

Angiogenic and lymphangiogenic microvessel density in breast carcinoma: correlation with clinicopathologic parameters and VEGF-family gene expression

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Angiogenesis and lymphangiogenesis are essential for breast cancer progression and are regulated by vascular endothelial growth factors (VEGF). To determine clinical and molecular correlates of these processes, we measured blood and lymphatic vascular microvessel density in 29 invasive carcinomas (22 ductal, six lobular, one papillary), using the vascular marker CD31 and the novel lymphatic marker D2-40. Microvessel density was assessed microscopically and by image cytometry, and was compared with tumor histology, grade, stage, lymph node metastasis, hormone receptors, HER2/neu status, and expression of VEGF, VEGF-C and VEGF-D by immunohistochemistry or quantitative RT-PCR. Strong correlation was observed between visual and image cytometric microvessel density using D2-40 but not CD31 ($P=0.016$ and 0.1521 , respectively). Image cytometric CD31 microvessel density correlated with tumor size, grade, stage and lymph node metastasis ($P=0.0001$, 0.0107 , 0.0035 and 0.0395 , respectively). D2-40 microvessel density correlated with tumor stage ($P=0.0123$ by image cytometry) and lymph node metastasis ($P=0.0558$ by microscopy). Immunohistochemical VEGF signal in peritumoral blood vessels correlated with image cytometric CD31 and D2-40 microvessel density ($P=0.022$ and 0.0012 , respectively), consistent with the role of VEGF in blood and lymphatic vascular growth. Intratumoral VEGF-C and VEGF-D expression by quantitative RT-PCR correlated with D2-40 ($P=0.0291$ by image cytometry) but not with CD31 microvessel density, which could suggest a selective role of VEGF-C and VEGF-D in lymphangiogenesis. CD31 and D2-40 microvessel density correlated significantly with several prognostic factors, including lymph node metastasis. Thus, measurements of angiogenesis and lymphangiogenesis may have utility for breast cancer pathology, particularly for estimation of metastatic risk.

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Angiogenesis (blood vessel growth) and lymphangiogenesis (lymph vessel growth) are critical processes for tumor growth, invasion and metastasis.^{1–3} Thus, measurement of vascular growth may be

clinically important in breast cancer specimens. The common pathologic approach to assessing angiogenesis and lymphangiogenesis involves microscopic estimation of vascular density or microvessel density on tissues probed for endothelial markers by immunohistochemistry. Several markers of blood vessel endothelium have been developed for routine use, including CD31/PECAM-1, CD34, and Factor VIII-related antigen (von Willebrand Factor or vWF).⁴ However, lymphangiogenesis studied have been limited by lack of specific lymphatic endothelial markers. Recently, monoclonal antibody D2-40 was shown in breast⁵ and tonsillar⁶ tissue to detect lymphatic vessels selectively.

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In these studies, lymphatic vessels were immunoreactive for D2-40, while, in contrast, blood vessels were reactive for CD31, CD34, vWF and PAL-E.^{5,6}

Studies of various tumors have shown the potential clinical significance of angiogenesis and lymphangiogenesis, suggesting that blood and lymphatic microvessel density correlate with tumor growth and metastasis.^{7,8} These promising results have launched subsequent research aimed at optimizing methods for microvessel density measurement. The two principal approaches are direct microscopic immunohistochemistry and semiautomated image cytometry. Direct immunohistochemical microvessel density is relatively inexpensive and widely available in diagnostic pathology departments, but is subjective and difficult to standardize within and between laboratories. In contrast, image cytometry is more conducive to standardized quantification but is relatively expensive.

In addition to quantification, interest is growing in the molecular regulation of angiogenesis and lymphangiogenesis. Breast cancers elaborate several angiogenic and lymphangiogenic growth factors, which represent candidate markers for diagnosis, prognosis and therapeutic strategies. For example, vascular endothelial growth factor (VEGF) appears to be critical primarily for blood vessel development in breast cancer, while the related factors VEGF-C and VEGF-D regulate lymphatic development.^{9,10} VEGF-C mRNA and protein localize to tumor cells of breast carcinoma, squamous cell carcinoma, lymphoma, melanoma and sarcoma.¹¹ VEGFR-3 has been shown to have a role in adult blood vessel angiogenesis.¹²

Previous studies in breast cancer suggest that VEGF-family gene expression correlates with tumor growth and metastasis.^{9,13} However, it is still unresolved whether VEGF, VEGF-C or VEGF-D expression can be related to blood or lymphatic microvessel density in clinical breast cancer specimens. Therefore, we sought to address this issue by using image cytometry to determine blood or lymphatic microvessel density on a cohort of well-annotated breast carcinoma samples stained with CD31 and D2-40. We analyzed these data together with VEGF, VEGF-C and VEGF-D expression using immunohistochemistry and quantitative RT-PCR.

Materials and methods

Clinicopathologic Data

The breast carcinoma samples were obtained from the archives of the Surgical Pathology Division of the Department of Pathology and Laboratory Medicine, Emory University Hospital and Veterans Affairs Medical Center, Atlanta, GA, USA. Cases were diagnosed between 1995 and 2000, and all were deidentified prior to the study. All tumors were invasive carcinomas from female patients. Tumors were subtyped as ductal or lobular carcinoma,

ma,¹⁴ and histologically graded as I–III based on tubule formation, nuclear grade and mitotic count.^{15,16} Pathologic staging T1–T4 was according to the American Joint Cancer Committee (AJCC) TNM Staging 2002. Regional lymph node, estrogen receptor, progesterone receptor and Her2/neu status were recorded. Pathologic parameters were obtained by review of hematoxylin and eosin (H&E)-stained slides, surgical pathology reports, and the Oncology Data Bank. This research was approved by the Emory University Institutional Review Board under protocol 015-2001.

Immunohistochemistry

All tissues were fixed in 10% neutral buffered formalin and embedded in paraffin using standard surgical pathology protocols. Diagnoses were established from H&E-stained slides using standard histopathologic criteria. Immunohistochemistry was performed on a single representative block from each case. Tissue sections (5 μ m) were dewaxed and antigen retrieval was performed in citrate buffer (pH 6), using an electric pressure cooker set at 120°C for 5 min.¹⁷ Sections were incubated for 5 min in 3% hydrogen peroxide to quench endogenous tissue peroxidase. Primary monoclonal antibodies were directed against CD31 (1:80 dilution, DAKO Corporation, Carpinteria, CA, USA), D2-40 antigen (1:5 dilution, Signet Laboratories, Dedham, MA, USA), and VEGF (1:160 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Tissue sections were incubated with appropriate primary antibody for 25 min at room temperature. After washing unbound primary antibody, sections were treated with commercial biotinylated secondary anti-immunoglobulin, followed by avidin coupled to biotinylated horseradish peroxidase, at room temperature, according to the manufacturer's instructions (LSAB2 kit, DAKO Corporation). Immunohistochemical reactions were developed with diaminobenzidine as the chromogenic peroxidase substrate. Sections were counterstained with hematoxylin after immunohistochemistry. Specificity was verified by negative control reactions without primary antibody, as well as appropriate cytoplasmic reactions for each antigen in positive control tissues. For VEGF expression, immunohistochemical reactions were assessed in areas of invasive carcinoma and non-neoplastic breast tissue. Cytoplasmic staining intensity was graded from 0 (no staining) to 3 (most intensely stained), and the percentage of positive cells was noted.

Microvessel Density Assessment

Direct microscopy

Immunohistochemical reactions for CD31 and D2-40 antigen were interpreted independently by two authors (WWLC, CC) using a two-headed microscope. The two most vascularized areas within

tumor ('hot spots') were chosen at low magnification ($\times 40$) and vessels were counted in a representative high magnification ($\times 400$; 0.152 mm²; 0.44 mm diameter) field in each of these two areas. The high-magnification fields were then marked for subsequent image cytometric analysis. Single immunoreactive endothelial cells, or endothelial cell clusters separate from other microvessels, were counted as individual microvessels. Endothelial staining in large vessels with tunica media, and nonspecific staining of nonendothelial structures, were disregarded in microvessel counts. Mean visual microvessel density for CD31 and D2-40 was calculated as the average of four counts (two authors and two microscopic fields).

Image cytometry

Tissue sections stained with anti-CD31 and D2-40 were analyzed with the Ariol SL-50™ system (Applied Imaging, Santa Clara, CA, USA). Ariol SL-50™ is an automated scanning microscope controlled by a computer with software that directs a motorized stage, filter-wheels and automated focusing system. The Kisight image analysis application was used to quantify microvessel density. The default Kisight assay executes three passes (at $\times 12.5$, $\times 50$ and $\times 200$ total magnifications). The initial low-magnification pass locates tissue on the slide and creates digital scans for the pass at intermediate magnification. Automated image analysis at this magnification identifies brown-stained ring-like structures based on color and morphometry. Areas marked during direct microscopic microvessel counting were selected on intermediate magnification images, using drawing tools for reprocessing. Microvessel density was calculated as the percentage of total field area positively stained for vascular marker.

Quantitative RT-PCR for VEGF-Family Gene Products

Reactions were performed on formalin-fixed paraffin-embedded tissues using published protocols with minor modifications.¹⁸ Histologic sections were deparaffinized with xylene and rehydrated with ethanol. Areas containing tumor were microdissected with a sterile scalpel into digestion buffer. Samples were heated at 95°C for 10 min and digested with proteinase K at 52°C for 4 h. Total RNA was isolated by phenol/chloroform extraction followed by ethanol precipitation. Quantitative RT-PCR assays were performed using standard SYBR-Green methodology on the I-cycler system (Bio-Rad). Intron-spanning PCR primers for the VEGF, VEGF-C and VEGF-D were designed with Primer Express software (Applied Biosystems) according to the manufacturer's instructions. Primers and amplicon sizes were as follows. VEGF: forward 5'-GCTGTCTTGGGTGCATTGG-3'; reverse 5'-GCAGCCTGGGACCACTTG-3'; Amplicon size

69 bp. VEGF-C: forward 5'-CAGTGTGTCAGGCAGC GAACA-3'; reverse 5'-TTCCTGAGCCAGGCATCTG-3'; amplicon size 78 bp. VEGF-D: forward 5'-TGGGATAGCAACAAATGTAATGTG-3'; reverse 5'-GCAACGATCTTCGTCAAACATC-3'; amplicon size 126 bp. Quantification was based on cycle number required to reach an SYBR-Green fluorescence threshold in the linear phase of amplification. Reaction specificity was assessed by comparing amplicon melting points and size with values predicted by amplicon sequences. Expression levels of VEGF, VEGF-C and VEGF-D were normalized to 28S ribosomal RNA. For all cases, total RNA was prepared from two independent, replicate sections to assess interassay variability. Interassay coefficients of variation were generally less than 10% (not shown).

Statistical Analyses

Three levels of statistics were performed using the SAS version 8.2 (Copyright© 1999–2001 by SAS Institute Inc., Cary, NC, USA): (a) Pearson's correlations; (b) 2×2 χ^2 and Fisher's exact tests; and (c) Kappa agreement tests. Pearson's correlations were validated using graphical methods such as scattergrams. Specifically, the relationship between mean manual CD31 and D2-40 microvessel density with their mean image cytometry counterparts, the relationship between VEGF immunohistochemical results and mean CD31 and D2-40 values (both manual and image cytometry), the relationship between VEGF family protein PCR results and mean CD31 and D2-40 values (both manual and image cytometry), and the relationship between clinicopathologic factors and mean CD31 and D2-40 values (both manual and image cytometry) were all analyzed by Pearson's correlation coefficients. Only Pearson's correlation coefficients with an accompanying *P*-value of <0.05 were considered statistically significant. χ^2 and Fisher's exact tests were used to compare VEGF, VEGF-C and VEGF-D (both immunohistochemical results and PCR results) with microvessel density and clinicopathologic parameters.

Results

Clinicopathologic parameters of the 29 invasive breast carcinoma studied are summarized as follows. Median patient age was 66 years (range 34–91 years). Histologic diagnoses included ductal ($N=22$), lobular ($N=6$) and papillary ($N=1$) carcinomas. Histologic grades included grade I ($N=7$), grade II ($N=16$) and grade III ($N=6$). Tumor stages included pT1 ($N=1$), pT2 ($N=9$), pT3 ($N=1$) and pT4 ($N=1$). Status of regional lymph nodes, hormone receptors and HER2/neu were: lymph node metastasis positive ($N=14$) and negative ($N=15$), estrogen receptor positive ($N=23$) and

negative ($N=6$), progesterone receptor positive ($N=14$) and negative ($N=15$), and HER2/neu positive ($N=15$) and negative ($N=14$). Case characterization is compiled in Table 1.

Correlation of Microvessel Density with Clinicopathologic Parameters of Breast Cancer

Angiogenesis and lymphangiogenesis were quantified by visual and image cytometric microvessel density, using CD31 and D2-40 as markers for vascular and lymphatic endothelium, respectively

(Figures 1 and 2). For CD31, mean microvessel density was 29.2 microvessels per $\times 200$ field visually (range 12.8–94.3), or 2.4% area per $\times 200$ field by image cytometry (range 0.0–8.2). For D2-40, mean microvessel density was 7.4 lymphatic microvessels per $\times 200$ field visually (range 0.0–24.0), or 2.1% area per $\times 200$ field by image cytometry (range 0.0–8.2). Table 2 shows Pearson's correlations between visual and image cytometric microvessel density for CD31 and D2-40. CD31 microvessel density showed similar trends using either technique, but the correlation was not statistically significant ($r=0.2779$; $P=0.1521$). In contrast, visual

Table 1 Clinicopathologic features of the specimen cohort

Patients	Age (years)	Histology	Primary tumor size (cm)/stage ^a	Grade	LN	ER	PR	Her2/neu
1	53	Ductal	5.5/pT3	3	POS	NEG	POS	NEG
2	72	Ductal	0.9/pT1b	3	POS	POS	POS	POS
3	78	Ductal	2.7/pT2	3	POS	NEG	NEG	NEG
4	50	Ductal	2.3/pT2	2	NEG	POS	POS	POS
5	37	Ductal	1.7/pT1c	2	POS	POS	POS	NEG
6	73	Ductal	0.5/pT1a	1	NEG	POS	POS	POS
7	66	Ductal	1.2/pT1c	3	NEG	POS	NEG	POS
8	66	Papillary	1/pT1b	1	NEG	POS	POS	NEG
9	58	Ductal	0.8/pT1b	1	POS	POS	POS	NEG
10	51	Lobular	1.3/pT1c	2	NEG	POS	POS	NEG
11	75	Ductal	0.3/pT1a	2	POS	POS	POS	NEG
12	55	Lobular (L&R)	1.1(L:0.9, R:1.1)/pT1c	2	POS	POS	NEG	POS
13	66	Ductal	1.4/pT1c	1	NEG	POS	NEG	NEG
14	70	Ductal	0.3/pT1a	2	POS	NEG	NEG	NEG
15	86	Ductal	2.9/pT2	2	NEG	NEG	NEG	POS
16	58	Lobular	1.5/pT1c	2	NEG	POS	NEG	POS
17	43	Ductal	0.9/pT1b	2	NEG	POS	NEG	NEG
18	46	Ductal	1.2/pT1c	1	NEG	POS	POS	NEG
19	67	Ductal	4.6/pT2	2	NEG	NEG	NEG	POS
20	52	Ductal	1.8/pT1c	3	NEG	NEG	NEG	POS
21	67	Ductal	2.3/pT2	2	NEG	POS	POS	POS
22	66	Lobular	2.7/pT2	2	POS	POS	NEG	POS
23	70	Ductal	2/pT1c	2	POS	POS	NEG	POS
24	34	Ductal	2.4/pT2	2	POS	POS	POS	POS
25	71	Lobular	2.2/pT2	2	POS	POS	POS	POS
26	69	Ductal	1.1pT1c	1	NEG	POS	POS	NEG
27	56	Lobular	2.3/pT2	2	POS	POS	NEG	NEG
28	53	Ductal	0.2/pT1a	1	NEG	POS	NEG	NEG
29	91	Ductal	10/pT4	3	POS	POS	NEG	POS

LN = lymph node status; ER = estrogen receptor status; PR = progesterone receptor status; POS = positive; NEG = negative.

^aPrimary tumor stage according to AJCC 2002; insufficient patient data for stage grouping.

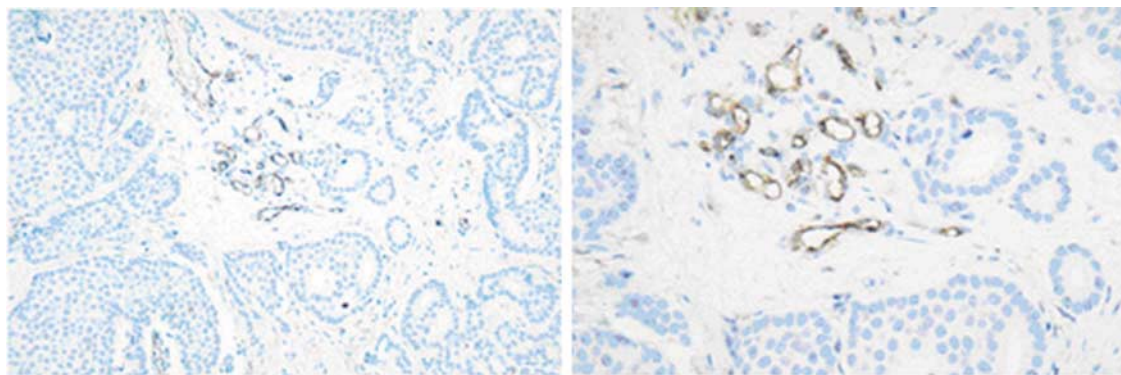


Figure 1 CD31 immunostained blood vessels in an invasive breast carcinoma 'hot spot', quantified visually (mean microvessel density = 54) and image cytometrically (mean microvessel density = 4.0% area), $\times 200$ (left) and $\times 400$ (right).

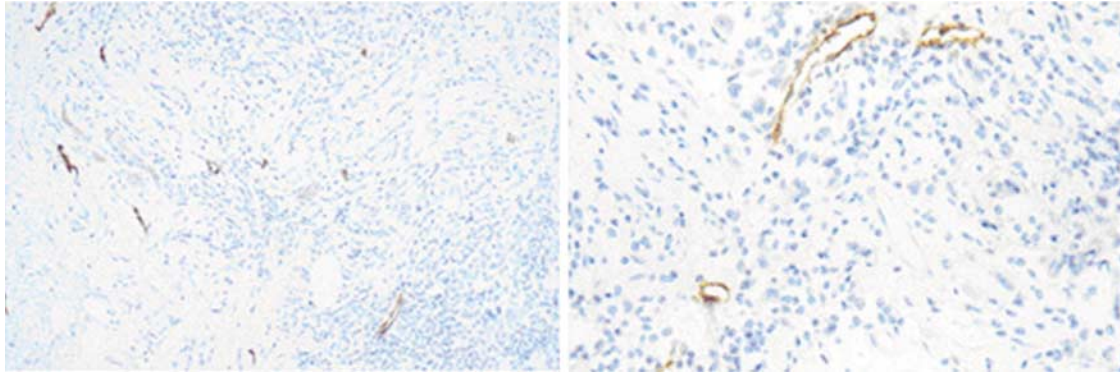


Figure 2 D2-40 immunostained lymphatics in an invasive breast carcinoma 'hot spot', quantified visually (mean microvessel density = 24) and image cytometrically (mean microvessel density = 5.5% area), $\times 200$ (left) and $\times 400$ (right).

Table 2 Comparison of CD31 and D2-40 mean microvessel density assessed visually and by image cytometry (expressed as Pearson's correlations with associated *P*-values)

	CD31 (visual)	D2-40 (visual)	CD31 (image cytometric)	D2-40 (image cytometric)
CD31 (visual)	1	0.1157 (<i>P</i> = 0.5502)	0.2779 (<i>P</i> = 0.1521)	0.1539 (<i>P</i> = 0.4343)
D2-40 (visual)	0.1157 (<i>P</i> = 0.5502)	1	0.5589 (<i>P</i> = 0.0020)	0.4503 (<i>P</i> = 0.0162)
CD31 (image cytometric)	0.2779 (<i>P</i> = 0.1521)	0.5589 (<i>P</i> = 0.0020)	1	0.3812 (<i>P</i> = 0.0498)
D2-40 (image cytometric)	0.1538 (<i>P</i> = 0.4343)	0.4503 (<i>P</i> = 0.0162)	0.3812 (<i>P</i> = 0.0498)	1

Significant correlations are in boldface.

and image cytometric measurements of D2-40-positive vessels correlated strongly ($r = 0.4503$; $P = 0.0162$). D2-40 microvessel density (using either technique) correlated significantly with image cytometric, but not visual, CD31 microvessel density ($r = 0.5589$, $P = 0.0020$ for visual D2-40; $r = 0.3812$, $P = 0.0498$ for image cytometric D2-40).

Table 3 shows Pearson's correlations of CD31 and D2-40 microvessel density with clinicopathologic parameters of breast cancer. Mean image cytometric CD31 microvessel density correlated significantly with tumor size ($P = 0.0001$), histologic grade ($P = 0.0107$), lymph node status ($P = 0.0395$), and stage ($P = 0.0035$). Mean image cytometric D2-40 microvessel density correlated significantly with stage ($P = 0.0123$), while visual D2-40 analysis trended with lymph node metastasis ($P = 0.0558$). Angiogenesis and lymphangiogenesis did not correlate significantly with estrogen receptor, progesterone receptor or HER2/neu immunoreactivity.

Correlation of Microvessel Density with VEGF, VEGF-C and VEGF-D Gene Expression

VEGF was detected immunohistochemically in breast carcinoma and vascular endothelial cells (Figure 3). The correlations of CD31 and D2-40 microvessel density with immunohistochemical

VEGF expression are shown in Table 4. Tumor angiogenesis and lymphangiogenesis, determined as mean CD31 and D2-40 microvessel density, correlated significantly with VEGF expression in the endothelial cells of peritumoral benign breast tissue ($P = 0.0217$ and 0.0012 , respectively, by image cytometry), but not with VEGF expression in carcinoma cells or intratumoral endothelial cells. VEGF antigen in peritumoral endothelial cells also correlated with pathologic stage pT2 or higher ($P = 0.0025$ and 0.0049 for χ^2 -test and Fisher's Exact Test). Table 5 shows the correlation of CD31 and D2-40 with VEGF, VEGF-C and VEGF-D mRNA expression by Q-RT-PCR. Visual mean D2-40 microvessel density correlated significantly with VEGF-C and VEGF-D mRNA ($P = 0.0291$ and 0.0432 , respectively), but not with VEGF. Although image cytometric D2-40 microvessel density also trended with VEGF-C and VEGF-D expression, the correlations did not achieve statistical significance. CD31 microvessel density did not correlate significantly with VEGF, VEGF-C or VEGF-D mRNA expression. In addition to their correlation with D2-40 microvessel density, VEGF-C and VEGF-D were associated with the histologic type of invasive ductal carcinoma ($P = 0.0218$ and 0.0469 , respectively, for χ^2 -test), and VEGF-C mRNA correlated significantly with lymph node status ($P = 0.0221$ and 0.0414 for χ^2 -test and Fisher's exact test).

Table 3 Correlations of mean CD31 and D2-40 microvessel density, visually and image cytometrically, with clinicopathologic parameters of breast carcinoma (expressed as Pearson's correlations with associated *P*-values)

Clinicopathologic parameter	Microvessel density technique			
	CD31 (visual)	D2-40 (visual)	CD31 (image cytometric)	D2-40 (image cytometric)
Patient age	-0.0878 (<i>P</i> =0.6507)	-0.3074 (<i>P</i> =0.1048)	0.1062 (<i>P</i> =0.5907)	-0.3553 (<i>P</i> =0.0635)
Tumor size	0.2998 (<i>P</i> =0.1141)	0.1213 (<i>P</i> =0.5308)	0.6669 (<i>P</i>=0.0001)	0.0879 (<i>P</i> =0.6567)
Histologic type	-0.0039 (<i>P</i> =0.9841)	-0.1418 (<i>P</i> =0.4633)	0.1255 (<i>P</i> =0.5247)	-0.0501 (<i>P</i> =0.8001)
Histologic grade	-0.0912 (<i>P</i> =0.6382)	0.0360 (<i>P</i> =0.8531)	0.4746 (<i>P</i>=0.0107)	-0.0077 (<i>P</i> =0.9690)
Lymph node status	-0.1840 (<i>P</i> =0.3393)	0.3590 (<i>P</i>=0.0558)	0.3912 (<i>P</i>=0.0395)	0.1713 (<i>P</i> =0.3835)
Tumor stage	0.3529 (<i>P</i> =0.0604)	0.2632 (<i>P</i> =0.1677)	0.5324 (<i>P</i>=0.0035)	0.4669 (<i>P</i>=0.0123)
Estrogen receptor status	-0.2516 (<i>P</i> =0.1880)	-0.0435 (<i>P</i> =0.8227)	-0.3425 (<i>P</i> =0.0744)	0.0442 (<i>P</i> =0.8230)
Progesterone receptor status	-0.1886 (<i>P</i> =0.3272)	0.0650 (<i>P</i> =0.7378)	-0.0553 (<i>P</i> =0.7799)	0.3430 (<i>P</i> =0.0739)
HER2/neu status	0.1576 (<i>P</i> =0.4141)	-0.1660 (<i>P</i> =0.3894)	-0.0260 (<i>P</i> =0.8957)	0.0152 (<i>P</i> =0.9389)

Significant correlations are in boldface.

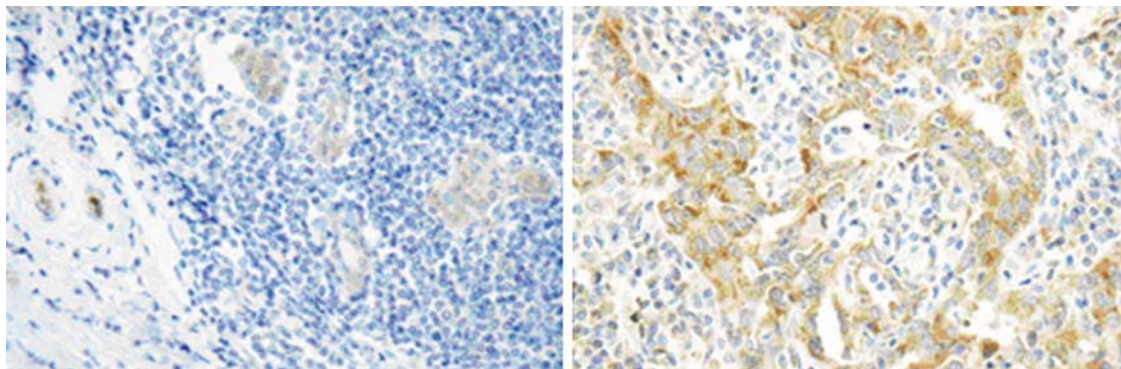


Figure 3 VEGF protein expression in blood vessels in normal breast tissue (left, × 400) and in breast carcinoma cells (right, × 400).

Discussion

This study is one of the first attempts to quantify breast cancer angiogenesis and lymphangiogenesis concurrently by microvessel density using CD31 and the novel lymphatic marker D2-40. We compared the performance of visual vs automated microvessel density for CD31 and D2-40, and related these measurements with clinicopathologic parameters of breast cancer. In addition, to explore mechanisms of angiogenesis and lymphangiogenesis in clinical breast cancer specimens, we compared CD31 and D2-40 microvessel density with VEGF, VEGF-C and VEGF-D expression by immunohistochemistry and fixed-tissue quantitative RT-PCR. Blood and vascular microvessel density correlated with prognostic parameters including lymph node metastasis, while VEGF family gene expression correlated with microvessel density. Thus, measurements of angiogenesis

and lymphangiogenesis may have clinical utility in the evaluation of breast cancer, particularly for estimation of metastatic risk, if our findings are confirmed in prospective studies. This suggestion is supported by several studies showing microvessel density to be a significant prognostic marker for breast cancer. For example, a recent Japanese study showed that microvessel density, determined visually with anti-Factor VIII-related antigen, was an independent predictor of disease-free and overall survival in a series of 252 cases.¹⁹ A meta-analysis of 43 independent studies determined that high microvessel density was associated significantly with breast cancer survival, particularly in node-negative cases.⁴ However, the relative risks defined in this meta-analysis were too small to make unequivocal conclusions on the clinical utility of microvessel counting. Differences in microvessel counting techniques and targeted endothelial antigens were

Table 4 Comparisons of mean CD31 and D2-40 microvessel density, visually and image cytometrically, with immunohistochemical expression of vascular endothelial growth factor (expressed as Pearson's correlations with associated *P*-values)

VEGF expression	Microvessel density technique			
	CD31 (visual)	D2-40 (visual)	CD31 (image cytometric)	D2-40 (image cytometric)
Malignant cells	0.235 (<i>P</i> = 0.2204)	-0.29 (<i>P</i> = 0.1333)	-0.1081 (<i>P</i> = 0.5841)	0.1517 (<i>P</i> = 0.4410)
Intratumoral vascular endothelium	0.275 (<i>P</i> = 0.1574)	0.07 (<i>P</i> = 0.7081)	0.1634 (<i>P</i> = 0.4154)	0.1914 (<i>P</i> = 0.3390)
Peritumoral vascular endothelium	0.493 (<i>P</i> = 0.0170)	0.35 (<i>P</i> = 0.1060)	0.4864 (<i>P</i> = 0.0217)	0.6325 (<i>P</i> = 0.0012)

Significant correlations are in boldface.
 VEGF = vascular endothelial growth factor.

Table 5 Correlation of mean CD31 and D2-40 microvessel density with log₂ relative expression of VEGF, VEGF-C and VEGF-D by quantitative RT-PCR (expressed as Pearson's correlations with corresponding *P*-values)

Gene product	Microvessel density technique			
	CD31 (visual)	D2-40 (visual)	CD31 (image cytometric)	D2-40 (image cytometric)
VEGF	-0.1126 (<i>P</i> = 0.5921)	0.0171 (<i>P</i> = 0.9352)	-0.0758 (<i>P</i> = 0.7247)	-0.2029 (<i>P</i> = 0.3307)
VEGF-C	-0.0388 (<i>P</i> = 0.8539)	0.4365 (<i>P</i> = 0.0291)	0.2772 (<i>P</i> = 0.1898)	0.3030 (<i>P</i> = 0.1409)
VEGF-D	-0.0020 (<i>P</i> = 0.9923)	0.4075 (<i>P</i> = 0.0432)	0.2629 (<i>P</i> = 0.2145)	0.2938 (<i>P</i> = 0.1540)

Significant correlations are in boldface.
 VEGF = vascular endothelial growth factor; RT-PCR = reverse transcriptase-polymerase chain reaction.

considered likely sources of variability among the analyzed studies. Furthermore, several studies have shown no significant relationship between microvessel density and clinicopathologic parameters of breast cancer. For example, Axelsson *et al* assessed angiogenesis visually with anti-Factor VIII-associated antigen and found no correlation with lymph node status or any other breast cancer parameter. A major factor limiting the strength of association was high interobserver variability in microvessel counting.²⁰ In a large Italian study, a significant correlation was shown between microvessel density (assessed visually with anti-CD34) and Her2/neu overexpression, but not lymph node metastasis or patient survival.²¹ Among node negative cases, this study focused on tumors negative for estrogen and progesterone receptors, which is distinct from the cohort in our research. Overall, the clinical significance of high microvessel density in breast cancer remains uncertain, and the variability in technical approaches and difficulty in distinguishing blood and lymphatic microvessels appears to contribute to this uncertainty.

Research on tumor lymphangiogenesis has lagged behind that of angiogenesis. Indeed, the existence of

intratumoral lymphatic channels in breast cancer remains a controversial topic.^{9,22} A major impediment to the study of lymphangiogenesis in solid tumor growth and spread was the lack of a specific lymphatic marker. Several candidate lymphatic endothelial markers have been established recently, including the integral membrane glycoprotein podoplanin;^{23,24} the desmosomal component desmoplakin;²⁵ the transcription factor Prox 1;²⁶ the transmembrane 4 superfamily protein CD9;²⁷ the receptor for VEGF-C and VEGF-D (VEGFR-3); and the LYVE-1 antigen.^{9,10} However, while each marker appears to be expressed preferentially in lymphatic endothelium, expression in other cell types has been described. Of particular relevance to the current study, VEGFR-3 and LYVE-1 may be expressed in certain blood vessel endothelia, complicating their use in differentiating lymphangiogenesis from angiogenesis.²⁸

D2-40 antibody was recently reported to detect a fixation-resistant epitope on a 40 kDa O-linked sialoglycoprotein expressed in lymphatic endothelium but not blood vessels.⁶ D2-40 was shown to immunostain lymphangiomas (benign tumors of undisputed lymphatic origin), but not benign neoplasms or tumor-like lesions of blood vessel origin.⁶ Although D2-40 reacted with malignant endothelial tumors with features of blood vessels, including angiosarcomas and Kaposi sarcomas, these tumors have been shown to coexpress lymphatic markers such as podoplanin and VEGFR-3 in a significant proportion of cases.²⁹⁻³³ In the setting of breast cancer, in which the associated blood and lymphatic vessels are nonmalignant, D2-40 immunoreactivity appears to be specific for lymphatic phenotype, making D2-40 an appropriate marker to assess lymphangiogenesis specifically.

Visual and image cytometric microvessel density counting methods are each associated with key advantages and limitations. For example, microscopic visual counting is less expensive and much more widely available among pathologists, but the inherent subjectivity of this method may limit interobserver reproducibility.²⁰ In contrast, image

cytometry is likely to be more objective and reproducible and can measure several unique morphometric parameters. Apart from the number of vessels within a predetermined area, many image cytometry software packages can also measure vessel luminal area, vessel luminal perimeter and the number of immunostained areas per microscopic field or scanned area.^{34,35} In the past few years, image cytometric microvessel area and microvessel perimeter have been demonstrated as independent prognostic factors in invasive ductal carcinoma.^{36,37}

In light of the frequent use of both visual and image cytometric microvessel density counting methods, a question that continues to arise is how well data from the two approaches correlate. In our study, while visual and image cytometric CD31 microvessel density gave similar data, direct correlation did not reach statistical significance, and only image cytometric data were associated significantly with clinicopathologic parameters of breast cancer. In contrast, direct correlation of visual and image cytometric D2-40 microvessel density was statistically significant in our study. Nevertheless, tumor stage correlated only with image cytometric lymphangiogenesis, while lymph node status and VEGF family gene expression correlated only with visual D2-40 data. Factors limiting the strength of correlations in our study may include the subjective nature of visual estimates, the confounding signals from nonendothelial structures (eg stromal connective tissues or plasma cells, which may react with CD31 and be counted by the image cytometer but not by experienced pathologists), and the study size. Previous reports in the literature also show conflicting correlation between visual and image cytometric analysis of vessel counts in breast carcinomas. When anti-CD31 was used for vascular staining, Barbareschi *et al*³⁸ demonstrated a statistically significant correlation between visual and computer image analysis microvessel counts in 91 breast carcinoma cases. The statistical power of this study may be accounted for by the greater case number than that of our study. Differences in methodologies between our study and Barbareschi's study might also play a role. For example, microvessels within tumors were counted in their study. This also reflects part of the subjective nature of the microvessel counting process. Fox *et al*³⁹ also used anti-CD31 as the tool for angiogenesis research and reported a statistically significant correlation between various vascular parameters (luminal area, luminal perimeter and microvessel number) with a subjective, visual three-tier vascular grading system under the microscope. While this is an innovative method of estimating angiogenesis, it is difficult to compare its results with our and other studies using more common methods of microvessel counting. In contrast to the aforementioned studies, Kohlberger *et al*,⁴⁰ using anti-factor VIII to assess angiogenesis, reported no significant correlation between manual microvessel counting and computer image analysis.

Thus, to summarize previous studies correlating visual and computer image analysis microvessel density, diversity exists in the degrees of correlation reported, likely related to differences in target vascular antigens, methods used for microvessel counting, sample sizes and possibly other factors.

Assays that probe molecular mechanisms of tumor angiogenesis and lymphangiogenesis have potential to increase the clinical utility of microvessel density studies, since they may allow the observer to distinguish varying signal transduction pathways with distinct prognostic or therapeutic implications. For example, the advent of specific VEGF inhibitors for antiangiogenic therapy makes it important to determine if expression of VEGF-family gene products correlates consistently with microvessel density.⁴¹ To explore the relationship of vascular and lymphatic microvessel density with VEGF, VEGF-C and VEGF-D expression, we developed immunohistochemical and quantitative RT-PCR assays for these growth factors that utilize formalin-fixed paraffin-embedded tissues, which are more generally accessible in routine diagnostic pathology settings. Immunohistochemical assays for all three growth factors have been described.^{42,43} However, most studies of VEGF-C and VEGF-D in cancer have employed RT-PCR,^{44,45} and we have only established reliable immunohistochemical assays for VEGF to date. Our data suggest that VEGF protein expression in blood vessels of benign tissue surrounding breast tumors correlates significantly with tumor angiogenesis and lymphangiogenesis determined by microvessel density. Similar relationships were not seen using quantitative RT-PCR, which may be due to the inability to localize the VEGF mRNA signals to specific cells.

Our ability to assay VEGF, VEGF-C and VEGF-D mRNA in formalin-fixed paraffin-embedded breast specimens is consistent with several recent studies, which have used fluorogenic quantitative RT-PCR to measure gene transcription in fixed tissues.⁴⁶⁻⁴⁹ Fluorogenic quantitative RT-PCR is designed to amplify very small sequences (200 bp or less), which can be extracted intact from fixed tissue with adequate efficiency for comparison among specimens. Assays for VEGF, VEGF-C and VEGF-D have potential clinical utility, based on growing evidence that these growth factors play important roles in lymphangiogenesis and lymph node metastasis in breast and other cancers.^{50,51} In our study, mRNA for VEGF-C and VEGF-D tended to correlate with D2-40 microvessel density and lymph node metastasis. Similarly, Skobe *et al*⁹ showed that VEGF-C overexpression in breast cancer cells increased intratumoral lymphangiogenesis, assessed by LYVE-1, and enhanced metastasis to regional lymph nodes and lungs. Mattila *et al*¹³ similarly demonstrated that VEGF-C overexpression stimulated tumor lymphangiogenesis and increased lymph node metastasis of the estrogen-dependent MCF-7 breast cancer cell line in a nude mice model. Stacker *et al*¹⁰ showed

that VEGF-D, but not VEGF, promoted the metastatic spread of tumor cells via the lymphatics. Studies performed on other cancer types have yielded comparable results. Thus, Kitadai *et al*⁵⁰ illustrated an association between VEGF-C expression in human esophageal squamous cell carcinomas with depth of tumor invasion, tumor stage, venous invasion, lymphatic invasion and lymph node metastasis. Likewise, both microvessel density and VEGF expression were significantly increased in nasopharyngeal carcinoma tissue with metastasis as compared with those without.⁵¹

To summarize, we used the recently described lymphatic endothelial immunomarker D2-40 to estimate lymphatic microvessel density in fixed breast tissues, and achieved comparable results by visual microscopy and image cytometry. In contrast, blood vascular microvessel density measurements with CD31 did not correlate using visual microscopy vs image cytometry. Microvessel density assays using CD31 and D2-40, and molecular assays for VEGF family gene expression, illustrated relationships of angiogenesis and lymphangiogenesis with tumor histology, grade, stage and lymph node metastasis. Based on these findings, prospective studies may be useful to determine if assays for blood and lymphatic microvessel density and VEGF family gene expression have clinical utility in defining risk for nodal metastasis in breast cancer.

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Duality of interest

The authors of this article declare no duality of interest.

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