

## Lymphatic Vessel Density and Lymphatic Vessel Invasion in Invasive Mammary Carcinomas As Identified By Immunohistochemistry Endothelial Lymphatic Marker D2-40 and Its Association with Other Indicators of Poor Prognosis

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## Abstract

## Original Research Article

**Background:** Metastatic spread of tumor cells is responsible for the majority of cancer-related deaths. Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females. **Aims and Objectives:** (i) To determine the peritumoral lymphatic vessel density and lymphatic vessel invasion using immunohistochemistry (endothelial lymphatic marker, D2-40) in invasive mammary carcinomas and (ii) To correlate peritumoral lymphatic vessel density and lymphatic vessel invasion with other indicators of poor prognosis (Age, tumor size, topography, histology, grade, total lymph nodes identified, no. of lymph nodes positive for metastasis and lymph node ratio). **Material and Methods:** Thirty prospective cases of invasive breast carcinoma diagnosed on histopathological examination of breast tissue included in the study. **Results:** Mean age of patients was 51±12.40 years. Sixteen patients of <13 p-LVD had mean tumor size 4±2.69 and 14 patients of >13 p-LVD had tumor size 6±2.37 which was found to be statistically significant (p <0.05). Six patients had 2 positive lymphnodes, 5 had 3 lymphnodes, 1 had four, 4 each had 6 and 7 lymphnodes and 5 patients had >7 lymphnodes. In 2 patients, no positive lymphnode found. A total of 53.33% cases found to be positive and 46.66% negative on D2-40 staining for LVI. With regard to peritumoral LVD, we found maximum 6 patients with 7 p-LVD followed by 4 patients with 8 p-LVD, 3 patients with 13 and 19 p-LVD. Two patients each had 15, 16 and 20 p-LVD and 1 patient each had 9, 10, 12, 14, 17, 18 and 23 p-LVD. **Conclusion:** This study supported the importance of peritumoral lymphatic vessel density and lymphatic vessel invasion assessment using D2-40 in breast cancer patients. The high positivity of peritumoral lymphatic vessel density and lymphatic vessel invasion correlated with other established prognostic measures like tumor size, number of lymphnode involved and lymphnode ratio. This highlights the use of this novel marker in identification of patients who will have poor prognosis even if they have early cancer without nodal involvement. Thus, targeted therapy against lymphatics is required to halt the process of lymphnode metastasis and hence improve prognosis of breast carcinoma.

**Keywords:** Lymphatic vessel, Mammary Carcinomas, Immunohistochemistry, Lymphatic marker.**Copyright © 2019:** This is an open-access article distributed under the terms of the Creative Commons Attribution license which permits unrestricted use, distribution, and reproduction in any medium for non-commercial use (NonCommercial, or CC-BY-NC) provided the original author and source are credited.

### INTRODUCTION

Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females worldwide, with an estimated 1.7 million cases and 521,900 deaths in 2012. Breast cancer alone accounts for 25% of all cancer cases and 15% of all cancer deaths among females. More developed countries account for about one-half of all breast cancer cases and 38% of deaths. Breast cancer incidence rates have been rising in many countries in South America, Africa, and Asia. The reason is not completely understood, but likely reflects changing reproductive patterns, increasing obesity, decreasing physical activity and some breast cancer screening activity. Mortality

rates in these countries are also increasing, most likely due to changes associated with westernization compounded by delayed introduction of effective breast cancer screening programs and, in some cases; limited access to treatment [1, 2].

In most cancers, lymph node (LN) metastasis is an important prognostic factor. However, LN status does not allow a solid prediction of prognosis for patients presenting with small tumours without LN involvement. Other reliable markers predictive of LN metastasis might improve prognostication and might be useful for therapeutic decision-making in these early cancers. The entry of tumor cells into lymphatic vessels is promoted by lymphangiogenesis and

lymphatic enlargement [3]. Therefore, lymphatic vessel density (LVD), a representation of lymphangiogenesis, can serve as an indicator of early lymphogenous spread. Some studies have suggested that lymphatic vessel density is associated with an increased risk of LNM; [4] however; this conclusion is not supported by all of the published studies [5].

Lymphovascular invasion (LVI), which refers to the invasion of lymphatic spaces, blood vessels, or both in the peritumoral area by tumor emboli, is one of the critical steps in metastasis. The prognostic value of Lymphovascular invasion in breast cancer was described first more than 4 decades ago. Routine assessment of Lymphovascular invasion is now part of the minimum data set for breast cancer pathology reporting produced by the United Kingdom Royal College of Pathologists, the European Commission, and College of American Pathologists [6].

Due to the lack of specific markers, the detection of lymphatic vessels has been hampered in previous studies. D2-40 is a recently available monoclonal antibody specific for human podoplanin and has been used in identifying lymphovascular density of tumors. Various studies have evaluated D2-40 expression in several malignant neoplasms. Immunostaining with D2-40 significantly increased the accuracy of detection of lymphatic density compared to conventional hematoxylin and eosin (H&E) staining in early breast cancer. Because lymphovascular invasion is a strong prognostic marker, D2-40 has potential as a key prognostic predictor for breast cancer and its effectiveness is actively investigated [7].

The data suggested that peritumoral lymphatics may have a role in the pathophysiology of lymphatic vessel invasion. If these lymphatics correlate with known prognostic factors then they might constitute a potential new target for development of anti-breast cancer therapeutic concepts. This study was planned to quantify the peritumoral lymphatic vessel density and the lymphatic vessel invasion and evaluate their association with other indicators of poor prognosis.

## MATERIALS AND METHODS

This study was conducted in the department of Pathology in collaboration with department of Surgery, Maharaja Agrasen Medical College, Agroha. The study comprised of a minimum thirty prospective cases of invasive breast carcinoma diagnosed on histopathological examination of breast tissue send to our department, after surgery. The relevant clinical history of the patient was collected. Tissue sections were assessed with H&E staining for histological type, histological grade (Nottingham modification of Bloom Richardson grading system), lymphovascular invasion, regional lymph node status and lymph node ratio. Then lymphatic vessel density analysis was performed on

paraffin embedded representative tissue section stained with monoclonal antibody D2-40. Patients of all aged with invasive mammary carcinoma who underwent surgical treatment as primary modality of treatment were included in the study. Special care was taken to include only specimens with sufficient amount of normal tissue surrounding the invasive tumor to evaluate peritumoral lymphatic vessel invasion. The patients who received any prior treatment like lumpectomy, neoadjuvant chemotherapy or radiotherapy were excluded. Cases of isolated tumor cells were also excluded.

### Procedure for IHC staining [8]

Mounting of 4-5µm sections on slides coated with poly-L-Lysine. Deparaffinization of sections in xylene and rehydration through graded alcohol with washing the slides in running tap water. Antigen retrieval using Tris EDTA buffer at PH 9 in a pressure cooker for 20 minutes followed by Rinsing the sections in Tris buffered saline (TBS) and draining off excess TBS. Blocking the endogenous peroxidase using methanol with 3% H<sub>2</sub>O<sub>2</sub> for 10 minutes and washing with TBS for 5 minutes. Incubation of sections with D2-40 (isotype:IgG 1, kappa) (Dako) for 60 minutes followed by washings in TBS. Incubation with polymerized horseradish peroxidase (HRP)-anti-mouse/rabbit immunoglobulin IgG (secondary antibody) (Dako) for 30 minutes followed by washings in TBS. Incubation in DAB reagent for 10 minutes for color development and rinsing the slides TBS and transferring to running water. Counter staining with Mayer's hematoxylin, Dehydration with graded alcohols and Clearing with xylene and mounting with DPX.

### Interpretation

Lymphatic endothelial cells: Brown, cytoplasmic and membranous staining.

### Positive control

Lymphatic vessels in sclerotic areas within tumor, where lymphatic vessels are sparse, and immediately adjacent areas of unaffected breast tissue was used as positive control for D2-40 antibody.

### Negative control

Was obtained by substituting the primary antibody with an antibody of irrelevant specificity

### Morphometric Analysis

The quantitative morphometric study was done by image analysis. Images provided by a device video camera coupled with Olympus CX21i microscope at a magnification of 400X were stored on a host computer based on Pentium [R] processor with operating system Microsoft windows XP through a digital frame grabber and processing was done by image analysis software Magnus Pro 4.1. Peritumoral lymph vessels were defined as D2-40 positive vessels located in pre-

existing mammary stroma at a maximum distance of 2 mm from the tumor periphery. Lymphatic vessel density was assessed by light microscopy in representative areas with highest number of lymphatic vessels “lymphatic hot spots” according to the method that was described by Weider *et al.* Any brown staining endothelial cell or endothelial-cell cluster that clearly separated from adjacent microvessels, tumour cells, and other connective tissue elements was considered a single, countable microvessel. Vessel lumen was not considered necessary for a structure to be defined as a microvessel, and red cells were not used to define vessel lumen. The sections were scanned first at low power (100X), and the most intense areas of lymphatic vessels (hot spots) was identified and lymphatic vessel counts was performed at 400X [9, 10].

### Results

Number of lymphatic vessels or hot spots stained with D2-40 in three fields at 400X magnification was counted and results were expressed as total number of vessels.

### Statistical analysis

At the end of the study, the data was analysed statistically by using Student t-test (Independent t-test) for mean comparison and One-way Analysis of Variance for multi-group mean comparisons. A p value of <0.05 was considered as significant by using Statistical Package for Social Sciences (SPSS) Version 20.0.

### Biomedical waste disposal [11]

The breast tissues submitted for histopathological study was used in wax blocks and slides. The representative blocks were preserved up to 10 years and slides preserved up to five years in the

department. All the biomedical waste generated during this study in the laboratory was discarded as per the Biomedical Waste Management and Handling Rules, 2016 guidelines.

## OBSERVATIONS AND RESULTS

In the present study, maximum number of women belonged to elderly age group i.e. >60 years. Mean age of patients was 51±12.40 years. A total of 9 patients had tumour size upto 3 cm, 13 had 3-5 cm size, 7 had 5-10 cm size and only 1 patient had >10 cm size. Topography examination of the patients showed that maximum 16 patients had mass in superolateral quadrant, 7 in inferolateral quadrant, 4 in inferomedial quadrant and 3 in superomedial quadrant. Maximum number of patients i.e. 66.66% had grade III tumour followed by grade II (26.66%) and grade I (6.66%). We found maximum number of 8 (26.66%) patients who identified with >10 lymphnodes followed by 5 (16.66%) who had 7 lymphnodes. With regard to total positive number of lymphnodes, six patients had 2 positive lymphnodes, 5 had 3 lymphnodes, 1 had four, 4 each had 6 and 7 lymphnodes and 5 patients had >7 lymphnodes. In 2 patients, no positive lymphnode found.

A total of 16 patients were found to be positive and 14 negative for lymphatic vessel invasion on D2-40 in the present study i.e. 53.33% and 46.66% respectively. With regard to total number examined through p LVD, we found maximum 6 patients with 7 p-LVD followed by 4 patients with 8 p-LVD, 3 patients with 13 and 19 p-LVD. Two patients each had 15, 16 and 20 p-LVD and 1 patient each had 9, 10, 12, 14, 17, 18 and 23 p-LVD.

**Table-1: Correlation of age with lymphatic vessel density (D2-40)**

	Age (range)	
	<50 years (n=15)	> 50 years (n=15)
Mean p-LVD*	14±4.56	12.26±5.58
Mean LVI**	8±3.35	10±4.52

p=0.357 (P>0.05 Not significant)\*; p=0.357 (P>0.05 Not significant)\*\*

Table 1 shows correlation of age with p-LVD that shows 15 patients with less than 50 years had mean p-LVD 14±4.56 and 15 patients with >50 years had mean p-LVD 12.26±5.58 (p >0.05). Correlation of age

with LVI that shows 15 patients with less than 50 years had mean LVI 8±3.35 and 15 patients with >50 years had mean LVI 10±4.52 (p >0.05).

**Table 2: Correlation of topography with lymphatic vessel density (D2-40)**

	Topography			
	Inferolateral N=7	Inferomedial N=4	Superolateral N=16	Superomedial N=3
Mean p-LVD	12.14 ±4.41	15.75 ±4.99	12.12 ±5.13	17.33 ±5.50

p=0.269 (P >0.05 Not significant)

Table 2 shows topography correlation with p-LVD. A total of 7 patients had inferolateral mass with mean p-LVD  $12.14 \pm 4.14$ , 4 observed inferomedial mass with  $15.75 \pm 4.99$  mean p-LVD, 16 had superolateral mass with  $12.12 \pm 5.13$  and 3 with superomedial mass with  $17.33 \pm 5.50$  mean p-LVD, respectively. Statistical analysis showed insignificant difference among all these groups. With regard to tumor

grade I/II (n=10) we found mean p-LVD  $14.2 \pm 5.59$  and tumor grade III (n=20) had less p-LVD i.e.  $12.6 \pm 4.88$ , which was found to be statistically insignificant ( $p > 0.05$ ). We found that 16 patients of  $<13$  p-LVD had mean tumor size  $4 \pm 2.69$  and 14 patients of  $>13$  p-LVD had tumor size  $6 \pm 2.37$  which was found to be statistically significant ( $p < 0.05$ ).

**Table-3: Correlation of LVI with lymphatic vessel density (D2-40)**

	LVI	
	Positive (n=16)	Negative (n=14)
Mean p-LVD	$16.81 \pm 3.08$	$8.92 \pm 3.33$

$p=0.0001$  ( $P < 0.001$  highly significant)

Table 3 shows correlation of LVI and p-LVD. We observed 16 patients with positive lymphatic vessel invasion whose p-LVD was  $16.81 \pm 3.08$  and 14 patients

with negative lymphatic vessel invasion had lesser p-LVD i.e.  $8.92 \pm 3.33$  ( $p < 0.001$ ).

**Table-4: Correlation of positive lymphnode identified with lymphatic vessel density (D2-40)**

	Positive lymphnode		
	Upto 3 (n=16)	4-9 (n=9)	$>10$ (n=5)
Mean p-LVD	$11.21 \pm 5.26$	$14 \pm 3$	$18.8 \pm 3.63$

$p=0.0001$  ( $P < 0.001$  highly significant)

Table 4 shows positive lymphnode identified cases correlation with p-LVD. A total of 16 patients who had upto 3 lymphnode with mean p-LVD  $11.21 \pm 5.26$ , 9 patients with 4-9 with p-LVD  $14 \pm 3$  and 5

had  $>10$  with mean p-LVD  $18.8 \pm 3.63$ . Statistical analysis showed significant difference ( $p < 0.001$ ) among all these three groups.

**Table-5: Correlation of lymphnode ratio with lymphatic vessel density (D2-40)**

	Lymphnode ratio		
	Upto 0.25 (n=7)	0.26-0.65 (n=10)	$>0.65$ (n=13)
Mean p-LVD	$7.57 \pm 1.13$	$12.1 \pm 4.62$	$16.92 \pm 3.37$

$p=0.0001$  ( $P < 0.001$  highly significant)

Table 5 shows correlation of lymphnode ratio with p-LVD. LN ratio upto 0.25 showed mean p-LVD of  $7.57 \pm 1.13$ , from 0.26-0.65 it was  $12.1 \pm 4.62$  and  $>0.65$ , it was  $16.92 \pm 3.37$ . Statistical analysis showed significant difference among all these groups.

International Consensus on The Methodology of Lymphangiogenesis Quantification in Solid Human Tumors', Podoplanin was considered to be the most reliable marker of lymphatic vessels currently available. In this study, D2-40 produced strong and specific lymphatic vessel immunoreactivity in breast cancer.

## DISCUSSION

Metastatic spread of tumor cells is responsible for the majority of cancer-related deaths. Breast cancer has a predilection to initially metastasize to the regional lymph nodes, most commonly via the lymphatic system. Lymphangiogenesis is considered to be a key process during lymphatic metastasis. Several lymphatic endothelial markers have been established recently like podoplanin, desmoplakin, Prox 1, receptors for VEGF-C and VEGF-D (VEGFR-3). The D2-40 antibody has been shown to specifically recognize the glomerular podocyte membrane protein Podoplanin, and is a very sensitive and specific marker for lymphatic endothelium in most tissues, especially breast cancer. In 'The First

In a study conducted by Zhao *et al.* [12], D2-40-stained lymphatic vessels were unevenly distributed throughout the breast tumors. The lymph vessels in the peritumoral areas were more frequent, larger and dilated. D2-40 immunostaining highlighted the presence of lymphatic invasion, which is usually present at the periphery of tumors. No significant difference was observed between the intratumoral LVD of breast carcinoma and the LVD of control tissues ( $5.47 \pm 2.03$  vs.  $5.25 \pm 1.73$ ,  $P > 0.05$ ). However, the peritumoral LVD ( $8.77 \pm 3.30$ ) was significantly higher than the intratumoral LVD and LVD of control tissues.

Age is strong risk factor for developing breast carcinoma. In our study we correlated age with the risk of LVD and LVI. However, no correlation has been found between age and LVD or LVI. (0.357). This result shows that age alone cannot predict the dissemination of tumor. Our study was in concordance with the study conducted by Wahal *et al.* [9] in which no significant association between age and vessel density either for lymphatics or blood vessels in tumor or peritumor could be seen. ( $p>0.05$ ). Zhao *et al.* [12] also showed comparable results in which data suggested no correlation between age and peritumoral lymphatic vessel density ( $p=0.62$ ).

Increasing tumor size is another important prognostic marker in breast cancer and predictor of local recurrence, regional and/or systemic spread and overall survival as increasing tumor size has been known to reflect increasing vascular and lymphatic dissemination. A study conducted by Ansari *et al.* [13] showed mean tumor size in patients with high lymphatic micro vessel density ( $>9$ ) was significantly more than those with low lymphatic micro vessel density ( $<9$ ) ( $p=0.003$ ). Similar observations were found in present study where mean tumor size in patients with high lymphatic vessel density was significantly more than those of low lymphatic vessel density.

In the study conducted by Wahal *et al.* [9] 30% tumors were multicentric and 30% were in upper-outer quadrant. Lymphatic vessel density, blood vessel density and lymphatic vessel invasion were not increased by the multicentricity of the tumor. Similar were the results shown by our study which showed that topography of tumor cannot predict the tumor dissemination potential of the tumor.

In the present study, LVD and LVI were correlated with different histological grades (modified bloom Richardson nothingham grading). However there was no significant increase in parameters with increasing grade of tumor. Our results were comparable to study conducted by Wahal *et al.* [9] in which no significant correlation was found between increasing grade of tumor and LVD, BVD and LVI.

In the study conducted by Ansari *et al.* [13], it was emphasized that a correlation between lymphatic microvessel density and lymphatic vessel invasion coexists. The study observed that mean lymphatic vessel density in lymphatic vessel invasion positive and negative cases to be  $22.85\pm 13.29$  and  $7.95\pm 2.05$  ( $p\leq 0.001$ ). This significant association between lymphatic microvessel density and lymphatic vessel invasion could be explained through a lymphangiogenesis induced increase of the "lymphatic window" providing tumor cells with more opportunities to enter into lymphatic vessels. Choi *et al.* [14] studied the lymphatic vessel density and blood vessel density

and its correlation with clinicopathological parameters in 29 invasive carcinomas using D2-40 and CD-31 antibody. The results showed that D2-40 lymphatic vessel density correlated with tumor stage and lymph node metastasis. Zhao *et al.* [12] concluded in their study that there was a significant correlation between peritumoral lymphatic vessel density and lymphatic vessel invasion, lymph node metastasis and TNM clinical stage, indicating that VEGF-C/D-induced peritumoral lymphangiogenesis leads to lymphatic invasion and lymph node metastasis.

The present study observed a similar results with mean peritumoral lymphatic vessel density in lymphatic vessel invasion positive cases of  $13.81\pm 3.08$  and in lymphatic vessel invasion negative cases of  $8.92\pm 3.33$  ( $p\leq 0.001$ ). This signifies that a higher mean lymphatic vessel density to be present in lymphatic vessel invasion positive cases as compared to lymphatic vessel invasion negative cases. The result suggests that breast cancers with high peritumoral lymphangiogenesis as measured with lymphatic vessel density more often invade these lymphatic vessels and have more chance of lymphatic metastasis as compared to low lymphatic vessel density. Further, a significant correlation between total number of positive lymph nodes and lymphatic vessel density supports the statement ( $P\leq 0.001$ ). Similar results were obtained by Ansari *et al.* [13] where they found correlation of lymphatic vessel density with the number of metastatic lymph node ( $p<0.0001$ ).

In the study conducted by Wahal *et al.* [9], high lymphatic vessel density was observed in cases with high lymph node ratio. A strong correlation between lymphatic vessel density in peritumoral zone, and lymph node ratio was observed. The mean lymphatic vessel density in peritumoral zone was seen to be significantly increasing with increasing lymph node ratio ( $p<0.001$ ). Our study showed similar results, with mean lymphatic vessel density in peritumoral zone was seen to be significantly increasing with increasing lymph node ratio ( $p<0.001$ ).

## CONCLUSION

D2-40 positive lymph vessel invasion is an independent predictor of recurrence in node negative breast cancer patients and is also associated with reduced survival and the present study supported the importance of peritumoral lymphatic vessel density and lymphatic vessel invasion assessment using D2-40 in breast cancer patients for prognostic purpose. The high positivity of peritumoral lymphatic vessel density and lymphatic vessel invasion correlated with other established prognostic measures like tumor size, number of lymphnode involved and lymphnode ratio. This highlights the use of this novel marker in identification of patients who will have poor prognosis even if they have early cancer without nodal involvement. Thus, targeted therapy against lymphatics

is required to halt the process of lymphnode metastasis and hence improve prognosis of breast carcinoma.

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