ORIGINAL ARTICLE

Lack of lymphangiogenesis during breast carcinogenesis

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Background: Recent evidence suggests that functional intratumorous lymph vessels may be absent from some human cancers. This could result from either the failure of tumours to induce lymphangiogenesis, or the collapse of lymph vessels, caused by high interstitial tumour pressure.
Methods: To differentiate between these two hypotheses, paraffin wax embedded clinical specimens from normal breast (n = 13), usual ductal hyperplasia (n = 11), ductal carcinoma in situ (n = 21), and invasive breast cancer (n = 40) were compared for lymphatic and blood vessel density by immunohistochemistry with antibodies to the lymphatic endothelial hyaluronan receptor (LYVE-1) and CD31, respectively.
Results: Lymph vessel density was lower than blood vessel density in normal breast tissue. Within breast lobuli, lymph vessels were absent. In premalignant lesions blood microvessel density increased, whereas no increase in lymph vessels could be seen intralesionally. In invasive cancers, lymph vessels were absent in all but a few cases, where probably some pre-existing lymph vessels remained, although blood microvessel density was once again increased.
Conclusion: Unlike angiogenesis, lymphangiogenesis is absent during breast carcinogenesis. This, and not rising interstitial pressure caused by an increase in the size of lesions, explains the absence of intratumorous lymph vessels in invasive breast cancer.

Metastasis of breast cancer occurs by the invasion of local tissue, by haematogenous spread, or by lymphatic spread. Much is known about the formation of new blood vessels as a mechanism to support haematogenous spread and facilitate tumour growth. In contrast, little is known about the mechanisms involved in lymphatic spread. For a long time, this was because of a lack of knowledge about lymphangiogenic factors and the lack of suitable markers to distinguish between blood endothelial and lymphatic endothelial cells.

Recently, however, two members of the vascular endothelial growth factor (VEGF) family, VEGF-C[1] and VEGF-D,[2] were found to be of importance in the induction of lymphangiogenesis. Vascular endothelial growth factor receptor-3 (VEGFR-3/Flt4) is the lymph vessel specific receptor tyrosine kinase specific for VEGF-C and VEGF-D.[3] Unfortunately, VEGFR-3 was also found to be expressed on intratumorous blood vessels, making it less useful for the detection of lymphatics in tumours.[4 5]

A more specific marker for lymph vessels is LYVE-1, a receptor for hyaluronan, which is expressed early during the development of lymphatic endothelium.[6 7] Furthermore, podoplanin,[8] a glycoprotein of podocytes, and Prox I, a homeobox transcription factor,[9 10] are used for the detection of lymphatics. Identification of these lymph vessel specific markers has facilitated the study of lymphangiogenic growth factors and intratumorous lymphangiogenesis.

It was found that VEGF-C expression is upregulated in many solid tumours, and its expression correlated with lymph node metastasis and lung metastases in breast cancer.[1] Furthermore, in transgenic and xenotransplanted mice, overexpression of the VEGF-C or VEGF-D ligands induced intratumorous lymphangiogenesis.[11–13] Investigation of lymph vessel patterns in spontaneously arising tumours, such as head and neck squamous cell carcinoma (HNSCC), showed that proliferating lymphatics occur in human cancers, and may in some cases contribute to lymph node metastasis.[14] Investigation of intratumorous lymphatics in HNSCC showed that a high intratumorous lymph vessel density (LVD) is associated with a higher risk of local relapse, in addition to a poor prognosis, and that peritumorous LVD is associated with a better prognosis.[14 15] The presence of intratumorous lymphatics was also shown in melanoma.[16 17] Other studies showed that peritumorous lymphatic vessel counts correlated with a poor prognosis in patients with breast cancer[18] and cervical cancer.[19 20]

> ''Our study was undertaken to investigate lymphangiogenesis during breast carcinogenesis and to evaluate two possible explanations for the putative absence of intratumorous lymphatics''

In contrast to the above findings, the absence of intratumorous lymph vessels was first described in a murine sarcoma model.[21] In this model, lymph vessels were absent, although all lymphatic factors necessary to induce lymphangiogenesis were present. The authors suggested that this was caused by high intratumorous pressure, which resulted in collapse of the proliferating lymph vessels. Recently Padera *et al* examined intratumorous lymph vessels in mouse tumours overexpressing VEGF-C.[22] Using functional and biochemical investigations, no functional intratumorous vessels could be found in these experimental tumours. Furthermore, the investigation of lymph vessels in human lung tumour specimens showed the absence of LYVE-1 positive vessels. Most of these tumours showed marginally raised intratumorous pressure, indicating that pressure might play a role in the absence of intratumorous lymphatics in vivo.

Abbreviations: BVD, blood vessel density/mm^2; DCIS, ductal carcinoma in situ; HNSCC, head and neck squamous cell carcinoma; LVD, lymph vessel density/mm^2; NHS, normal human serum; TBS, Tris buffered saline; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor

Our study was undertaken to investigate lymphangiogenesis during breast carcinogenesis and to evaluate two possible explanations for the putative absence of intratumorous lymphatics: increased interstitial pressure causing collapse of lymph vessels or failure to induce lymphangiogenesis as a result of other factors. To differentiate between these two hypotheses during breast cancer carcinogenesis, we compared lymph vessel with blood vessel patterns in normal breast tissue and different proliferative breast lesions, including neoplastic ones (ductal hyperplasia, ductal carcinoma in situ (DCIS), and invasive breast cancer).

MATERIALS AND METHODS

Patients

Tissue sections containing breast tissue were obtained from tissue blocks of randomly selected patients, which had been deposited in the tissue banks of the pathology departments of the VU University Medical Centre, Amsterdam and the Gooi-Noord Hospital, Blaricum, the Netherlands. Normal breast tissue (n = 13) was taken from reduction mammoplasties performed on premenopausal patients without proliferative breast disease. Specimens of pure ductal hyperplasia (n = 11), pure DCIS (n = 21), and stage I/II invasive ductal carcinoma (n = 40) were obtained from excision biopsy procedures or mastectomies. The DCIS and invasive cancer grades did not seem to influence correlations with blood or lymph vessels, so different grade lesions were simply grouped as DCIS or invasive cancer for further analysis.

None of the patients with invasive breast cancer had received preoperative treatment. All specimens were fixed in neutral 4% buffered formaldehyde. In our hospitals, anonymous use of left over patient material for scientific purposes is a standard item in the treatment contract, although the wishes of those patients who object to this are respected.

Immunohistochemistry

Immunohistochemistry was performed on 4 μm thick slides. After dewaxing and rehydration, endogenous peroxidase activity was blocked for 30 minutes in methanol containing 0.3% hydrogen peroxide. For CD31 staining, antigen retrieval was performed by autoclave treatment for 20 minutes in citrate buffer (pH 6.0). After antigen retrieval, a cooling off period of 20 minutes preceded incubation with the primary antibody. Tissue sections were incubated with saturating concentrations of primary antibody (mouse monoclonal JC-70; Dako, Glostrup, Denmark), diluted at 1/40 in phosphate buffered saline with 5% bovine serum albumin. Thereafter, the Labvision system (Dako) was used according to the manufacturer's instructions.

For LYVE-1 staining, antigen retrieval was performed by microwave treatment (four minutes at 750 W, four minutes at 360 W) in a 0.1M Tris/HCl, pH 9.0, 2mM EDTA solution. After antigen retrieval, a cooling off period of 20 minutes preceded preincubation for 15 minutes in Tris buffered saline (TBS) containing 5% normal human serum (NHS), after which the primary antibody (anti-LYVE-1; rabbit polyclonal; kindly provided by DJ Jackson, Oxford, UK) was added at a dilution of 1/100 in TBS with 5% NHS. Next, an enhanced peroxidase staining method (Envision; Dako) was used according to the manufacturer's instructions. All sections were developed with diaminobenzidine (Dako) and then briefly counterstained with haematoxylin.

Quantification

Blood and lymph vessels were counted using an ocular grid at a magnification of ×400. To this end, in four adjacent fields of vision in the most vascularised area (the hot spot; total area, 0.6 mm^2), microvessels were counted and expressed as the blood vessel density (BVD) or LVD/mm^2, as described previously.[23 24] These assessments were carried out by two observers (MMV and PJvD).

Statistical analysis

The non-parametric Mann Whitney test for independent samples (SPSS for Windows version 9.01, 1999; SPSS Inc, Chicago, Illinois, USA) was used to compare LVD and BVD between the different groups. Correlations between BVD and LVD on the one hand and lesion size on the other were compared with Fisher's exact test. Two sided p values < 0.05 were considered to be significant.

RESULTS

Lymphatic vessel patterns in the normal breast

Stromal breast lymph vessels strongly expressed LYVE-1 (fig 1A). LYVE-1 positive lymph vessels were large irregular vessels with empty lumina covered with one layer of non-atypical endothelium. Occasionally, LYVE-1 positive tissue macrophages were seen in the interlobular stroma. Blood vessels were negative for LYVE-1, as can be seen from the surrounding LYVE-1 negative vessels with intraluminal erythrocytes (fig 1A, arrow). This observation confirms the specificity of LYVE-1 for human breast lymph vessels.

LYVE-1 positive lymph vessels were dispersed in the interlobular stroma, smooth muscle, and adipose tissue. In these areas, lymph vessels were often arranged in linear patterns, probably leading to local draining lymph nodes. Most of the lymph vessels were seen in conjunction with blood vessels. In some cases, lymph vessels were seen lying around a blood vessel. Remarkably, no lymph vessels were seen within the breast lobuli.

CD31 positive blood vessels were seen throughout the tissue in normal breast. In the interlobular stroma, larger vessels were present, often accompanied by numerous microvessels. Around the lobuli and in the intralobular stroma, mainly microvessels were seen (fig 1B).

Premalignant breast lesions show no increase of intralesional lymph vessels

Similar results were obtained for usual hyperplasia as those seen for normal breast, with no increase in LVD or BVD (fig 1C; table 1). In DCIS lesions, breast lymph vessels were present in only the interlobular stroma and the adipose tissue surrounding the lesions (fig 1D). These vessels showed similar morphology to the lymph vessels seen in normal breast tissue.

In DCIS lesions, no LYVE-1 positive lymph vessels were found lying directly around the ducts, in contrast to blood vessels, which were often seen as a vascular rim around the DCIS lesions (fig 1E), as noted previously.[24] Indeed, the LVD was not significantly different in DCIS compared with normal breast ($p = 0.06$), but BVD was much higher ($p < 0.001$; table 1). The size of the DCIS lesion correlated inversely with LVD ($p = 0.02$). This association was not found for BVD (table 2).

Intratumorous lymph vessels are absent from most invasive breast carcinomas

Thirty one of 40 invasive breast cancer cases showed complete absence of intratumorous lymphatics (fig 1F). In nine of 40 cases, some intratumorous lymph vessels were seen with comparable morphology to normal breast lymphatics (fig 1G). In most of these cases, peritumorous lymphatics were present at the tumour margin, with infiltrating tumour islands between these vessels. The surrounding ''normal'' tissue showed normal lymphatics also. Occasionally, these peritumorous lymphatics appeared to be somewhat enlarged and more complex than normal breast lymphatics. However, other cases just showed small lymph vessels with decreased

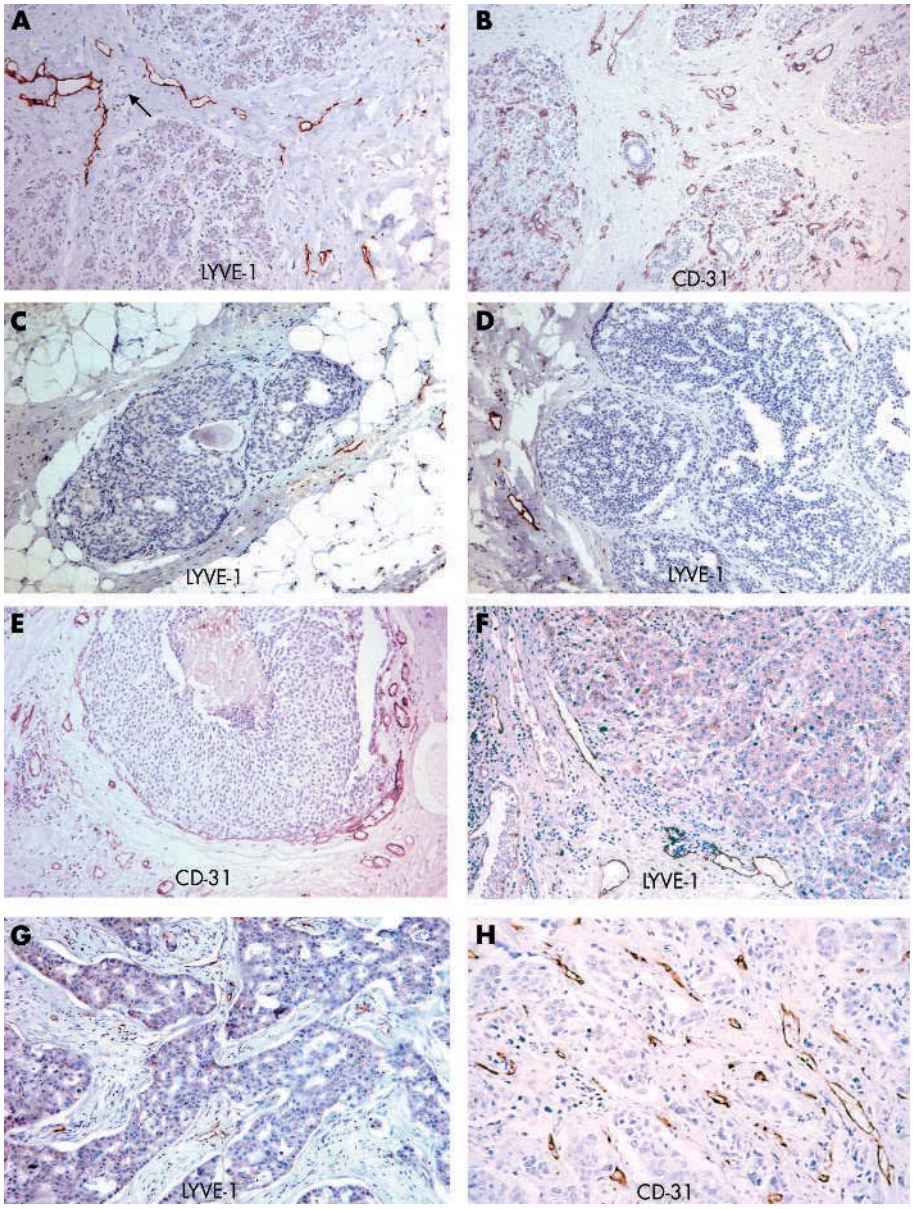

Figure 1 Lymph and blood vessel patterns in the normal human breast, premalignant lesions, and invasive breast cancer. (A) In the normal breast, scattered lymph vessels are seen in the interlobular stroma. No intralobular lymph vessels are seen. Arrow: unstained blood vessel associated with lymph vessels. (B) In the normal breast, numerous blood vessels are seen, also in the intralobular space. (C) Lymph vessels are present at some distance in the adipose tissue surrounding usual ductal hyperplasia. (D) Ductal carcinoma in situ with lymph vessels in the surrounding stroma at some distance from the neoplastic ducts. (E) Blood vessels are present as a vascular rim around a ductal carcinoma in situ. (F) In invasive breast cancer, lymphatics are seen at the tumour margin, but are absent from the intratumorous stroma in most cases. (G) In invasive breast cancer, intratumorous lymphatics are seen occasionally in the intratumorous stroma in some cases. (H) In invasive breast cancer, intratumorous blood vessels are dispersed within the tumour.

numbers of lymphatics compared with normal breast tissue. Peritumorous lymphatics were often seen in association with LYVE-1 positive tissue macrophages. In some cases, invasion of the lymphatic vessels by tumour cells was seen intratumorously at the periphery, or peritumorously. Intratumorous LVD was much lower than that seen in normal breast tissue ($p < 0.001$), and showed no relation to tumour size. Intratumorous BVD was significantly higher ($p < 0.001$) than that seen in normal breast tissue (table 1; fig 1H). Peritumorous LVD was not significantly different from normal breast tissue ($p = 0.07$), and showed no correlation with tumour size ($p = 0.364$; table 3).

DISCUSSION

The spread of tumour cells via the lymphatic system is one of the major causes of tumour metastasis. However, a functional intratumorous lymphatic system seems to be absent from most cancer types.[22] To date, there have been many

Table 1 BVD and LVD counted in the "hot spots" in normal breast and in (pre)malignant breast lesions

Tissue	N	BVD	p Value*	LVD	p Value*	Lesion size (cm)	p Value*
Normal breast	13	41 (2.8)	NA	21 (19)	NA	NA	
Hyperplasia	11	54 (12.0)	0.417	16.8 (10.0)	0.691	NA	
DCIS	21	140.9 (58.6)	<0.001	11.5 (11.0)	0.062	0.43 (0.26; 0.1–1.4)	
Invasive cancer (intratumorous)	40	110.1 (57.2)	<0.001	1.9 (4.6)	<0.001	2.8 (1.25; 1.0–6.0)	0.001

*Mann-Whitney test, in relation to normal breast.
Values are mean (SD; range (for lesion size)).
BVD, blood vessel density (in mm^2); DCIS, ductal carcinoma in situ; LVD, lymph vessel density (in mm^2); NA, not applicable.

Table 2 Correlation between lesion size and vessel density of breast DCIS (n = 21)

	LVD		BVD	
	Low (<10)	High (≥10)	Low (<75)	High (≥75)
DCIS size				
0–4 mm	2	7	6	3
4–14 mm	9	3	9	3
	p = 0.017		p = 0.676	

BVD, blood vessel density (in mm^2); DCIS, ductal carcinoma in situ; LVD, lymph vessel density (in mm^2).

hypotheses to explain the absence of intratumorous lymphatics.[25–33] To differentiate between the hypothesis of total collapse of newly formed lymph vessels because of a rise in interstitial pressure and the total absence of lymphangiogenesis, we investigated lymph vessel patterns in normal breast, premalignant breast lesions, and invasive breast cancers.

> ''The inverse correlation between lymph vessel density and the lesion size in ductal carcinoma in situ could be explained by the replacement of stroma by tumour epithelium, rather than the collapse of lymph vessels''

For the first time, lymphangiogenesis was studied in premalignant breast lesions. In ductal hyperplasia, an early proliferative breast lesion,[34 35] neither LVD nor BVD was increased in comparison with normal breast tissue. In DCIS, the final non-invasive stage of breast cancer development, BVD was increased, with extensive vascular rimming, indicating that the angiogenic switch had taken place. These results are in accordance with those from other groups (the importance of angiogenesis in DCIS is reviewed in Rice and Quinn[36]). However, LVD was not increased. Because it is unlikely that interstitial pressure is increased in these small premalignant lesions, the collapse of newly formed lymph vessels can hardly explain the absence of lymph vessels. Furthermore, even collapsed lymph vessels would be expected to retain the expression of LYVE-1 in the endothelium. Therefore, the inverse correlation between LVD and the lesion size in DCIS could be explained by the replacement of stroma by tumour epithelium, rather than the collapse of lymph vessels. Thus, lymphangiogenesis simply seems to be lacking during breast carcinogenesis. This idea is supported by the complete absence of intratumorous lymphatics in most invasive breast cancers. We hypothesise that this is the result of a completely destructive growth pattern, where the pre-existing stroma is destroyed and replaced by newly formed tumour stroma lacking lymph vessels. LVD was present in a few invasive cancers, but it was much lower than in normal breast tissue, pointing to an incompletely destructive growth. In addition, at the peritumorous margin, LVD was not higher than in normal tissue. These results are in agreement with the observation of other groups for breast[37] and other cancer types.[21 22] All these results indicate that the absence of intratumorous lymphatics can be explained by the absence of lymphangiogenesis.

The absence of lymphangiogenesis during breast cancer progression might be explained by the absence of lymphangiogenic growth factors, such as VEGF-C and VEGF-D, or the absence of expression of their receptors. Because overexpression of VEGF-C/D has been found in many tumour types, including breast cancer,[38] this is not a plausible explanation. This also holds true for the expression of the lymph vessel specific VEGF-C/VEGF-D receptor, VEGFR-3 (flt-4), which was also found to be expressed on blood vessels in various cancer types, including breast cancer.[5]

Invasion related proteins are involved in matrix degradation to support processes such as angiogenesis,[39] although whether or not matrix degradation occurs in lymphangiogenesis is still unclear. However, the invasion related proteins involved in this process are different from those thought to be important for blood vessel formation.[40] Therefore, changes in matrix composition, as seen in cancer, may lead to disturbances in the initiation of lymphangiogenesis, as a result of the absence of appropriate invasion related proteins.

Other inhibitors of lymphangiogenic growth might be factors released by the tumour cells themselves, or properties of the dividing tumour cells that directly cause destruction of lymph vessels. This has also been proposed by Williams *et al*.[37] In their study, an inverse relation was found between tumour aggressiveness and the absence of intratumorous lymphatics in invasive breast cancer. Furthermore, low LVD was found to be predictive of poor prognosis. Their results suggest that lymph vessels are invaded and destroyed by naturally occurring breast carcinomas. These findings are in agreement with our observations, which have shown that intratumorous lymphangiogenesis does not occur during breast carcinogenesis. However, because intratumorous lymphangiogenesis is seen in some other tumour types, such as head and neck cell carcinoma and melanoma,[14–18] tissue specific tumour features might also be responsible for the presence or absence of a ''lymphangiogenic switch''. Further studies on the molecular basis of lymphangiogenic inhibitors during carcinogenesis should consider this.

The absence of intratumorous lymphangiogenesis in most cancer types has important consequences. First, because no functional lymphatics are present, lymphatic metastasis should occur via the pre-existing peritumorous lymphatics. These will mostly only be present at or outside the tumour margin. Indeed, it has long be suggested that lymph vessel invasion as a diagnostic feature should only consider tumour emboli outside the tumour margin to be reproducible.[41] In retrospect, this is a fortunate decision because intratumorous lymphatic invasion is now known to be too rare to be diagnosed reliably. Second, intratumorous blood vessels are often leaky.[42] In view of the absence of a functional intratumorous lymphatic network, it is surprising that

Table 3 Correlation between lesion size and vessel density of invasive carcinomas (n = 40)

	LVD		LVD-IT		BVD-IT	
	Low (<25)	High (≥25)	Absent	Present	Low (<75)	High (≥75)
Tumour size						
0–2 cm	8	6	12	2	5	9
2–5 cm	16	6	17	5	9	13
>5 cm	4	0	2	2	0	4
	p = 0.235		p = 0.320		p = 0.287	

BVD, blood vessel density (in mm^2); DCIS, ductal carcinoma in situ; IT, intratumorous; LVD, lymph vessel density (in mm^2).

Take home messages

- By studying premalignant and malignant breast lesions we have shown that, in contrast to angiogenesis, lymphangiogenesis is absent during breast carcinogenesis
- The lack of lymphangiogenesis, rather than rising interstitial pressure caused by an increase in the size of lesions, probably explains the absence of intratumorous lymph vessels in invasive breast cancer
- The factors that contribute to lymphangiogenic growth inhibition should be investigated to explain the role of this phenomenon in tumour progression

tumours do not show extremely high interstitial pressures. We know from the sentinel node procedure that peritumorously injected colloid tracers are efficiently transported to the lymph nodes.[43] Apparently, interstitial fluid finds its way to the lymphatics through tissue spaces driven by the interstitial pressure gradient. This suggests that the breast acts as a sort of sponge.

In conclusion, although angiogenesis parallels the progression of premalignant breast lesions, lymphangiogenesis appears to be lacking during breast carcinogenesis, resulting in the complete absence of lymph vessels in most invasive breast cancers. This points either to a lack of lymphangiogenic growth stimuli or to the presence of inhibitors of lymphangiogenic growth. The identification of factors other than interstitial pressure rise that contribute to lymphangiogenic growth inhibition might increase our knowledge about the mechanisms of decreased tumour lymphangiogenesis and the role of this phenomenon in tumour progression.

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REFERENCES

1 **Karpanen T**, Egeblad M, Karkkainen MJ, *et al.* Vascular endothelial growth factor C promotes tumor lymphangiogenesis and intralymphatic tumor growth. *Cancer Res* 2001;**61**:1786–90.
2 **Achen MG**, Jeltsch M, Kukk E, *et al.* Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). *Proc Natl Acad Sci U S A* 1998;**95**:548–53.
3 **Kaipainen A**, Korhonen J, Mustonen T, *et al.* Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. *Proc Natl Acad Sci U S A* 1995;**92**:3566–70.
4 **Valtola R**, Salven P, Heikkila P, *et al.* VEGFR-3 and its ligand VEGF-C are associated with angiogenesis in breast cancer. *Am J Pathol* 1999;**154**:1381–90.
5 **Partanen TA**, Alitalo K, Miettinen M. Lack of lymphatic vascular specificity of vascular endothelial growth factor receptor 3 in 185 vascular tumors. *Cancer* 1999;**86**:2406–12.
6 **Banerji S**, Ni J, Wang SX, *et al.* LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. *J Cell Biol* 1999;**144**:789–801.
7 **Mouta CC**, Nasser SM, di Tomaso E, *et al.* LYVE-1 is not restricted to the lymph vessels: expression in normal liver blood sinusoids and down-regulation in human liver cancer and cirrhosis. *Cancer Res* 2001;**61**:8079–84.
8 **Breiteneder-Geleff S**, Soleiman A, Kowalski H, *et al.* Angiosarcomas express mixed endothelial phenotypes of blood and lymphatic capillaries: podoplanin as a specific marker for lymphatic endothelium. *Am J Pathol* 1999;**154**:385–94.
9 **Wilting J**, Papoutsi M, Christ B, *et al.* The transcription factor Prox1 is a marker for lymphatic endothelial cells in normal and diseased human tissues. *FASEB J* 2002;**16**:1271–3.
10 **Rodriguez-Niedenfuhr M**, Papoutsi M, Christ B, *et al.* Prox1 is a marker of ectodermal placodes, endodermal compartments, lymphatic endothelium and lymphangioblasts. *Anat Embryol (Berl)* 2001;**204**:399–406.
11 **Mattila MM**, Ruohola JK, Karpanen T, *et al.* VEGF-C induced lymphangiogenesis is associated with lymph node metastasis in orthotopic MCF-7 tumors. *Int J Cancer* 2002;**98**:946–51.
12 **Stacker SA**, Caesar C, Baldwin ME, *et al.* VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. *Nat Med* 2001;**7**:186–91.
13 **Skobe M**, Hawighorst T, Jackson DG, *et al.* Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nat Med* 2001;**7**:192–8.
14 **Beasley NJ**, Prevo R, Banerji S, *et al.* Intratumoral lymphangiogenesis and lymph node metastasis in head and neck cancer. *Cancer Res* 2002;**62**:1315–20.
15 **Maula SM**, Luukkaa M, Grenman R, *et al.* Intratumoral lymphatics are essential for the metastatic spread and prognosis in squamous cell carcinomas of the head and neck region. *Cancer Res* 2003;**63**:1920–6.
16 **Skobe M**, Hamberg LM, Hawighorst T, *et al.* Concurrent induction of lymphangiogenesis, angiogenesis, and macrophage recruitment by vascular endothelial growth factor-C in melanoma. *Am J Pathol* 2001;**159**:893–903.
17 **Dadras SS**, Paul T, Bertoncini J, *et al.* Tumor lymphangiogenesis: a novel prognostic indicator for cutaneous melanoma metastasis and survival. *Am J Pathol* 2003;**162**:1951–60.
18 **Schoppmann SF**, Birner P, Studer P, *et al.* Lymphatic microvessel density and lymphovascular invasion assessed by anti-podoplanin immunostaining in human breast cancer. *Anticancer Res* 2001;**21**:2351–5.
19 **Birner P**, Schindl M, Obermair A, *et al.* Lymphatic microvessel density as a novel prognostic factor in early-stage invasive cervical cancer. *Int J Cancer* 2001;**95**:29–33.
20 **Birner P**, Obermair A, Schindl M, *et al.* Selective immunohistochemical staining of blood and lymphatic vessels reveals independent prognostic influence of blood and lymphatic vessel invasion in early-stage cervical cancer. *Clin Cancer Res* 2001;**7**:93–7.
21 **Leu AJ**, Berk DA, Lymboussaki A, *et al.* Absence of functional lymphatics within a murine sarcoma: a molecular and functional evaluation. *Cancer Res* 2000;**60**:4324–7.
22 **Padera TP**, Kadambi A, di Tomaso E, *et al.* Lymphatic metastasis in the absence of functional intratumor lymphatics. *Science* 2002;**296**:1883–6.
23 **Weidner N**, Folkman J, Pozza F, *et al.* Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma. *J Natl Cancer Inst* 1992;**84**:1875–87.
24 **de Jong JS**, van Diest PJ, Baak JP. Hot spot microvessel density and the mitotic activity index are strong additional prognostic indicators in invasive breast cancer. *Histopathology* 2000;**36**:306–12.
25 **Clarijs R**, Ruiter DJ, de Waal RM. Lymphangiogenesis in malignant tumours: does it occur? *J Pathol* 2001;**193**:143–6.
26 **Karpanen T**, Alitalo K. Lymphatic vessels as targets of tumor therapy? *J Exp Med* 2001;**194**:F37–42.
27 **Pepper MS**. Lymphangiogenesis and tumor metastasis: myth or reality? *Clin Cancer Res* 2001;**7**:462–8.
28 **Jackson DG**, Prevo R, Clasper S, *et al.* LYVE-1, the lymphatic system and tumor lymphangiogenesis. *Trends Immunol* 2001;**22**:317–21.
29 **Karkkainen MJ**, Makinen T, Alitalo K. Lymphatic endothelium: a new frontier of metastasis research. *Nat Cell Biol* 2002;**4**:E2–5.
30 **Jain RK**, Fenton BT. Intratumoral lymphatic vessels: a case of mistaken identity or malfunction? *J Natl Cancer Inst* 2002;**94**:417–21.
31 **Alitalo K**, Carmeliet P. Molecular mechanisms of lymphangiogenesis in health and disease. *Cancer Cell* 2002;**1**:219–27.
32 **Stacker SA**, Baldwin ME, Achen MG. The role of tumor lymphangiogenesis in metastatic spread. *FASEB J* 2002;**16**:922–34.
33 **Jackson DG**. The lymphatics revisited: new perspectives from the hyaluronan receptor LYVE-1. *Trends Cardiovasc Med* 2003;**13**:1–7.
34 **van Diest PJ**. Ductal carcinoma in situ in breast carcinogenesis. *J Pathol* 1999;**187**:383–4.
35 **Mommers EC**, van Diest PJ, Leonhart AM, *et al.* Expression of proliferation and apoptosis-related proteins in usual ductal hyperplasia of the breast. *Hum Pathol* 1998;**29**:1539–45.
36 **Rice A**, Quinn CM. Angiogenesis, thrombospondin, and ductal carcinoma in situ of the breast. *J Clin Pathol* 2002;**55**:569–74.
37 **Williams CS**, Leek RD, Robson AM, *et al.* Absence of lymphangiogenesis and intratumorous lymph vessels in human metastatic breast cancer. *J Pathol* 2003;**200**:195–206.
38 **Salven P**, Lymboussaki A, Heikkila P, *et al.* Vascular endothelial growth factors VEGF-B and VEGF-C are expressed in human tumors. *Am J Pathol* 1998;**153**:103–8.
39 **Duffy MJ**, Maguire TM, Hill A, *et al.* Metalloproteinases: role in breast carcinogenesis, invasion and metastasis. *Breast Cancer Res* 2000;**2**:252–7.

40 **Wernert N**, Okuducu AF, Pepper MS. Ets 1 is expressed in capillary blood vessels but not in lymphatics. *J Pathol* 2003;**200**:561–7.
41 **Clemente CG**, Boracchi P, Andreola S, *et al*. Peritumoral lymphatic invasion in patients with node-negative mammary duct carcinoma. *Cancer* 1992;**69**:1396–403.
42 **Dvorak HF**. Rous-Whipple award lecture. How tumors make bad blood vessels and stroma. *Am J Pathol* 2003;**162**:1747–57.
43 **Borgstein PJ**, Pijpers R, Comans EF, *et al*. Sentinel lymph node biopsy in breast cancer: guidelines and pitfalls of lymphoscintigraphy and gamma probe detection. *J Am Coll Surg* 1998;**186**:275–83.