation were established by a series of preliminary titration experiments using liver, kidney, breast, and skin specimens as controls. Antibodies to factor VIII and type IV collagen were recorded as positive or negative on immunostaining.

Continued on next page

metastasis. Invasion of lymphatic microvessels may involve the same molecules required for invasion of blood vessels, although, to our knowledge, such comparative studies have not been reported.

A mechanical component of the multistep process of successful hematogenous metastasis is passage through the systemic circulation and the relatively poor survival of tumor cells exposed to traumatic shearing forces in blood vessels. The number of cells entering the bloodstream depends on the total area of available vascular endothelium. The density of the microvessel bed within a tumor is a direct measure of the size of this "vascular window" through which tumor cells pass to spread to distant body sites. The larger the window, the greater the number of circulating tumor cells and the greater the likelihood of hematogenous metastases. It is conceivable that the larger the analogous "lymphatic window," the greater the probability that tumor cells will access the lymphatic microvessels and reach the axillary lymph nodes in sufficient numbers to establish metastases.

Microvessel density, identified by counting endothelial-stained vessels in clusters (the so-called hot spots), is associated with an increased incidence of regional lymph node metastasis. This association is unlikely to be causal unless the vessels identified by non-specific stains are lymphatic microvessels. Lymphatic microvessels may constitute a varying proportion of the immunostained vessels, and it is possible that in some patients whose tumors secrete increased quantities of the lymphangiogenic cytokine, vasoactive endothelial growth factor C (VEGF-C), more lymphatic microvessels may be present. Standard pathological processing of breast cancer biopsy specimens does not allow an accurate distinction between blood vessel and lymphatic endothelium. A recently identified epitope, vasoactive endothelial
growth factor receptor 3 (VEGFR-3), is found on lymphatic endothelial cells and not on adult blood vessel endothelium. Immunostaining with specific antibodies to VEGFR-3 may distinguish lymphatic vessels from small blood vessels in fixed, paraffin-embedded tissue.

In this study, we assumed that the factor VIII-immunostained vessels represented blood vessels and lymphatic vessels. We hypothesized that some VEGFR-3-immunostained microvessels in the hot spots of breast cancers are lymphatic vessels and not blood vessels. We reasoned that an increased number of lymphatic vessels might be associated with regional lymph node metastasis, a postulate that could explain the correlation between increased microvessel density and regional lymph node metastasis. We chose to study patients with T2 lesions because approximately 50% of these patients have metastases to regional lymph nodes. This article describes our results in a selected group of patients with breast cancer.

**RESULTS**

The study population comprises 60 women with stage II breast cancer, in whom a diagnosis was made between January 1991 and December 1997, who were treated with lumpectomy and axillary lymph node dissection or modified radical mastectomy at the Henry Ford Health System. The median age at diagnosis was 53 years (range, 28-81 years). Approximately half the women (33 [55%] of 60) had positive nodes at diagnosis. In all but 1 patient, the tumor was classified as T2; the single exception is a woman with a 13-mm tumor and positive nodes.

**STATISTICAL METHODS**

This is an observational study of women with angiogenic hot spots identified by initial HE screening. Our objective was to determine whether VEGFR-3 and type IV collagen immunostaining techniques could distinguish lymphatic vessels from blood vessels and aid in the identification of women whose breast tumors have metastasized to their axillary lymph nodes. Hot spots were identified by factor VIII immunostaining; a VEGFR-3 immunostain was used to identify putative lymphatic vessels, while a type IV collagen immunostain was used to confirm blood vessels that stained with factor VIII. Pathological reports were used to determine lymph node status, and pathologists were blind to the results of the angiogenic analyses.

Two-sample t tests for continuous variables and χ² tests for categorical variables were used to compare demographic and disease characteristics of women with positive lymph nodes with those of women with negative nodes. The association between the proportion of nodes examined that were positive, proportions of vessels stained by VEGFR-3 and by type IV collagen, tumor size, and the patient’s age was estimated using Pearson correlation coefficients. The Sidak method for multiple comparisons was used to assess statistical significance of the correlation coefficients.

A logistic regression model was used to assess simultaneously the prognostic value for positive lymph node status of the proportion of vessels stained with VEGFR-3 and the proportion stained with type IV collagen. The fit of the model was assessed using the Pearson χ² statistic. A full range of regression diagnostics, residuals, and deletion and leverage statistics was used to identify outlying and influential observations.
Clinical features of lymph node metastases of breast cancer were studied in 60 women with 33 node-positive and 27 node-negative cases. The microvessels identified as putative lymphatic vessels by factor VIII immunostaining were counted in the hot spots of each node-positive or node-negative breast cancer. Patients with positive regional lymph nodes had significantly more factor VIII–immunostained vessels (P<.001). There is also a moderate linear increase with the percentage of VEGFR-3–immunostained microvessels than patients with negative nodes.

Table 1. Demographic Characteristics of the Population Studied

<table>
<thead>
<tr>
<th>Characteristic*</th>
<th>Lymph Node Status</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive (n = 33)</td>
</tr>
<tr>
<td>Age, mean (SE), y</td>
<td>54 (2.4)</td>
</tr>
<tr>
<td>Size, mean (SE), mm</td>
<td>32 (1.6)</td>
</tr>
<tr>
<td>No. of nodes examined, mean (SE)</td>
<td>17 (1.0)</td>
</tr>
<tr>
<td>No. of factor VIII–stained vessels, mean (SE)</td>
<td>51 (2.7)</td>
</tr>
<tr>
<td>Microvessels, mean (SE), %</td>
<td></td>
</tr>
<tr>
<td>VEGFR-3 stained</td>
<td>16 (1.4)</td>
</tr>
<tr>
<td>Type IV collagen-stained</td>
<td>38 (3.9)</td>
</tr>
<tr>
<td>Unstained</td>
<td>46 (4.1)</td>
</tr>
<tr>
<td>ER positive, No. (%)</td>
<td>21 (64)</td>
</tr>
<tr>
<td>PR positive, No. (%)</td>
<td>22 (67)</td>
</tr>
<tr>
<td>Nuclear grade 3, No. (%)</td>
<td>25 (76)</td>
</tr>
</tbody>
</table>

*VEGFR indicates vasoactive endothelial growth factor receptor; ER, estrogen receptor; and PR, progesterone receptor.
†χ² Test for equality of proportions. All the other statistical analyses in this column were done using 2-sample t tests with Satterthwaite’s corrected degrees of freedom for unequal variances.

Figure 1. Patients with positive regional lymph nodes had significantly (P<.001) more vasoactive endothelial growth factor receptor 3 (VEGFR-3)–immunostained microvessels than patients with negative nodes. (r = −0.46, P = .001). There are no statistically significant correlations between the percentage of putative lymphatic vessels or the percentage of putative blood vessels and the age of the patient or the size of the tumor (r<0.2, P>.90 in all cases).

There is no statistically significant difference in the total number of microvessels immunostained with factor VIII between node-positive and node-negative women (Table 1). There are, however, large and statistically significant differences in the number and percentage of microvessels identified as putative lymphatic vessels by VEGFR-3 immunostaining (Figure 1 and Figure 2) and in the number and percentage of microvessels identified as blood vessels by type IV collagen immunostaining (Figure 2 and Figure 3). The percentage of putative lymphatic vessels is approximately 4 times greater (P<.001) in node-positive women than in node-negative women. On the other hand, the percentage of blood vessels in node-positive women is approximately half the percentage in node-negative women (P<.001).

We fitted a logistic model with 2 predictors, percentage of putative lymphatic vessels and percentage of putative blood vessels. The odds ratio (SE) for the percentage of VEGFR-3–stained vessels was 1.40 (0.20) (95% confidence interval, 1.20-1.80; P = .001); for type IV collagen–stained vessels, 0.90 (0.02) (95% confidence interval, 0.89-0.98; P = .006). The fitted model indicated that the odds of a woman having metastatic spread to her axillary lymph nodes increase linearly as the percentage of VEGFR-3–immunostained microvessels increases, and decrease linearly as the percentage of type IV collagen–immunostained microvessels increases. There is also a moderate linear increase with the total number of factor VIII immunostained microvessels. The model fits well (log likelihood = −14.6; χ² = 53.4; P<.001; area under the receiver

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operation characteristic curve = 0.97). There are, however, 2 individuals with high influence on the fitted model. One is a node-positive woman with a high percentage of blood vessels (90%), even higher than the mean for node-negative women. The percentage of putative lymphatic vessels (8%) for this individual is intermediate compared with the means of node-negative and node-positive women. The other individual is a node-negative woman whose percentage of lymphatic vessels (19%) is more consistent with that of node-positive women than node-negative women; her percentage of putative blood vessels (76%) is consistent, however, with the values of other node-negative women. Because both of these women were misclassified by the model, deletion of their observations increases the magnitude of the associations but does not change the conclusions drawn from the model, and they are included in all analyses.

Predicted probabilities estimated from the logistic model are combined to produce groupings of high (≥75%), moderate (25%-75%), and low (<25%) probabilities of positive lymph nodes (Figure 2). There were 24 women predicted to be at low risk of positive nodes and all but 1 are, in fact, node negative (Table 2). Similarly, 27 women were predicted to be at high risk and all but 1, in fact, have positive nodes. These women represent the 2 influential observations previously described. Nine women were predicted to be in the intermediate-risk category, and two thirds of them had positive axillary nodes.

![Figure 2.](image1)

**Figure 2.** The relative proportions of vasoactive endothelial growth factor receptor 3 (VEGFR-3)– and type IV collagen–immunostained microvessels predicted the likelihood of regional lymph node metastasis. The predicted risk of positive lymph nodes was low (A), moderate (B), or high (C). Solid circles indicate negative lymph nodes; squares, positive lymph nodes (see the "Microvessel Density in Primary Breast Cancers With Lymph Node Metastases" subsection of the "Results" section).

![Figure 3.](image2)

**Figure 3.** Patients with positive regional lymph nodes had significantly (P = .004) fewer type IV collagen–immunostained microvessels than patients with negative nodes (see the "Microvessel Density in Primary Breast Cancers With Lymph Node Metastases" subsection of the "Results" section).

<table>
<thead>
<tr>
<th>Predicted Risk*</th>
<th>Patients With Positive Nodes/Total Patients*</th>
<th>Putative Lymphatic Vessels†</th>
<th>Putative Blood Vessels†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low (&lt;25)</td>
<td>1/24 (4)</td>
<td>3.2 (0.6)</td>
<td>69.3 (2.7)</td>
</tr>
<tr>
<td>Moderate (25-75)</td>
<td>6/9 (67)</td>
<td>6.1 (0.8)</td>
<td>41.9 (6.9)</td>
</tr>
<tr>
<td>High (&gt;75)</td>
<td>26/27 (96)</td>
<td>18.4 (1.3)</td>
<td>31.2 (4.2)</td>
</tr>
</tbody>
</table>

*Percentages are given in parentheses. †Data are given as mean (SE) percentage of vessels.

![Table 2. Predicted Risk of Lymph Node Metastasis](image3)
Patients with primary invasive breast cancers whose tumors metastasized to ipsilateral axillary lymph nodes had statistically more VEGFR-3–immunostained vessels than patients with tumor-negative nodes. These vessels also immunostained with endothelium-selective antibodies to factor VIII but failed to immunostain with type IV collagen found in blood vessel basement membranes. The immunostained vessels, blood vessels and lymphatic vessels, were commonly found at or near the surface of the tumor, mostly aggregated in hot spots. Typical blood vessels, found in the angiogenic hot spots, immunostained with antibodies to factor VIII and, in many instances, also with antibodies to type IV collagen. Patients whose tumors metastasized to the lymph nodes had significantly fewer type IV collagen–immunostained vessels, while the total number of factor VIII–immunostained vessels was the same whether the nodes showed tumor metastasis or not. These data suggest that an increase in VEGFR–immunostained microvessels is associated with an increased risk of node metastasis in patients with breast cancer.

The probability of node metastasis was assessed by an analysis of simultaneous importance of the relative proportion of blood vessels and putative lymphatic vessels in angiogenic hot spots. Risk classifications derived from the statistical model were accurate predictors of axillary node metastasis. Patients with the highest relative numbers of VEGFR–immunostained microvessels and the lowest relative numbers of blood vessels had the highest probability of having node metastasis. Those with the lowest relative numbers of VEGFR–immunostained microvessels and the highest relative number of blood vessels had the lowest probability of having node metastasis. An intermediate group of patients with neither high nor low relative numbers of VEGFR–immunostained microvessels or blood vessels had a moderate predicted risk of node metastasis. These relations have potentially interesting clinical implications since an accurate marker for predicting node metastasis could help determine which patients might benefit from axillary lymphadenectomy. To date, to our knowledge, none of the clinical, radiological, pathological, or molecular methods of predicting node metastasis have proved sensitive or specific enough to supplant standard or sentinel axillary lymphadenectomy in the accurate staging of breast cancer.

Lymphatic vessels have been described in ductal carcinoma in situ, in invasive breast cancers, and in various other tumors. It is unclear whether these were residual lymphatic channels or newly formed (lymphangiogenesis). Another study failed to find lymphangiogenesis in melanoma, but this was not based on direct staining of lymphatic endothelium but on differential double-staining techniques not yet proved to be lymphatic specific.

Vasoactive endothelial growth factor receptor 3, part of the FLT gene family, belongs to the class III receptor tyrosine kinases, which also include 2 proto-oncogenes (c-FMS and c-KIT), the α and β chains of the platelet-derived growth factor receptors, and the product of the FLT3–FLK2 gene. Vasoactive endothelial growth factor receptors 1, 2, and 3 differ from other members of class III receptor tyrosine kinases by having 7 instead of 5 immunoglobulin loops in their extracellular domains. Vasoactive endothelial growth factor receptors 1 and 2 bind VEGF with high affinity and also bind placental growth factor. Vasoactive endothelial growth factor receptor 2 is a major regulator of vasculogenesis and angiogenesis. Vasoactive endothelial growth factor receptor 3 is closely related in structure to the products of the VEGF-1 and VEGF-2 genes. Vasoactive endothelial growth factor receptor 3 is related to the other VEGF receptors but does not bind VEGF-A and VEGF-B, and its expression becomes restricted mainly to lymphatic endothelia during embryonic development. During late embryonic development, VEGF-3 messenger RNA (mRNA) becomes restricted to developing lymphatic vessels. Only lymphatic endothelia and some high endothelial venules express VEGF-3 mRNA in normal adult human tissues. Vasoactive endothelial growth factors A and B bind to VEGF-1 (FLT1) and VEGF-2 (FLK or KDR) on blood vessel endothelial cells, and do not bind to VEGF-3.

A recent investigation shows significantly increased density of VEGFR–immunostained vessels and VEGF-C expression in breast cancers but suggests that anti–VEGFR-3 antibodies bind to lymphatic vessels and blood vessels. Some investigators concluded that VEGF-C is involved in angiogenesis, but not lymphangiogenesis, in breast cancer, a surprising conclusion given the compelling evidence for VEGF-C–induced subdermal lymphangiogenesis in mouse embryos from the same group of investigators. Vasoactive endothelial growth factor C, when deliberately overexpressed in transgenic mouse embryos, induces marked lymphangiogenesis and lymphangiectasis. The pattern of expression of VEGF-C in relation to its major receptor, VEGF-3, during the sprouting of the lymphatic endothelium in embryos suggests a paracrine mode of action and that one of the functions of VEGF-C may be in the regulation of angiogenesis in the lymphatic vasculature. Vasoactive endothelial growth factor C can also bind to VEGF-2 expressed in blood vessel endothelia, induce capillary endothelial cell migration in culture, and induce angiogenesis in ischemic rabbit hind limbs and mouse corneas. Vasoactive endothelial growth factor C mRNA is detectable in some, but not all, tumor cell lines, suggesting there may be a variable propensity for stimulation of lymphatic and some blood vessel endothelial cells. A logical extension of these findings in tumors is that secretion of larger amounts of VEGF-C could induce more lymphatic vessels to sprout, as occurs with angiogenesis induced by angiogenic cytokines such as fibroblast growth factor β or VEGF-A.

Angiogenesis is a complex process regulated by VEGFs, angiopoietins, and other protein ligands via their cognate transmembrane tyrosine kinase receptors, VEGFR-1, VEGFR-2, Tie1, and Tie2, expressed on endothelial cells. Vasoactive endothelial growth factor receptor 3 is expressed in primitive blood vessels,
but this expression is lost during later differentiation and development when this epitope is expressed on mature lymphatic endothelium.\textsuperscript{17-22} The pattern and complexity of epitope expression in tumor angiogenesis is not yet clearly defined, but the coexistence of blood vessel and lymphatic epitopes in VEGFR-3–expressing tumor endothelial cells\textsuperscript{25} is reminiscent of lymphatic development during vasculogenesis.\textsuperscript{21,22} Blood vessel markers may be expressed in primitive lymphatic endothelia and disappear as the embryonic microvessels mature.\textsuperscript{17,22} It is possible that this pattern of epitope expression may be reproduced during the sprouting of cytokine-induced microvessels in tumors. Differentiation of endothelial cell phenotypes may follow pathways similar to those seen in differentiating hemopoietic cells.\textsuperscript{41,43}

The association between tumor microvessel density and hematogenous metastasis is measurable by investigating overall and disease-free survival.\textsuperscript{13,16} Microvessel density is the pathobiological correlate of angiogenesis, a vital part of tumor progression and metastasis.\textsuperscript{44} Tumor cells exposed to increased numbers of proliferating microvessels are more likely to metastasize to visceral sites and to lymph nodes.\textsuperscript{45,47,15} There is a logical association between increased blood vessel density and hematogenous metastasis because invading tumor cells are exposed to a potentially larger vascular window through which they gain access to the systemic circulation.\textsuperscript{13} However, the association between blood vessel density and node metastasis is unlikely to be causal because tumor microvessels do not drain to axillary lymph nodes unless they are lymphatic vessels. An increased size of the lymphatic window, by lymphangiogenesis, through which breast cancer cells can metastasize to the nodes is an attractive possibility. Lymphangiogenesis occurs during embryonic development,\textsuperscript{36} after wounding,\textsuperscript{36} and in angiosarcomas.\textsuperscript{46} Lymphangiomas,\textsuperscript{47} and tissue culture\textsuperscript{48} but has not been reported, to our knowledge, in normal adult tissues.\textsuperscript{36}

While our data suggest lymphangiogenesis occurs in patients with breast cancer, the study is small and highly selective and needs confirmation in a larger cohort of patients. A proportion of lymph node micrometastases that are missed using standard H&E staining may be identified by immunohistochemistry or by polymerase chain reaction.\textsuperscript{1,10} The individual patient in our study in whom microvessel density strongly predicted lymph node metastasis, but in whom H&E staining failed to show metastasis, may have had positive nodes. If this were proved to be the case, our study would be even more significant.

In conclusion, we showed an association between increased numbers of VEGFR-3–immunostained microvessels in breast cancer and axillary lymph node metastasis. This information, if confirmed by larger studies, could prove useful to clinicians treating patients with breast cancer.

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REFERENCES

DISCUSSION

Mark M. Connolly, MD, Chicago, Ill: Dr Nathanson has presented a very interesting pilot study posing a novel mechanism for axillary lymph node metastases. He has utilized a valid approach modeled on extensive work by multiple investigators, previously demonstrating microvascular density as an independent and highly significant prognostic indicator for relapse-free and overall survival in early breast cancer. Several of these studies also showed a significant correlation with axillary lymph node metastases. He correctly suggests that this does not have an inherent direct causal association with nodal metastasis and theorizes an increased “lymphatic window” secondary to lymphangiogenesis as an explanation, seemingly validated by his findings.

Risk classifications derived from his statistical models were accurate predictors of axillary nodal metastases, the current most important prognostic factor that is available to us. This appears to address the first requirement of a new prognostic indicator in possessing a clear biologic significance. The hypothesis is based on antibody to vasoactive endothelial growth factor 3, staining only the lymphatics.

The role of prognostic factors has changed with the widespread strategy of administering systemic adjuvant therapy to patients regardless of nodal status, the significant trend for decreased tumor size associated with screening, and the availability of sentinel lymph node biopsies. There is presently no factor or combination that completely separates patients who are cured by local treatment from those who are destined to recurrence. Although the list of potential prognostic factors is long, the list of established factors is short and relatively unchanged until the emergence of angiogenesis as a biomarker of invasion, as well as providing a target for a novel therapeutic intervention. This study serves to reemphasize the value of exploring mechanisms of metastases, which potentially may offer new approaches to therapy as well as prognosis. Dissemination to axillary lymph nodes is still the most powerful prognostic indicator, and this study appears to actively predict nodal status in the high- and low-risk groups.

The current role of complete axillary dissection is controversial and such a predictor could help direct management, but I question its role compared to sentinel lymph node biopsy. Trials using angiogenic factors are in progress, and it remains to be determined to what degree, if any, these agents effect lymphangiogenesis, or if that remains important if the tumors are induced to their prevascular phase, or will this potentially be a new target for therapeutic modulation.

I have several questions. Do you really know what you are observing? Might you also stain new blood vessels that have different characteristics than the normal adult vessels? The methodology is very work intensive and not user friendly; lack of interobserver concordance reproducibility can significantly alter results. Can this be done outside a controlled research setting? Would utilization of image analysis technology be feasible to standardize counts?

Finally, although this is not a survival study, in light of your conclusions that there was no statistical difference in the total number of microvessels immunostained between node negative and node positive, and intuitively we would predict a worse prognosis for the node positive, how do you reconcile this with previous studies reporting microvessel count as an independent risk factor for both relapse-free and overall survival? Basically, lymph node metastases have served as an indicator of hematogenous metastases; does this cohort of node-negative women behave differently than node-negative women without hot spots, or could this possibly differentiate subsets for early regional vs systemic failure?

Anton Bilchik, MD, Santa Monica, Calif: I have 2 questions. First, was there any correlation between microvessel immunostaining and other prognostic characteristics of the primary tumor, such as HER2-NEU and ploidy? Second, since we are in the era of micrometastases, particularly with the sentinel node technique, what percentage of tumor-positive axillary nodes contained micrometastases correlated with immunostaining?

James Goodnight, MD, Sacramento, Calif: Did you study lymph nodes that had metastases or not for the presence of the VEGF-C? In other words, was there any correlation between...
the appearance in the primary tumor and the appearance in the axillary nodes?

Dr Nathanson: First, Dr Connolly, the specificity of the antibody is an important question. The specificity shown with lymphangiommas may not apply to newly developing microvessels in tumors. This is an area that needs further study. We cannot tell you for sure that all tumor microvessels that stain with VEGFR-3 are lymphatics, nor can we tell you that those non-stained specimens that I mentioned did not contain lymphatics. That is an important question and will be a fruitful area to investigate. Your second question was about the methodology and whether this would be transportable to a routine pathology lab. This is a very labor-intensive effort, and I have tried to interest my pathology collaborators to do all of the studies with me and they petered out when we got to 33 blocks per patient. I doubt very much that it would be practical to do it by counting microvessels under the microscope. Image analysis may overcome the practical hurdles.

Your next question probably relates to how accurate is assessment of microvessel density in determining angiogenesis and its relationship to survival in our cohort. We did not do that in our study, but there are certainly many studies in many different solid tumors showing MVD (microvessel density) to be the best prognosticator or predictor of survival.

Dr Bilchik asked about other prognostic indicators, HER2-NEU, etc. Our study was a multivariate analysis, which included well-known predictors of axillary metastasis, and lymphatic vessel density was independently predictive. You asked about axillary micrometastases; there is a lot of interest in upstaging the percentage of micrometastases, particularly, for example, with RTPCR (reverse transcription polymerase chain reaction) and with immunohistochemistry. We did not look at either of these methods in this group of patients.

Dr Goodnight, you asked about the VEGF-C. We considered doing VEGF-C immunostaining on primary tumors to relate its expression to node metastasis. However, we soon discovered that all the tumor cells expressed VEGF-C. We tried to quantify the expression of VEGF-C but discarded this effort because it is too crude. It did not make too much sense to study VEGF-C in tumor cells in nodes because there was no control.

JAMA

The Relationship Between Cyclooxygenase-2 Expression and Colorectal Cancer

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Context: Epidemiological studies have implicated the inducible form of cyclooxygenase (COX-2) in the pathogenesis of colorectal cancer; however, its role is not fully understood.

Objective: To examine the relationship between the expression of COX-2 in human colorectal cancer and patient survival.

Design: Patients diagnosed as having colorectal cancer were evaluated and followed up for up to 9.4 years (median follow-up, 2.7 years). Tumor sections were stained for COX-2 using a rabbit polyclonal antibody raised against human COX-2. The extent of COX-2 staining was graded by 2 observers blinded to outcome. Preabsorption of the anti–COX-2 antibody with a COX-2 peptide abolished the staining, demonstrating the specificity of the assay.

Setting: Gastrointestinal unit of a large general teaching hospital in Dublin, Ireland.

Participants: Seventy-six patients (median age, 66.5 years) with colorectal cancer (Dukes tumor stage A, n = 9; Dukes B, n = 30; Dukes C, n = 25; Dukes D, n = 12) whose diagnosis was made between 1988 and 1991. Fourteen normal colon biopsies were stained for COX-2 as controls.

Main Outcome Measures: Survival in years following diagnosis compared by extent of COX-2 epithelial staining (grade 1, <1%; grade 2, 1%-19%; grade 3, 20%-49%; grade 4, ≥50%), Dukes stage, tumor size, and lymph node metastasis.

Results: COX-2 was found in tumor epithelial cells, inflammatory cells, vascular endothelium, and/or fibroblasts. The extent of epithelial staining was heterogeneous, varying markedly among different tumors. Normal tissue adjacent to the tumors also stained weakly for COX-2. No COX-2 was detected in control tissue samples. The Kaplan-Meier survival estimate was 68% in patients who had grade 1 tumor epithelial staining compared with 35% in those with higher grades combined (log-rank $\chi^2 = 5.7; P = .02$). Greater expression of COX-2 correlated with more advanced Dukes stage (Kendall $\tau$-b, 0.22; $P = .03$) and larger tumor size (Kendall $\tau$-b, 0.21; $P = .02$) and was particularly evident in tumors with lymph node involvement (Kendall $\tau$-b, 0.26; $P = .02$).

Conclusions: Our data indicate that COX-2 expression in colorectal cancer may be related to survival. These data add to the growing epidemiological and experimental evidence that COX-2 may play a role in colorectal tumorigenesis. (1999;282:1254-1257) www.jama.com

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