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ASSESSMENT OF LYMPHATIC VESSEL DENSITY IN BREAST CARCINOMA USING IMMUNOHISTOCHEMICAL MARKER D2-40

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Abstract:

Background: Breast carcinoma is the most common malignant tumor in females and the incidence of this disease has been significantly increased. Metastasis is the leading cause of mortality in patients diagnosed with breast cancer. It relies heavily on development of new blood vessels (angiogenesis) and lymphatics (lymphangiogenesis)

Objective: The aim of the present study was to assess the significance of lymphatic vessel quantitation in breast carcinoma.

Methods: The study included 50 cases of invasive breast cancer. Lymphatic microvessels were identified by using immunohistochemical stain D2-40 in tumoral and peritumoral area. The microvessels were counted within a 400 x magnification field in the area of highest microvessel density.

Result: -Intratumoral lymphatic vessel density and peritumoral lymphatic vessel density in node positive cases was significantly correlated.

Conclusion: A significant intra and peritumoral lymphatics vessel density in node positive patients indicate that their expression have clinical utility in defining risk for nodal metastasis in breast cancer.

Key words: Breast, carcinoma, metastasis, lymphatic vessel density,D2-40

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Introduction

Breast carcinoma is the most common malignant tumor and leading cause of carcinoma death in women, with more than 1,00,000 cases occurring worldwide annually.[1] Metastasis is the leading cause of mortality in patients diagnosed with breast cancer. It relies heavily on development of new blood vessels (angiogenesis) and lymphatics (lymphangiogenesis). Compared to blood vessels a lymphatic vessel pathway offers many advantages for invasion and transport of pre-metastatic cells.[2] Tumor cell emboli in lymph and blood vessels are considered to be the morphological correlates of breast cancer metastasizing to loco-regional lymph nodes and distant haematogenous sites, respectively. Lymphovascular invasion has also been shown to be a predictor of axillary lymph node metastasis.[3] The aim of the present study was to assess the significance of lymphatic vessel quantitation in breast carcinoma and correlate it with various parameters.

Material And Methods

The study included 50 cases of breast carcinoma undergone radical or modified

radical mastectomy. Patients with breast carcinoma other than primary adenocarcinoma such as lymphoma, sarcoma, stromal tumor and metastasis were excluded. Specimens submitted were examined grossly for tumor size, consistency, margins, and cut surface along with axillary lymph node status. Representative blocks were prepared from tumor, peritumoral tissue, tumor margins, overlying skin, deepest resection margin and axillary lymph nodes. Histopathological diagnosis was established on routine haematoxylin and eosin (H&E) stain and all the histologic prognostic parameters including histologic type, histologic grade, tumor necrosis, lymphatic vessel invasion and lymph node metastasis were assessed. Histologic grading was done by Modified Bloom-Richardson system (MBR). Lymphangiogenic profile of the tumors and peritumoral tissues were assessed by subjecting one section each from a representative block to D2-40 immunostains. Immunohistochemistry (IHC) was performed by peroxidase-antiperoxidase method. Positive and negative controls were run with each batch of immunohistochemical stain.

Interpretation of results

Lymphatic endothelium showed brown membranous positivity with D2-40. Microvessel was defined as any highlighted endothelial cell or endothelial cell cluster clearly separated from adjacent microvessels, tumor cells and other connective tissue elements. Vessel lumen was not necessary for a structure to be defined as microvessel.

Lymphatic vessel density was calculated using Olympus BX51 microscope with image analysis software, image Pro Plus Version 6.3. The four most vascularized areas within tumor and peritumoral area was identified at low magnification (40X) and vessels were counted in a representative high magnification field (400X), in each of these four areas.(Fig 1a,1b,2a,2b) Mean lymphatic microvessel density (LVD) was calculated by taking the average of four counts.

Results

The patients included were in the age group of 21 to 80 years with a mean age of 48.3±11.8 years. All 50 cases were graded using Modified Bloom Richardson grading system. 66% of the cases belonged to grade II followed by grade I (30%). Grade III tumors were least common constituting only 4% of cases. Sixty percent cases were lymph node positive (LN+) with 32% falling into stage II (1-3 positive lymph nodes) and 28% falling into stage III (≥4 positive lymph nodes). 40% of cases did not show lymph node involvement. (LN-)

The mean intratumoral lymphatic vessel density (I-LVD) and peritumoral lymphatic vessel density (P-LVD) of 3.60±0.73 and 11.66±17.07 vessels per mm^2 respectively. There was a positive correlation between I-LVD and P-LVD with a p value of 0.000, which was statistically significant. The mean intratumoral and peritumoral LVD of lymph node negative breast carcinoma was 2.44±3.06 and 6.97±10.73 vessels per mm^2 with a p value of 0.44, which was statistically not significant.

The mean I-LVD of lymph node positive and lymph node negative breast carcinoma was 3.60±0.73 and 2.44±3.06 vessels per mm^2 respectively with a p value of 0.47, which was statistically not significant. The mean P-LVD of lymph node positive and lymph node negative breast carcinoma was 11.66±17.07 and 6.97±10.73 vessels per mm^2 with a p value of 0.28, which was statistically not significant.

The mean I-LVD of Grade I, Grade II and Grade III breast carcinoma was 1.45±2.32, 4.00±7.60 and 1.56±2.20 per mm^2 respectively with a p value of 0.35, which was statistically not significant. The mean P-LVD of Grade I, Grade II and Grade III breast carcinoma was 7.63±14.42, 10.12±15.35 and 20.30±12.52 vessels per mm^2 respectively with a p value of 0.33, which was statistically not significant.

Discussion

Breast cancer mortality rates are declining due to early detection and systemic adjuvant therapy. However, recurrence and distant metastasis, rather than a primary tumor, are the leading causes of death.[4] In similar manner to other carcinomas, breast cancer has a predilection to initially metastasize to the regional lymph nodes, most commonly via the lymphatic system.[1] Studies of lymphatic vessels and lymphatic metastasis have been hampered by the lack of specific lymphatic markers.[5] More recently, monoclonal antibody D2-40 was shown to selectively detect lymphatic vessels in breast and tonsillar tissue.[6] Breast cancer in particular has been studied with regard to the clinical impact of microvessel density and the initial

studies provided promising results. Our study included 50 cases of breast carcinoma. The patient's age ranged from 28-76 years. Majority (34%) of the cases were between 41-50 years and the mean age was 48.3±11.8 years. In present study, 54% patients were premenopausal and 46% were in postmenopausal group. In present study most of the cases (90%) were infiltrating ductal carcinoma (NOS). One case (2%) each comprised medullary carcinoma and metaplastic carcinoma. 60% of cases showed nodal involvement with 32% falling into stage II (1-3 positive lymph nodes) and 28% into stage III (≥4 positive lymph nodes). 40% of cases were node negative.

In our study, 26 out of 50 cases showed complete absence of intratumoral lymphatics where as in most of the cases the lymphatic vessels were present at the peritumoral area as highlighted by D2-40 with 16 cases showing complete absence of peritumoral lymphatics. These findings were in agreement with studies done by Vander shaft et al[7], Vleugel et al[8], Bono et al[9], Zhao et al[1], El Gohary et al[10] and Schoppmann et al[11] who also observed complete absence or only a few intratumoral lymphatic vessels and presence of peritumoral lymphatic vessels. Kato et al[12] suggested that the reasons for this selective localisation of lymphatic vessels in tumors is unknown but one possibility is that they are collapsed in expanding tumors because of the high interstitial pressure. That would suggest that they are present, but difficult to detect because of their flattened morphology. In our study the peritumoral lymphatics were widely opened which was in concordance with study of Saba El-Gendi & Mona Abdel-Hadi.[13]

Thus, the absence of the intratumoral lymphatics in the breast cancer suggest that this may be a result of a completely destructive growth pattern where the pre-existing stroma is destroyed and replaced by newly formed tumor stroma, lacking lymphatic vessels. The absence of lymphangiogenesis during breast cancer progression may be due to absence of lymphangiogenic growth factors, absence of expression of their receptors or the change in native composition in cancer which may lead to disturbances in the initiation of lymphangiogenesis.

In our study the mean I-LVD of lymph node positive and negative cases was 3.60±0.73 vessels per mm^2 and 2.44±3.06 vessels per mm^2 respectively with a p value of 0.47, which was statistically not significant. Our study was concordant with Zaho et al[1], Bono et al[9] and Vander shaft et al[7] who also observed that I-LVD had no significant association with the number of axillary lymph node metastasis (Table 1). This suggested that tumor-derived VEGF-C/D induces lymphangiogenesis around tumor, but not within tumor. They reported that the contradictory results on the role of intratumoral-lymphatic vessels and peritumoral lymphatic vessels in tumor reflects the fact that tumor lymphangiogenesis and lymphatic metastasis are complex mechanisms, which can differ significantly in different tumor types or in tumors at different anatomic location.

Table 1:
Comparison of intra tumoral LVD with different studies

	I-LVD		P-value
	LN +	LN -	
Present Study	3.60±0.73	2.44±3.06	0.47
Zaho et al[1]	5.38±2.15	5.58±1.92	0.74
Vander shaft et al[7]	0.29 ± 1.06	0.04 ± 1.44	>0.05
Bono et al[9]			0.83

In present study the mean P-LVD in lymph node positive and negative cases was 11.66±17.07 vessels per mm^2 and 6.97±10.73 vessels per mm^2 respectively with a p value of 0.28, which was statistically not significant. Our study was in concordance with Kato et al[12] and Vander shaft et al[7] who also suggested that P-LVD did not correlate with lymph node status and suggested that the tumor grows around the pre-existing vessels rather than stimulating the generation of new lymphatics. (Table 2)

Table 2:
Comparison of peritumoral lymphatic vessel density with different studies

	P-LVD		P-value
	LN+	LN-	
Present study	11.66±17.07	6.97±10.73	0.28
Kato et al[12]	6.3±4.5	6.3±4.5	0.08
Vander shaft et al[7]	4.59 ± 4.29	4.74 ± 3.80	>.05

In present study the mean intratumoral LVD of Grade I, Grade II and Grade III breast carcinoma was 1.45±2.32, 4.00±7.60 and 1.56±2.20 vessels per mm^2 respectively with a p value of 0.35, which was statistically not significant. Our study was in concordance with the study done by Zaho et al[1] and Saba El-Gendi & Mona Abdel-Hadi.[13] (Table 3)

Table 3:
Comparison of intratumoral lymphatic vessel density with tumor grade

	I-LVD			P-value
	Grade I	Grade II	Grade III	
Present study	1 . 4 5 ± 2.32 (n=15)	4.00± 7.60 (n=33)	1.56± 2.20 (n=2)	0.35
Zaho et al[1]	5.56 ± 2.05		5.23 ± 2.02	0.58
Saba El-Gendi & Mona Abdel-Hadi[13]	19.2 (0.00 - 35.00)	7.3 (0.00 - 45.00)	11.3 (0.00 - 22.00)	0.26

n =Number of cases

In present study the mean peritumoral LVD of Grade I, Grade II and Grade III breast carcinoma was 7.63±14.42, 10.12±15.35 and 20.30±12.52 per mm^2 respectively with a p value of 0.33, which was statistically not significant. Our study was in agreement with the study done by Zaho et al[1], Mohammed et al[14] and Vander shaft et al[7] who observed that peritumoral LVD did not correlated with histological grades. (Table 4) A possible explanation for variation in the results may be the inter observer variation in selecting criteria of grading such as tubule formation, nuclear pleomorphism and mitotic count and also inclusion of different histological types of tumors in individual studies.

Table 4:
Comparison of peritumoral lymphatic vessel density with tumor grade

	P-LVD			P-value
	Grade I	Grade II	Grade III	
Present study	7.63± 14.42 (n=15)	10.12± 15.35 (n=33)	20.30±12.52 (n=2)	0.33
Zaho et al[1]	8.17 ± 2.88		8.97 ± 3.42	0.87
Mohammed et al[14]				>0.05
Vander shaft et al[7]	5.86 ± 6.87	4.63 ± 3.75	5.00 ± 2.71	>0.05

n - number of cases

The mean intratumoral and peritumoral LVD of lymph node positive breast carcinoma was 3.60±0.73 and 11.66±17.07 vessels per mm^2 respectively with a p value of 0.000. The mean intratumoral and peritumoral LVD of lymph node negative breast carcinoma was 2.44±3.06 and 6.97±10.73 vessels per mm^2 with a p value of 0.44. Thus, there was a significant positive correlation between intratumoral & peritumoral LVD in lymph node positive breast carcinoma suggesting role of lymphangiogenesis and in metastasis.

Amongst all prognostic factors lymph node status remains the most important factor. Immunohistochemical techniques using D2-40 for lymphatics and provided a better assessment, than on conventional haematoxylin and eosin stain. D2-40 antibody specifically recognizes podoplanin and is the most sensitive and specific antibody for the detection of lymphatic endothelium.

A significant intra and peritumoral lymphatics and microvessel density in node positive patients indicate that their expression have clinical utility in defining risk for nodal metastasis in breast cancer and the identification of these features in early stage of disease in node negative patients would be of great interest allowing for a more effective antiangiogenic drugs.

However, on cautionary notes these parameters needs to be correlated with follow up of patients specially in node negative cases and a study with large cohort of patients with a larger follow up is desirable.

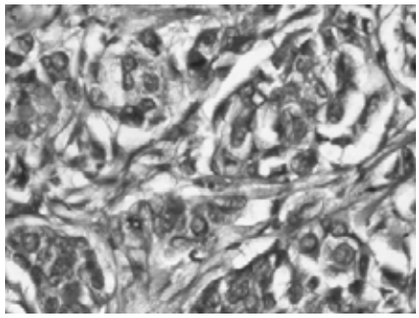

Fig.1a Photomicrograph showing intratumoral area of infiltrating duct carcinoma.(H&E, 200X)

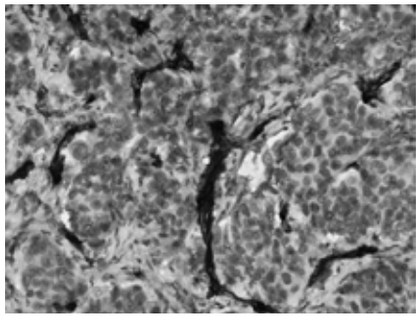

Fig.1b Photomicrograph showing intratumoral lymphatic microvessels stained with D2-40.(IHC, 200X)

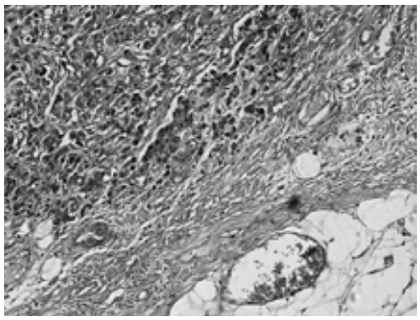

Fig.2a Photomicrograph showing peritumoral area of infiltrating duct carcinoma.(H&E, 100X)

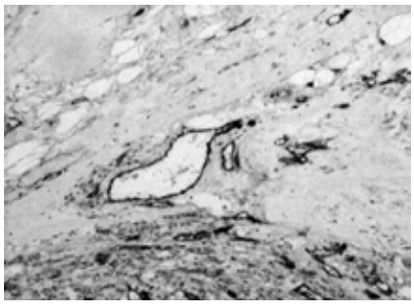

Fig.2b Photomicrograph showing peritumoral lymphatic microvessels stained with D2-40.(IHC, 100X)

Abbreviations

H&E: Haematoxylin and eosin
IHC: Immunohistochemistry
I-LVD: Intratumoral lymphatic vessel density
LN+: Lymphnode positive
LN- : Lymphnode negative
LVD: Lymphatic vessel density
MBR: Modified bloom Richardson scoring
NOS: Not otherwise specified
P-LVD: Peritumoral lymphatic vessel density

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