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RESEARCH

Can Lymphatic Vascular Density Be Used in Determining Metastatic Spreading Potential of Tumor in Invasive Ductal Carcinomas?

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Abstract Regional lymph node status is the primary parameter determining treatment strategies and prognoses in breast cancer. Lymphatic vessels in primary tumor tissue play a significant role in lymphatic metastasis. The aim of this study was to investigate the correlation of intra- and peritumoral lymphatic microvessel densities (LVD) with prognostic parameters in breast cancer, including lymphatic invasion (LI). Lymphangiogenesis was investigated using D2-40 monoclonal antibody in 69 invasive ductal carcinoma cases who underwent mastectomy and axillary lymph node dissection. Positively stained microvessels were counted at 400× in dense lymphatic vascular foci (hot-spots). Tumor LI was established when at least one neoplastic cell cluster was clearly visible inside a D2-40-positive lymph vessel. Relationships were sought between clinicopathological parameters and mean LVD and LI in primary tumor tissue. Peritumoral LVD was markedly higher than intratumoral LVD ($p<0.001$). No significant relationship was found between intratumoral LVD and clinicopathological parameters ($p>0.05$). However, significant relationships were detected between peritumoral LVD and LVI [H&E] ($p=0.04$), number of lymphatic invasion [n/mm2, D2-40] ($p=0.001$), tumor size ($p=0.01$), lymph node status ($p=0.03$), and tumor stage ($p=0.04$). The immunohistochemical determination of LI and LVD can contribute to the prediction of a tumor's biological behavior in invasive ductal carcinomas. Peritumoral LVD in primary tumor tissue is closely related to parameters influencing the prognosis of a tumor.

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Abbreviations

H&E	Hematoxylin and eosin
IDC	invasive ductal carcinomas
LECs	lymphatic endothelial cells
LI	lymphatic invasion
LVI	lymphovascular invasion
LVD	lymphatic vessel density
MVD	microvessel density
mAb	monoclonal antibody
AEC-DAB	3-amino-9-ethylcarbazole–3,3′-diaminobenzidine

Introduction

Angiogenesis is an important step in the progression of malignant neoplasms. In breast carcinomas, the microvessel density (MVD) of a tumor is closely related to local recurrence and survival [1, 2]. The presence of lymphovascular invasion (LVI) has been related to high proliferation index and low hormone receptor levels [3]. These results were obtained by morphological analysis and the examination of blood-vessel endothelium-specific immune markers; such methods are ineffective for determining the prognostic value of lymphatic vessel density (LVD) and lymphatic invasion (LI) in tumors. The newly developed D2-40 monoclonal antibody (mAb) is a safe immunohistochemical marker for lymphatic vessels that can be used on paraffin-embedded and formalin-fixed human tissues [4].

In this study, LVD and LI status were analyzed in invasive ductal carcinomas (IDC) using the D2-40 mAb.

The relationship between LVD and clinicopathological parameters in primary (peri- and intratumoral) tumor tissues was assessed. We also evaluated whether the LVD status of tumor tissue can be used to determine a tumor's metastatic spreading potential. The relationship of LI to clinicopathological parameters was also analyzed.

Materials and Methods

Patient Selection

All invasive breast carcinoma cases included in this retrospective study derived from patients who had undergone partial or total mastectomy and axillary lymph node dissection following diagnosis at the Department of Pathology, School of Medicine, Zonguldak Karaelmas University, between 2000 and 2010. Only patients with histologically typed IDC [not otherwise specified (NOS)] tumors were included in the sample. Sufficient tissue for immunohistochemical survey was obtained and estrogen receptor (ER), progesterone receptor (PR), and HER2/neu protein expressions were reliably detected in a final sample of 69 cases. Clinical and pathological parameters were obtained by review of hematoxylin and eosin (H&E)-stained slides, surgical pathology reports, and the Oncology Data Bank. No distant metastasis was detected and surgical margins were confirmed negative for all cases included in this study.

The cases were reassessed to determine tumor size, histological grade (using the modified Nottingham-Bloom-Richardson grading system) [5], regional lymph nodes, ER, PR, HER2/neu, LVI status, and clinical stage [using the 2002 American Joint Cancer Committee (AJCC) Tumor-Node-Metastases (TNM) Classification] [6]. No neoadjuvant chemotherapy or radiotherapy was administered before the surgical treatment.

Histopathological Examination

H&E-stained slides of all cases were reviewed to confirm the diagnosis and histopathological characteristics. For the immunohistochemical survey of primary tumor tissue, blocks containing sufficient peritumoral benign stroma and maximum quantities of living tumor tissue were chosen.

Immunohistochemistry

All tissues were fixed in 10% neutral buffered formalin and embedded in paraffin using standard surgical pathology protocols. Tissue sections (4 μm) were dewaxed and antigen retrieval was performed in citrate buffer (pH=6) for 5 min using a microwave oven. Sections were incubated for 5 min in 3% hydrogen peroxide to quench endogenous tissue peroxidase. Primary mAbs were directed against ER (1:50 dilution; DAKO Corporation, Carpinteria, CA, USA), PR (1:50 dilution; DAKO Corporation, Carpinteria, CA, USA), HER2 /neu (1:80 dilution; DAKO Corporation, Carpinteria, CA, USA), Ki-67 (Clone MIB-1, 1:50 dilution, DAKO Corporation, Carpinteria, CA, USA) and D2-40 antigen (1:50 dilution; Signet Laboratories, Dedham, MA, USA). Tissue sections were incubated with the appropriate primary mAb for 25 min at room temperature. After washing to remove unbound primary mAb, the 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. The sections were counterstained with hematoxylin after immunohistochemistry.

Specificity was verified by negative control reactions without primary mAb and by the appropriate reaction for each antigen in positive control tissues. Non-neoplastic tumor-adjacent lymphatic endothelial cells (cytoplasmic reaction) and benign tumor-adjacent ductal epithelial cells (nuclear reaction) were used as internal positive controls for D2-40 and ER plus PR, respectively.

Assessment and Scoring of Immunohistochemical Results

Immunostaining for hormone receptors was scored as negative (0–2) or positive (3–8), following Harvey et al. [7]. Immunostaining for HER-2/neu was scored as negative or positive (1+ to 3+), according to previously described parameters [8]. Positivity in at least 10% of tumor-cell nuclei was required for the tumor to be accepted as ER- or PR-positive, regardless of cytoplasmic staining.

Assessment of Lymphatic Microvessel Density, Lymphatic Endothelial Cell Proliferation and Lymphatic Invasion

The method of quantifying LVD has been reported previously [9–23]. The stained sections were first scanned at low magnification (100×) to select ten "hotspots" (areas with the greatest amount of distinct brown staining) in each tumor [13]. These hotspots were then counted at 400× with a microscope ocular grid corresponding to an examination area of 0.1885 mm2 (i.e., 40× objective lens and 10× ocular lens; 0.1885 mm^2 per field). Intratumoral lymphatic vessels were defined as vessels within the main tumor mass, surrounded by tumor cells. The peritumoral region was defined as the area outside of the carcinoma tissue at the tumor margin [9, 10]. Any immunostained cells or separate

clusters of endothelial cells, with or without an identifiable lumen, were considered and counted as a single vessel. Depending on the size of the hot spot, 1 to 3 readings were taken. In the absence of apparent hot spots, 10 randomly selected areas were counted [9, 11, 14, 15]. The LVD for each case was expressed by the mean value (total number of vessels in 10 hot spot microscopic fields/10). The average of intratumoral or peritumoral lymph vessel count was regarded as intratumoral or peritumoral LVD, respectively. The slides were reviewed independently by two pathologists (NOK, FB) without knowledge of the patients' clinicopathological details. When disagreement arose, the slides in question were jointly reviewed.

LI of the tumor was established when at least one neoplastic cell cluster was clearly visible inside a D2-40-positive lymph vessel, following Yamauchi et al. [18]. LI was considered to be positive only when both observers agreed. Total LI number was determined for each case as whole slide being treated with D2-40 immunohistochemically. Tissue areas in sections D2-40 was applied were measured using digital planimeter (Visitrak, Smith&-Nephew, 00 00 00 42 17) in each case as previously described [24]. LI number in a mm2 was determined via dividing total LI number in each case by tissue area. Total tissue area measured in lymph node positive cases is 4434 mm2 (mean±SD; 158.34±72.11, min. 50- max. 388) whereas it is 2535 mm2 (mean±SD; 175.65±88.91 mm2, min. 62- max. 364) in those with lymph nodes negative cases.

Proliferative activity in lymphatic endothelial cells was assessed in consecutive slides of tissues treated with D2-40. Lymphatic vessels whose proliferative activity was assessed via Ki-67 were compared morphologically and immunohistochemically to those treated with H&E and D2-40 (23). Lymphatic nuclei were only scored as proliferating (Ki-67 positive) if they fulfilled features of an lymphatic endothelial cell nucleus i.e. plump oval nuclei lying within the lymphatic vasculature. Cells that did not fulfil such criteria, were excluded as they could representa proliferating tumour or inflammatory cell infiltrating through the lymphatic vessel wall [25].

Statistical Analyses

Clinicopathologic data including patient age, tumor grade, tumor stage, tumor size, lymph node status, LVI (H&E), LI (D2-40), and number of LI in tissue area (n/mm2, D2-40) were correlated with intra- and peritumoral LVD. Mean differences in lymphatic microvessel counts were compared with the use of the paired t test and the chi-square contingency test. A P value of less than 0.05 was considered to represent a significant difference. All statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS, ver. 13.0; SPSS, Inc., Chicago, IL, USA).

Results

The study group was comprised of female patients with a mean age of 54.8±9.8 years (range: 39–85 years). Twenty-three (33%) patients were premenopausal and 46 (67%) were postmenopausal. Axillary lymph node results were negative in 26 (38%) cases and positive in 43 (62%) cases. A sufficient number ($n \geq 10$) of axillary lymph nodes were dissected in all cases; the mean number of extracted lymph nodes was 27.4 ±11.5 (range: 10–66). According to AJCC criteria, 26 (38%) breast tumors were in stage pN0, 28 (40%) were in pN1, 9 (13%) were in pN2, and 6 (9%) were in pN3. The mean number of positive lymph nodes was 7.4±8.5 (range: 1–30).

All cases were histologically typed as IDC (NOS). The mean tumor size was 3.7±2.9 cm (range: 0.8–15 cm). According to AJCC criteria, 15 (22%) breast tumors were in stage pT1, 46 (67%) were pT2, and 8 (11%) were pT3. Three (4%) cases were placed in histological grade I, 56 (81%) in grade II, and 10 (15%) in grade III. Pericapsular spread was observed in 14 (33%) cases of lymph-node positivity. Evaluation of H&E-stained slides detected LVI in 33 (48%) cases, whereas noninvasive (in situ) DC accompanied the tumor in 27 (39%) cases. The following hormone receptor statuses were determined: ER+, 54 (78%); ER–, 15 (22%); PR+, 50 (72%); PR–, 19 (28%). Immunohistochemical analysis obtained the following HER2/neu scores: 0, 17 (25%); 1+, 16 (23%); 2+, 23 (33%); 3+, 13 (19%).

LI (D2-40) was detected by D2-40 in 15 (58%) lymph-node negative cases and 28 (65%) lymph-node positive cases (Figs. 1 and 2). LI (D2-40) was found in 62% ($n=43$) of cases. A statistically significant association was found between LI (D2-40) and LVI [H&E] ($p=0.01$), tumor size ($p=0.04$) and histological grade ($p=0.03$). We found a 74% consistency between LVI (H&E) and LI (D2-40). Presence of multiple LI (D2-40) ($n>1$) was observed in 74% ($n=32$) of LI detected cases. In lymph node positive cases, mean LI number [n/mm2, D2-40] was determined as 0.10±0.17/mm2 (min.0.04-max. 0.8). In lymph node negative cases, mean LI number [n/mm2, D2-40] was determined as 0.05± 0.10/mm2 (min. 0.01-max. 0.5). A statistically significant association was found between LI number [n/mm2, D2-40] and lymph node involvement ($p=0.01$), tumor size ($p=0.03$), histological grade ($p=0.04$) plus stage ($p=0.02$). Distribution of lymphatic invasion numbers [n/mm2, D2-40] with respect to histological grade, tumor size and lymph node status in LI detected cases is presented in Table 1.

All lymphatic endothelial cell nuclei were negative for Ki-67, the proliferation marker; strong positivity for Ki-67

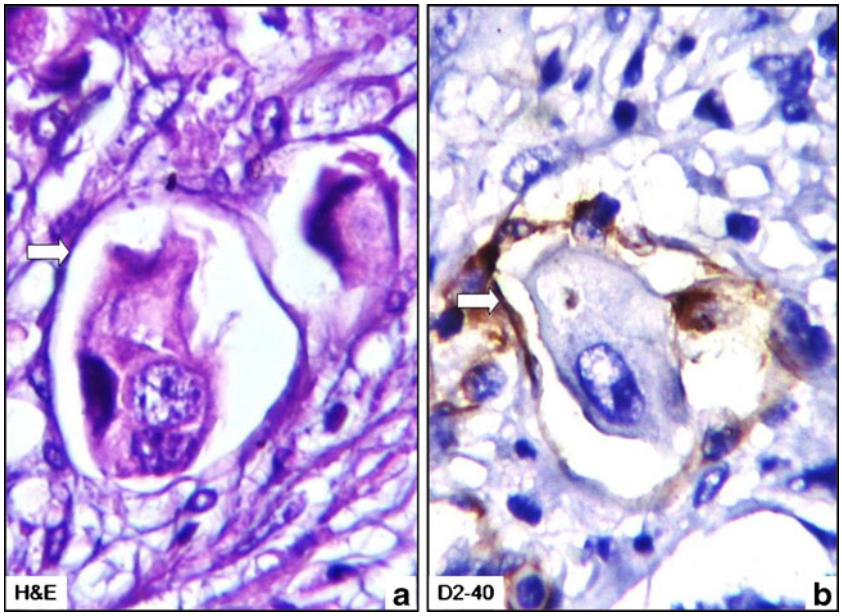

Fig. 1 D2-40 highlighting a lymphatic vessel around a tumor embolus (arrows). Note that this can be easily misinterpreted on an H&E-stained slide as a stromal retraction artifact **a.** H&E, 1000×. **b.** D2-40, 3-amino-9-ethylcarbazole–3,3′-diaminobenzidine (AEC-DAB), 1000×

in adjacent tumor cells and blood endothelial cells served as a positive internal control (Fig. 3b). Of the 69 breast IDC cases, D2-40 immunostaining showed intratumoral lymphatics in 18 (26%) cases and 25 (36%) cases demonstrated positive lymph-vessel staining in fibroadipose tissue at the leading edge of the tumor margin (peritumoral lymphatics) (Fig. 4).

(At all cases) The mean±SD/mm2 density of intratumoral lymphatic vessels was 16.3±9.7 (range, 0.0–24.7), whereas for peritumoral lymphatic vessels, it was 66.3±20.5 (range, 0.0–104). Intratumoral and peritumoral LVD was found to be 14.7±5.5 and 79±14.3, respectively, in lymph node positive cases. Values in question are 7.4±1.3 and 52.5±11.5, respectively, in lymph node negative cases. LVD values at tumor in study groups were presented in Tables 2, 3 and 4. Intratumoral LVD was markedly lower than peritumoral LVD in all groups (all study groups, lymph-node positive group, lymph-node negative group; (all p-values <0.001). However, no significant relationship was detected between intratumoral LVD and other clinicopathological parameters (all p-values >0.05). Significant relationships were detected between peritumoral LVD and LVI [H&E] (p=0.04), LI [D2-40] (p=0.01), tumor size (p=0.01), lymph node status (p=0.03), number of lymphatic invasion [n/mm2, D2-40]

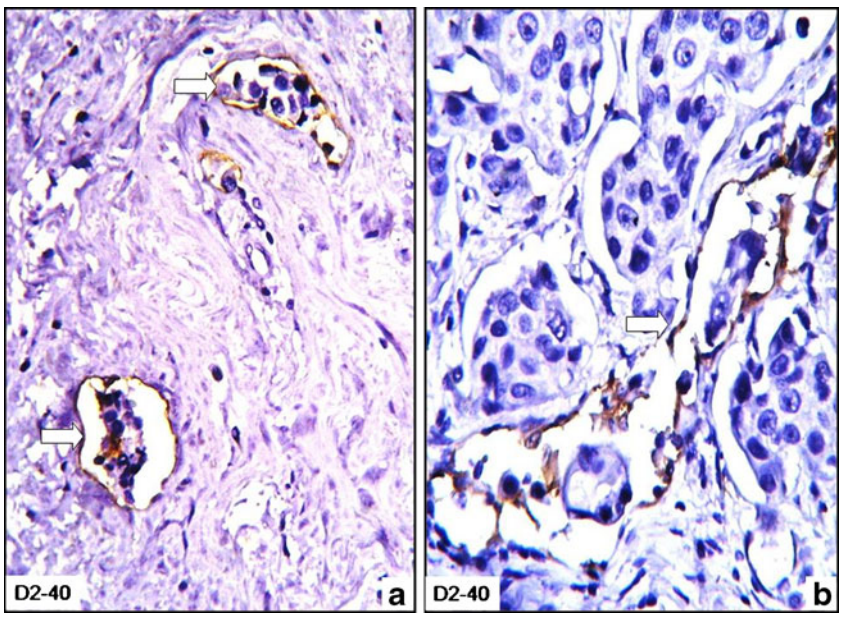

Fig. 2 Tumor embolus within peritumoral lymphatic vessels in a grade-II invasive ductal carcinoma (arrow). A strong cytoplasmic reaction to D2-40 is visible within the intratumoral lymphatic endothelium (arrow) **a.** D2-40, AEC-DAB, 200×. **b.** D2-40, AEC-DAB, 400×

Table 1 Distribution of lymphatic invasion numbers [n/mm2, D2-40] with respect to histological grade, tumor size and lymph node status in lymphatic invasion detected cases (n=43)

Variable		Cases	Number of lymphatic invasion [n/mm2, D2-40]	
		N (%)	Mean±SD	min.–max.
Histological grade (G)	G1	0 (0)	–	–
	G2	33 (77)	0.08±0.09	0.01–0.25
	G3	10 (23)	0.32±0.25	0.02–0.8
Tumor size (T)	T1	0 (0)	–	–
	T2	35 (81)	0.07±0.09	0.01–0.25
	T3	8 (19)	0.38±0.22	0.1–0.8
Lymph node status (N)	N0	15 (35)	0.06±0.05	0.01–0.2
	N1	14 (32)	0.09±0.12	0.01–0.5
	N2	8 (19)	0.22±0.21	0.04–0.5
	N3	6 (14)	0.36±0.25	0.1–0.8

(p=0.001) and tumor stage (p=0.04). Table 5 presents the results of statistical analysis seeking associations between LVD and clinicopathological parameters.

Discussion

The metastatic spread of tumor cells is the primary cause of cancer-related mortality. Cells leaving the primary focus form distant metastases via hematogenous spread; lymphatic spread causes the development of regional lymph-node metastasis. Lymph node status is the primary parameter used for the determination of a patient's prognosis, tumor stage, and treatment modality [10, 12–14, 16–18]. Experimental studies have shown that the inhibition of lymph-node metastasis prevented the development of distant organ metastases [19–21].

LVI is defined as the presence of tumor cells inside blood vessels or lymphatic canals. The LVI of tumors is conventionally evaluated on H&E slices. Because retraction artifacts and morphological appearances may cause confusion with blood vessel invasion, true LI cannot be reliably assessed with this method [14, 16, 18, 28, 30–32, 34, 35, 44]. The use of lymphatic endothelium-specific mAbs allows the reliable determination of LI and LVD [18–21]. D2-40, developed against the oncofetal membrane antigen M2A, is a mAb with an immunoglobulin-G2a (IgG2a) structure that is sensitive to lymphatic endothelial podoplanin [4]. D2-40 is the preferred mAb for investigating intratumoral and peritumoral lymphatics because it is more convenient and more sensitive than other lymphatic markers [22]. In our study, we used podoplanin (clone D2-40) to determine LI and LVD; its reliability has been proven in previous studies [23, 25–28].

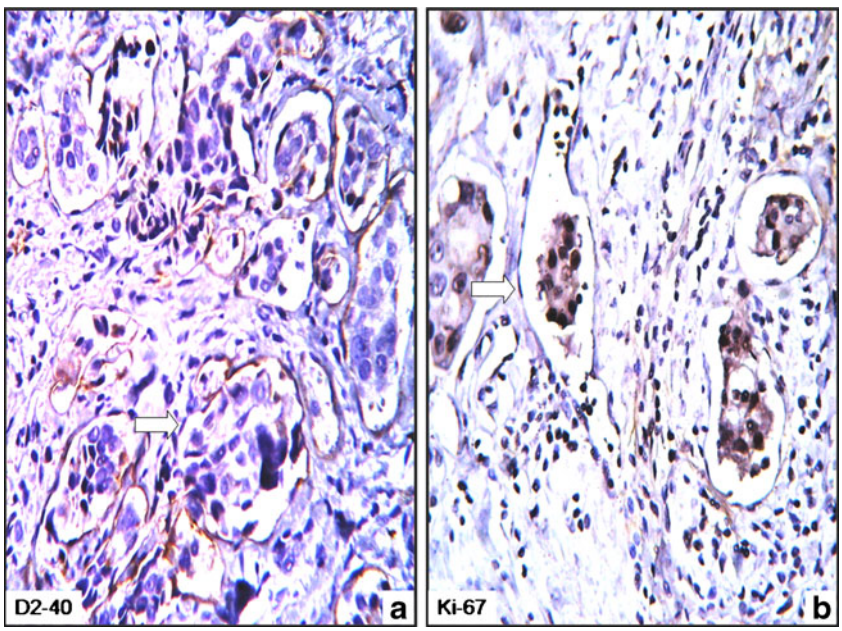

Fig. 3 **a.** Tumor emboli (arrow) are seen within lumen of lymphatic vessels reacting with D2-40 in an invasive ductal carcinoma cases in which widespread lymphatic invasion was detected. **b.** Widespread nuclear reaction is seen via Ki-67 in tumor cells within lumen of lymphatic vessels while no reaction is noted in lymphatic endothelium (arrow). **a.** D2-40, AEC-DAB, 200×. **b.** Ki-67, AEC-DAB, 200×

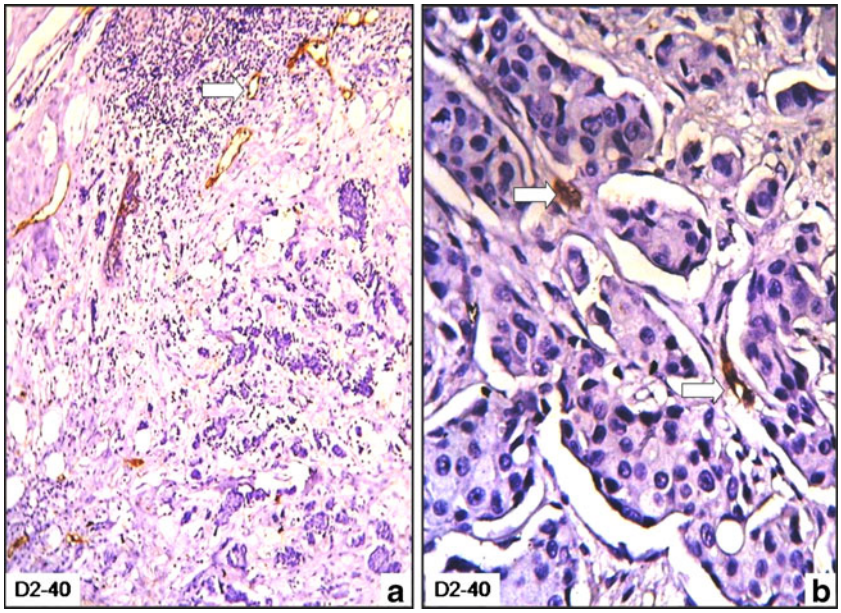

Fig. 4 Microphotos of intra- and peritumoral lymphatic vessels. **a.** The amount of lymphatic vessels within the peritumoral stroma is increased and the lumens are widened (arrow). **b.** The lymphatic vessels within tumor tissue appear collapsed and are few in number (arrow). **a.** D2-40, AEC-DAB, 100×. **b.** D2-40, AEC-DAB, 200×

Immunohistochemically determined frequency of LI in invasive breast cancers ranges between 23% and 74% [2, 3, 14, 18, 28, 31, 32]. It was concluded by most of studies that LI correlates positively with LVI [2, 3, 14, 30, 32]. However, it has been reported by some studies that LI is a parameter related to young age, large tumor size and high histological grade [30, 32–35]. Yamauchi et al. found that LI is associated with high local recurrence rates and shortened disease-free survival, and yet it is independent of both hormone receptor and nodal status [18]. Likewise, Britto et al. advocated that LI could not be used for predicting involvement of sentinel lymph node in early breast cancer [30]. LI was detected in 62% of all cases in our study. Despite lack of statistical significance, the frequency of detection of LI was found to be higher in lymph node positive cases (65%) compared to negative ones (58%) ($p>0.05$). In our study, LI was significantly associated with parameters that inform tumor prognosis, such as LVI, tumor size and histological grade ($p\leq0.05$). We found a 74% consistency between LVI (H&E) and LI (D2-40). Our results show that the immunohistochemical determination of LI provides more objective results than the evaluation of LVI using conventional methods. The determination of true LI in tumors and the inclusion of these data in pathology reports facilitate further research.

Presence of multiple LI was shown to be associated with shortened survival in studies in which the number and extensity of LI had been analyzed in breast carcinomas [3, 14, 18, 32]. Mascarel et al. found that presence of multiple LI was detected in 77% of all LI detected cases [14]. In was concluded in a different study that the presence of multiple LI was associated with tumor recurrence, lymph node involvement, and shortened survival (32). Presence of multiple LI was detected in 74% of the cases in which LI was observed in our study. In our study, number of LI [n/mm2, D2-40] was found significantly higher in lymph node positive cases compared to negative ones ($p<0.05$). A statistically significant association was detected between number of LI [n/mm2, D2-40] and tumor size, histological grade plus stage ($p<0.05$).

Development of lymph node metastasis in tumors is a complex and multifactorial process. Invasion of tumor-related lymphatic vessels located in the most proximal of lymphatic flow by tumor cells may not necessarily result in development lymph node metastasis. Many anti-tumoral defense mechanisms may prevent tumor emboli from

Table 2 Relationship between lymphatic vessel density and tumor size ($n=69$)

Tumor size (T)	Cases	Mean lymphatic vessel density ±SD/mm2 (min–max)	
	N (%)	Intratumoral	Peritumoral
T1	15 (22)	13. 3±2.8 (0.0–17.6)	32. 4±6.4 (0.0–20)
T2	46 (66)	18. 1±4.2 (0.0–23.5)	70. 5±13.9 (0.2–75.2)
T3	8 (12)	21. 5±10.5 (0.0–24.7)	86. 1±21.3 (0.2–104)
Total	69 (100)	16. 3±9.7 (0.0–24.7)	66. 3±20.5(0.0–104)

Table 3 Relationship between lymphatic vessel density and nodal status (n=69)

Lymph node status (N)		Cases	Mean lymphatic vessel density ±SD/mm2 (min–max)	
		N (%)	Intratumoral	Peritumoral
Negative	N0	26 (38)	7.4±1.3 (0.0–11.9)	52.5±11.5 (0.6–74.2)
	N1	28 (40)	15.6±4.1 (0.0–19.7)	71.8±10.3 (17.6–95.1)
Positive	N2	9 (13)	11.6±5.3 (0.0–20.8)	74.2±8.9 (28.5–92.7)
	N3	6 (9)	16.2±6.9 (4.3–24.7)	92.1±11.4 (58.4–104)
Total		69 (100)	16. 3±9.7 (0.0–24.7)	66. 3±20.5(0.0–104)

resulting in development of lymph node metastasis. Another factor is that a part of tumor related lymphatic vessels have abnormal functional properties and chaotic architectures. That many of these vessels are not in connection with normal lymphatic system may prevent tumor cells from reaching lymph nodes (19–21, 30). This explains why presence or absence of LI in tumor tissue is not sufficient alone for predicting lymph node involvement. A rise in number and extensity of LI in a tumor will lead more tumor cells to reach lymph nodes and thus form metastatic foci. Results of our study indicate that determination of the number and extensity of LI in breast carcinomas may provide important data for prediction of biological behavior of a tumor—particularly lymph node involvement.

It is well known as in many other tumor types that tumor angiogenesis and its indicator MVD are closely associated with biological behaviour of tumor in breast carcinomas [1, 2, 9, 17, 34–41]. In contrary to angiogenesis, the contribution of de novo lymphatic vessels on metastatic dissemination and whether tumor related lymphangiogenesis is present is not clear yet [15, 21–23, 25–27]. Methods which are used for measurement of angiogenesis are taken as basis in assessment of lymphangiogenesis in breast cancer [1, 2, 9, 11, 22, 29]. It is MVD method, defined by Weidner et al. in 1991, which is most widely used among these methods [9]. Via this method, mean vessel number is determined in these fields as vascular hot spots being indicated on which the densest immunostaining was detected. Tumor angiogenesis and tumor lymphangiogenesis are quite different in structural and functional terms. Thus, the way LVD is determined and its biological significance is still controversial [1, 2, 9, 11, 13–15, 17, 22, 23, 28, 33, 36–39, 41, 43]. However, in studies conducted on various tumor types, it was concluded that LVD has well correlation to lymphangiogenic growth factors and that LVD is a good indicator of tumor lymphangiogenesis [22, 23, 25, 40–42].

The number of hot spots evaluated for calculation of LVD ranges between 2 and 10 in different studies [9, 12, 17, 33, 37]. In studies regarding tumor angiogenesis, it is reported that 3 hot spots are sufficient enough to measure MVD [1, 9]. However, studies indicated that tumor related lymphatic vessels are more scattered and less in number compared to blood vessels [22, 33, 36]. Hence, it may provide a more accurate evaluation of LVD in tumors that more hot spots to be analyzed which enable larger tumor areas to be observed [10, 12, 13, 22, 25, 27]. So, 10 hot spots were assessed in our study for determination of lymphatic vessel profile of tumor tissue.

In some studies, lymphangiogenesis is assessed as limited by proliferating lymphatic endothelium [15, 17, 22, 27, 36]. All vascular structures that react immunologically with lymphatic endothelial markers have been included in most studies [9, 10, 12, 13, 17, 22, 29]. Experimental models in which lymphangiogenesis was inhibited have shown that the previously present lymphatic vessels also contributed to the development of lymphatic metastasis [19–21, 26]. Proliferative activity in lymphatic endothelial cells (LECs) was searched via Ki67 mAb in our study. Immunoreaction by Ki-67 was not detected in LECs in any of the cases. In some studies conducted regarding breast carcinomas, proliferative activity could not be detected in LECs [15, 17, 22, 27, 36]. Those researchers advocate that lymphangiogenesis does not exist in breast carcinomas. However, proliferative activity was found very low (0.1–2.2%) in LECs in some studies [37, 38, 40, 42].

Table 4 Relationship of lymphatic vessel density with lymphovascular invasion [LVI; H&E] and lymphatic invasion [LI; D2-40] (n=69)

Variable		Cases	Mean lymphatic vessel density ±SD/mm2	
		N (%)	Intratumoral	Peritumoral
LVI (H&E)	Positive	33 (48)	17.04±0.4	71.88±2.5
	Negative	36 (52)	12.29±0.1	59.44±1.8
LI (D2-40)	Positive	43 (62)	22.13±0.9	91.02±2.5
	Negative	26 (38)	15.41±0.1	55.29±1.6

Table 5 Results of statistical analysis seeking associations between lymphatic vessel density [LVD], and number of lymphatic invasion [n/mm2, D2-40], and clinicopathological parameters

Clinicopathological parameter	*P* value		
	Number of LI	Intratumoral LVD	Peritumoral LVD
Age (year)	0.11	0.22	0.24
Menopausal status	0.62	0.51	0.27
Tumor diameter (cm)	0.03	0.54	0.01
Histological grade	0.04	0.12	0.08
Lymph node status	0.01	0.21	0.03
Number of metastatic lymph nodes	0.08	0.09	0.09
TNM stage	0.02	0.81	0.04
Estrogen receptor status	0.61	0.68	0.25
Progesterone receptor status	0.45	0.57	0.56
HER2/ neu expression	0.09	0.62	0.13
Lymphovascular invasion (H&E)	0.07	0.44	0.04
Number of lymphatic invasion (n/mm2, D2-40)	–	0.06	0.001

Age (<50 *vs*. ≥50); menopausal status (premenopausal *vs*. postmenopausal); lymph node status (+/−); tumor size (T1: <2 cm, T2: 2–5 cm, T3: metastatic lymph nodes (Group I: <10, Group II: 10–20; Group III: >20); lymphovascular invasion [LVI] (+/−); estrogen and progesterone receptor status (+/−), HER2/ neu expression (+/−), number of LI (n/mm2, D2-40), lymphatic invasion (LI).

Those researchers explain this lowness by pointing out that lymphangiogenesis is a very slow process. Some, however, advocate that currently used mAbs may be insufficient to determine proliferative activity in LECs [15, 22, 36, 38]. Consequently, an important part of lymphatic spread in beast carcinomas occurs via preexisting lymphatic vessels. Contribution of newly developed lymphatic vessels to lymphatic spread of tumor is limited. In our study, LECs were evaluated indepedently from proliferative activity by taking into consideration that all mature and immature lymphatic vessels play a role at metastatic spread.

Studies investigating tumor lymphangiogenesis have focused on primary tumor tissue. Peritumoral LVD has often been higher than intratumoral LVD [10, 28, 33, 40], suggesting that peritumoral lymphangiogenic activity has a more important effect on metastatic spread. Studies have also associated peritumoral LVD with tumor grade, tumor stage, lymph-node metastasis, and short survival in patients with breast carcinoma [33, 40, 45]. LVD has been positively correlated with LVI (H&E) and LI (D2-40) [22, 23, 33, 40]. Agarwal et al. [15] found that intratumoral LVD was lower in breast carcinomas than in normal and benign breast lesions. Some other researchers advocate that interstitial pressure within tumor tissue is higher than that in periphery of tumor and thus, lymphatic vessels collapse. The fact that intratumoral lymphatic vessels are immature and less in number is reported to provide an advantage of growth to tumor by decreasing drainage of interstitial fluid rich of protein [42, 43]. The consensus of many studies is that intratumoral lymphatics do not play an important role in metastatic spread.

Due to methodological differences, intra- and peritumoral LVD values in breast cancers have been variable. In different studies, intratumoral LVD has ranged from 0.4–24.6/mm2 and peritumoral LVD has ranged from 3.1–51.8/mm2 [28, 29, 33, 40, 41, 45]. The contradicting results about LVD in breast carcinoma may be due to differences in patient selection, sample size, method, and/or the types of tumors included in the analyses. Differences in microvessel counting techniques and targeted lymphatic endothelial antigens were considered likely sources of variability among the analyzed studies [38–45]. El-Gohary et al. analyzed LVD by determining 3 hot spots in breast carcinomas of different histological types [33]. Mean intratumoral and peritumoral LVD were found by 21.8/mm2 and 51.8/mm2, respectively in this study. Agarwal et al. detected mean intratumoral and peritumoral LVD by 0.4/mm2 and 3.3/mm2; respectively in a study they designated 10 hot spots [15]. In our study, mean intra- and peritumoral LVD were 16. 3/mm2 and 66.3/mm2 ($p \leq 0.05$), respectively. Our results support the argument that peritumoral LVD is typically higher than intratumoral LVD in IDC. Although we found no relationship between intratumoral LVD and clinicopathological prognostic parameters ($p > 0.05$), significant relationships were detected between peritumoral LVD and LVI [H E], LI [D2-40], tumor size, lymph node status, number of lymphatic invasion [n/mm2, D2-40] and tumor stage (all p-values ≤0.05). When our results are assessed in concert with those of other studies, is it obvious that the effect of intratumoral lymphangiogenesis on prognosis is limited. However, peritumoral LVD may be a useful parameter for the prediction of a tumor's potential metastatic spread.

Our analysis of the lymphatic profiles of IDCs has found that the immunohistochemical determination of true LI and LVD in tumor tissue significantly informs the assessment of tumor aggressiveness. D2-40 highlights lymphatics and outlines the tumor emboli that are otherwise unrecognizable in H&E-stained sections. Peritumoral LVD and number of LI in primary tumor tissue is closely related to parameters influencing the tumor's biological behavior.

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