ORIGINAL ARTICLE

Immunohistochemical evaluation of lymphovascular invasion in carcinoma breast with CD34 and D2-40 and its correlation with other prognostic markers

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ABSTRACT

Background: Carcinoma breast is ever-evolving and becoming increasingly prevalent in India. Numerous prognostic factors based on morphology and immunohistochemistry (IHC) have been established which need to be interconnected to give patients best possible treatment. **Aims:** This study aims to confirm and analyze lymphovascular invasion (LVI) detected by hematoxylin and eosin (H and E) using IHC with CD34 and D2-40 and its correlation with other biologic and morphologic prognostic markers. **Settings and Design:** This was a prospective study. **Materials and Methods:** Fifty mastectomy specimens diagnosed as infiltrating ductal carcinoma breast on histopathology selected for the study. Evaluation of formalin-fixed paraffin-embedded sections was done using H and E and IHC for estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 HER2/*neu* receptors, CD34, and D2-40 endothelial markers. Correlation of LVI done with prognostic markers of Carcinoma Breast, namely, age of the patient, tumor size, Nottingham grade, lymph node ratio (LNR), Nottingham prognostic index (NPI), ER/PR status, and HER2/*neu* status. CD34 and D2-40 utilized to distinguish blood vessel, lymph vessel, and retraction artifacts and to calculate lymphatic microvessel density (LMVD) and blood microvessel density (BMVD). **Statistical Analysis Used:** SPSS Software Package. **Results:** LVI was associated with younger age ($P = 0.001$), greater tumor size ($P = 0.007$), higher Nottingham grade ($P = 0.001$), higher LNR ($P = 0.001$), higher NPI ($P = 0.001$), Negative ER Status ($P = 0.001$), Negative PR Status ($P = 0.002$), Positive HER2/*neu* status ($P = 0.021$), Higher Intratumoral BMVD ($P = 0.016$), Peritumoral BMVD ($P = 0.001$), and Intratumoral LMVD ($P = 0.009$). Blood vessels more commonly invaded than lymph vessels. Retraction artifacts can be mistaken for LVI without IHC. **Conclusions:** D2-40 is a promising marker for lymphatic endothelium. LVI is a poor prognostic marker hence should be evaluated imperatively in all cases of carcinoma breast.

KEY WORDS: Carcinoma breast, CD34/D2-40, lymph node ratio, lymphovascular invasion, Nottingham prognostic index, retraction artifacts

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INTRODUCTION

Breast cancer is a global disease, and statistics reveal that it is now the most common cancer in Indian females, in most cities, as well as second most common in the rural areas, after outpacing carcinoma of the uterine cervix.[1]

The management of carcinoma breast needs to be tailored for every patient according to their prognosis, which depends on numerous biologic and morphologic parameters. A well-recognized but relatively unexplored parameter is the presence of lymphovascular invasion (LVI), which is defined as the presence of tumor emboli inside an endothelium-lined space in the peritumoral area.[2] Detection of these endothelial cells on hematoxylin and eosin (H and E)-based morphology can be laborious and still unrewarding, as distinction of blood vessels, lymph vessels, and retraction artifacts cannot be made. Because the presence of lymphovascular space invasion is a strong predictor of patient outcome, its precise identification by immunohistochemical confirmation is highly desirable.

Tumor angiogenesis has been contemplated as a prognostic indicator in carcinoma

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breast. Epithelial cancers require the formation of a supporting stroma and vascular supply if they are to grow beyond the size of 1–2 mm and therefore induce the formation of new blood vessels that supply the tumor with nutrients and also provide a means for gas exchange and waste disposal. However, accurate identification and quantification of these vessels also require the application of immunohistochemistry (IHC).

The present study attempts to comprehensively evaluate LVI and tumor angiogenesis using IHC for the vascular endothelial marker CD34 and the novel lymphatic endothelial marker D2-40, as retraction artifacts can be mistaken for LVI without these IHC markers. Furthermore, the interplay between various prognostic markers of carcinoma breast is studied thoroughly, and reveals that LVI is associated with other poor prognostic markers of carcinoma breast.

MATERIALS AND METHODS

Fifty mastectomy specimens of invasive ductal carcinoma (IDC) breast in females were chosen for the study conducted at a tertiary care center in North India, based on the following:

Inclusion criteria

1. Primary epithelial malignancies of breast; diagnosed initially by fine needle aspiration cytology or trucut biopsy; 2. Previously untreated naive cases; 3. Adequate material available in the paraffin blocks for IHC.

Exclusion criteria

1. If the histopathological sections showed: a) secondary metastatic tumor, b) lymphoma, c) malignant mesenchymal tumors of the breast or d) predominantly necrosis; 2. If the relevant clinical history was not available.

The tumor size and number of lymph nodes (LNs) received was noted on gross examination. After processing and making of formalin-fixed paraffin-embedded blocks, sections of 3–4 μ thickness were cut with a microtome and stained with H and E.

The sections from tumor were examined for the extent of tubule formation, nuclear pleomorphism, and mitotic activity, to allocate a score for modified Bloom–Richardson scoring system and subsequently allotted the Nottingham grade. Sections from the peritumoral tissue were assessed for the presence of LVI and from the LNs for the presence of carcinomatous deposits.

Sections from the tumor tissue were subjected to IHC with estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2)/*neu.* Sections from tumor along with peritumoral tissue showing LVI were subjected to IHC with CD34 and D2-40.

ER and PR show nuclear positivity and Allred scoring was done for the same. Scores can have a maximum value of 8; 0/8 and 2/8 interpreted as negative; ≥3/8 interpreted as positive. HER2/*neu* receptors show membranous positivity and based on the intensity of staining are interpreted as negative (0, 1+), weak positive (2+), and positive (3+).

CD34 shows strong positivity in vascular endothelium and weak positivity in lymphatic endothelium. D2-40 is a specific marker for lymphatic endothelium. Any vascular space with phenotype CD34+, D2-40- interpreted as blood vessel, as shown in Figure 1; CD34±, D2-40 + as lymph vessel, as shown in Figure 2; and CD34−, D2-40 + as retraction artifact, as shown in Figure 3.

CD34 and D2-40 immunostained sections were scanned at low magnification (×40), and the area of tissue with the greatest number of distinctly highlighted microvessels ("angiogenic hot spot") was selected. Manual counting of five such "hot spots" in intratumoral and peritumoral areas was done at a magnification of ×400, for both blood vessels and lymph vessels, and average was taken to calculate blood microvessel density (BMVD) and lymphatic microvessel density (LMVD), respectively. Please refer to Figures 4 and 5.

All the data obtained were analyzed in context of its correlation with the other. Statistical analysis of these correlations was done using the SPSS Software Package, Version 22 (Trial), a product of IBM Corp., Armonk, New York, USA; and a value of $P < 0.05$ was taken as significant.

Ethics

Approval of the institutional thesis and ethics committee was obtained before the commencement of the study.

DISCUSSION

LVI is a harbinger of the distant metastasis in carcinoma breast. Detection of early stage of disease and its metastatic potential is very important for the treatment and patient care. The present study was therefore undertaken with the aim to scrupulously evaluate LVI in IDC breast and to see its correlation with various prognostic markers of breast cancer pathology and their role in determining patient outcome.

The status of estrogen, progesterone, and HER2/*neu* receptors is pivotal in deciding the administration of neoadjuvant therapy

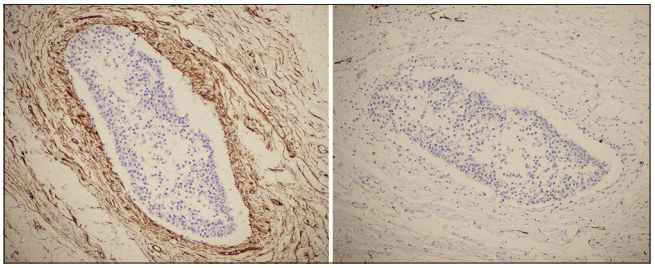

Figure 1: Microphotograph showing blood vessel invasion with CD34 positivity (left) and D2-40 negativity (right) around tumor embolus (×100)

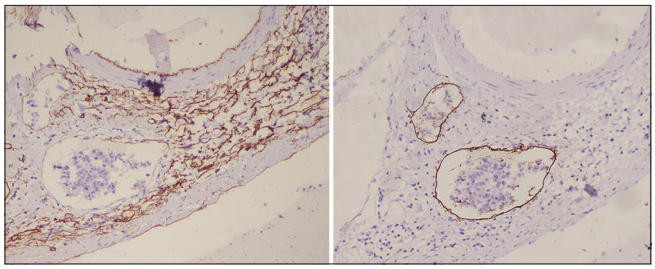

Figure 2: Microphotograph showing lymph vessel invasion with CD34 negativity (left) and D2-40 positivity (right) around tumor embolus (×200)

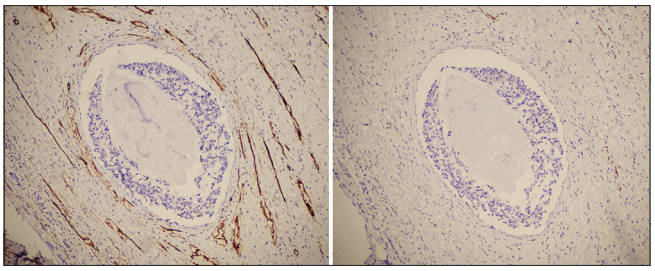

Figure 3: Microphotograph showing retraction artifact with CD34 negativity (left) and D2-40 negativity (right) around tumor embolus (×100)

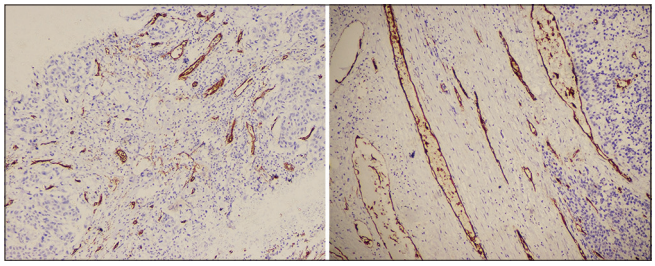

Figure 4: Microphotograph showing intratumoral (left) and peritumoral (right) blood microvessel density (CD34, ×100)

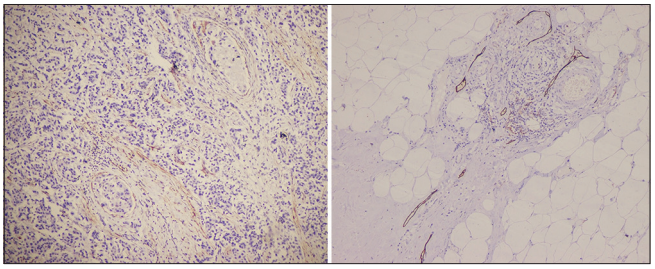

Figure 5: Microphotograph showing intratumoral (left) and peritumoral (right) lymphatic microvessel density (D2-40, ×100)

in carcinoma breast. It is therefore recommended that IHC be routinely done for these three markers. The role of LVI as a prognostic marker has been studied; however, its authenticity can be established only with the application of IHC for the endothelial markers, two of which, namely CD34 and D2-40, are utilized in the present study.

With the results of our study, we emphasize that H and E-based morphology even though essential may not be adequate to report LVI. There are two issues with this methodology. First, LVI can be mistaken for retraction artefacts, and second, LVI as a generic entity does not distinguish between blood vessels and lymph vessels. Both of these issues are successfully addressed with the help of IHC in our study, as we demonstrated that 28% cases thought to be LVI on H and E-based morphology were actually retraction artifacts. We were also able to subclassify LVI with IHC and observed that 52% cases had blood vessel invasion, while 20% cases had lymph vessel invasion.

The preferential route of tumor dissemination may be explained by the fact that capillaries are feeder vessels that ensure tissue viability and promote tumor growth and nutrition in malignant tumors. Lymphatic vessels, on the other hand, are draining vessels that are not essential for tumor metabolism and therefore would not provide any advantage for tumor growth or survival.[3,4]

Previous studies have also established the prognostic significance of retraction artifacts that even though are not true LVI may still be the forerunners of lymphatic space involvement and that they correlate with lymphangiogenesis and lymphatic tumor spread and thence predict a poor outcome.[5,6]

Besides the detailed assessment of LVI, the other parameters that define tumor biology were also elaborated, and these were age at diagnosis, tumor size, LN status, Nottingham grade, ER/PR hormonal status, HER2/*neu* status, and microvessel densities. All the data obtained during the course of the study have been summarized in Table 1.

Breast cancer at an early age is more likely to be associated with an increased familial risk, especially in women harboring a germline *BRCA1, BRCA2, TP53,* and *PTEN* mutations and more likely to have an aggressive phenotype of the disease.[7,8] In our study, the age of patients varied from 32 to 75 years, with mean age of presentation as 48.74 years. A large proportion of our study sample were quadragenarians, who were found to have larger tumors ($P = 0.001$), high risk LN ratio (LNR) ($P = 0.001$), higher Nottingham grade ($P = 0.001$), higher Nottingham prognostic index (NPI) ($P = 0.001$), negative ER and PR receptors ($P = 0.001$), and presence of True LVI, with blood vessels being invaded more often than lymph vessels ($P = 0.032$). Our results are concordant with those of Schoppmann *et al*., 2004; Mohammed *et al*., 2011; Rakha *et al*., 2012.[9-11]

Numerous studies have shown that the measured gross size represented by the largest dimension of a mammary carcinoma is one of the most significant prognostic variables, as it is positively correlated with decreased survival, increased frequency of axillary nodal metastases, and increased vascular and lymphatic dissemination.[12,13] Similarly in our study, greater tumor size was associated with younger age of presentation ($P = 0.001$), high risk LNR ($P = 0.001$), higher Nottingham grades ($P = 0.001$), negative ER status ($P = 0.047$), negative PR Status ($P = 0.015$) and increased incidence of true LVI, with blood vessels invasion seen

Table 1: Distribution of data

Parameter	*Categorization*	*Number of patients*
Age	≤50 years	35
	>50 years	15
Tumor size	<5 cm	14
	≥5 cm	36
LNR	≤0.25	13
	0.25-0.65	12
	≥0.65	25
Nottingham grade	1	13
	2	15
	3	22
NPI	≤3.40	3
	3.41-4.40	8
	4.41-5.40	5
	≥5.41	34
ER status	Positive	15
	Negative	35
PR status	Positive	14
	Negative	36
HER2/*neu* status	Positive	20
	Negative	30
LVI (IHC)	Positive	36
	Negative	14
LVI (IHC)	BV	25
	LV	9
	Both	2
	RA	14

LNR: Lymph Node Ratio; NPI: Nottingham Prognostic Index; PR: Progesterone receptor; ER: Estrogen receptor; LVI: Lymphovascular Invasion; IHC: Immunohistochemistry; BV: Blood Vessel; LV: Lymph Vessel; RA: Retraction Artefact

more commonly than lymph vessels invasion ($P = 0.007$). Our results are concordant with the studies conducted by Gurleyik *et al.*, 2007; Song *et al.*, 2011; and Mohammed *et al.*, 2011.[10,14,15]

In the present study, 48 out of 50 cases were found to have carcinomatous deposits in the LNs. The LN status was assessed as the LNR, which is defined as the ratio of number of LNs having carcinomatous deposits and the total number of LNs recovered on grossing. LNR is easy to calculate and has been found superior to the relatively simple count of number of positive peripheral LNs.[16] Based on LNR, patients were stratified into risk groups, and it was observed that 26% cases had LNR <0.25 (low risk), 24% cases had LNR between 0.25 and 0.65 (intermediate risk), and 50% cases had LNR more than 0.65 (high risk). Furthermore, LNR was positively correlated with tumor size ($P = 0.001$), Nottingham grade ($P = 0.001$), blood vessel invasion ($P = 0.001$), lymph vessel invasion ($P = 0.045$), and HER2/*neu* status ($P = 0.003$) and negatively correlated with age of the patient ($P = 0.001$), ER status ($P = 0.027$), and PR status ($P = 0.002$). Our results are reinforced by Schoppmann *et al.*, 2004; Gurleyik *et al.*, 2007; and Marinho *et al.*, 2008.[9,14,17]

Two cases in our study did not show metastatic deposits in the LNs but showed definite LVI, with the presence of tumor emboli in lymph vessels. Rakha *et al.*, 2011 elaborated on the significance of presence of definite LVI in LN-negative tumors and concluded that the prognostic value of these cases was equivalent to that provided by the presence of 1–3 positive LNs (pN1) if the tumor was LVI-negative.[11] Node-negative patients with LVI were also recommended to be candidates for neoadjuvant therapy.[18-20]

All the cases in our study belonged to the category of IDC – not otherwise specified. Hence, a correlation between histological type and LVI or other parameters could not be devised.

Nottingham combined histologic grade describes the microscopic growth pattern, as well as the cytologic features of differentiation of IDC breast. In the present study, majority of the cases presented with histological Grade 3 (44% of cases) followed by Grade 2 (30%) and Grade 1 (26%); and higher Nottingham grade was associated with younger age group ($P = 0.001$), larger tumor size ($P = 0.001$), ER/PR Hormonal receptor negativity ($P = 0.001$), HER2/*neu* receptor positivity ($P = 0.013$), high risk LNR ($P = 0.001$), blood vessel invasion ($P = 0.001$), and lymph vessel invasion ($P = 0.023$). Our results are compatible with the results of Song *et al.*, 2011; Rakha *et al.*, 2011; and Mohammed *et al.*, 2013.[11,15,21] High histological grade has been associated with decreased survival time in numerous studies.[22-24]

NPI encompasses three aspects of malignancy and was calculated using the formula: [Nottingham grade (1–3)] + [LN status (1–3, where 1 indicates negative; 2 indicates 1–3 positive LNs; 3 indicates ≥4 positive LNs)] + [tumor size in cm multiplied by 0.2]. Higher NPI in our study was seen to be associated with younger age ($P = 0.001$), negative ER/PR status ($P = 0.001$), positive HER2/*neu* status ($P = 0.024$), and blood vessel invasion ($P = 0.001$), as also observed by Rakha *et al.*, 2011.[11]

In our study, ER status was found to be positive in 30% cases and negative in 70% of the cases. Except for one case, which was ER+ PR−, PR status was found to run parallel to ER status and seen to be positive in 28% cases and negative in 72% cases. Cork *et al.*, 2012 attributed the ER+ PR− phenotype to the alternative splicing that may generate functional truncated PR variant proteins which are not detected by breast cancer screening using N-terminally targeted antibodies, leading to misclassification as PR negative.[25]

We observed ER and PR expression to be associated with younger age group ($P = 0.001$), larger tumors ($P = 0.05$), higher Nottingham grades ($P = 0.001$), intermediate and high risk LNR groups ($P = 0.05$), and tumors with definite LVI compared to retraction artifacts ($P = 0.001$). ER and PR status were found to be inversely related to HER2/*neu* status ($P = 0.012$ and $P = 0.021$ respectively). Our results are compatible with the results of Azizun-Nisa *et al.*, 2008; Mohammed *et al.*, 2013; and Krishnamurthy and Kumar 2016.[21,26,27]

Although we were not able to evaluate the status of ER/PR receptors in the positive LNs, it is recommended to be done routinely, to ensure that patients are receiving the most effective treatment at all times.[28]

Table 2: Interrelationship between numerous prognostic markers of carcinoma breast

Prognostic Marker of CA Breast	*Tumor size*	*LNR*	*Nottingham grade*	*NPI*	*ER status*	*PR status*	*HER2/neu status*	*LVI*
Age	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.120	0.032
Tumor size		<0.001	<0.001	NA	0.047	0.015	0.662	0.007
LNR			<0.001	NA	0.027	0.002	0.003	<0.001
Nottingham grade				NA	<0.001	<0.001	0.013	<0.001
NPI					<0.001	<0.001	0.024	0.001
ER status						ND	0.012	<0.001
PR status							0.021	<0.001
HER2/*neu* status								0.021

LNR: Lymph node ratio; NPI: Nottingham Prognostic Index; PR: Progesterone receptor; ER: Estrogen receptor; LVI: Lymphovascular Invasion; NA: Not Applicable; ND: Not Done

In the present study, HER2/*neu* receptor status was found to be positive in 44% cases and negative in 56% cases. Negative HER2/*neu* status was found to be seen in majority of the patients in the older age group and in tumors with size <5 cm. However, these results were not sufficient to achieve a significant statistical correlation. However, we found statistically significant results between negative HER2/*neu* status and lower Nottingham grade ($P = 0.013$), low risk LNR ($P = 0.003$), in patients with better NPI ($P = 0.024$), in patients with positive ER status ($P = 0.012$), and in patients with positive PR status ($P = 0.021$). Positive HER2/*neu* status was seen in tumors with definite LVI ($P = 0.021$). Our results are concordant with Krishnamurthy and Kumar 2016.[27]

Interrelationships among numerous variables as described above have been summarized in Table 2.

Manual counting of angiogenic hotspots showed that both LMVD and BMVD in the peritumoral zones were higher compared to the intratumoral zones. The presence of LVI was positively correlated with peritumoral BMVD ($P = 0.001$), intratumoral BMVD ($P = 0.016$), and intratumoral LMVD ($P = 0.009$). Our results are concordant with Schoppmann *et al.*, 2004 and El-Gohary *et al.*, 2008.[9,29] However, there was no significant correlation between peritumoral LMVD and presence of LVI in our study.

The results of the present study [summarized in Table 3] showed that LVI is associated with established indicators of tumor prognosis such as young age at diagnosis ($P = 0.032$), larger tumors ($P = 0.007$), higher Nottingham grades ($P = 0.001$), intermediate and high risk LNRs ($P = 0.001$), higher NPI ($P = 0.001$), negative ER/PR status ($P = 0.001$), and positive HER2/*neu* status ($P = 0.021$). Our results are concordant with numerous studies conducted previously, including Schoppmann *et al.*, 2004; Gurleyik *et al.*, 2007; Marinho *et al.*, 2008; Mohammed *et al.*, 2010; Rakha *et al.*, 2011; Song *et al.*, 2011; Panagiotopoulos *et al.*, 2016; and Krishnamurthy and Kumar 2016.[9-11,14,15,17,27,30]

Table 3: Correlation of lymphovascular invasion with other variables

Parameter	*Categorization*	*Number of patients*	*P*
Age	≤50 years	31	0.001
	>50 years	5	
Tumor size	<5 cm	4	0.007
	≥5 cm	32	
LNR	≤0.25	3	0.001
	0.25-0.65	10	
	≥0.65	23	
Nottingham grade	1	3	0.001
	2	13	
	3	20	
NPI	≤3.40	2	0.001
	3.41-4.40	1	
	4.41-5.40	3	
	≥5.40	30	
ER status	Positive	5	<0.001
	Negative	31	
PR status	Positive	4	<0.001
	Negative	32	
HER2/*neu* status	Positive	18	0.021
	Negative	18	
Intratumoral LMVD	NA	NA	0.009
Peritumoral LMVD	NA	NA	0.474
Intratumoral BMVD	NA	NA	0.016
Peritumoral BMVD	NA	NA	0.001

LMVD: Lymphatic microvessel density; BMVD: Blood microvessel density; NA: Not available; LNR: Lymph node ratio; NPI: Nottingham Prognostic Index; PR: Progesterone receptor; ER: Estrogen receptor

CONCLUSIONS

The present study successfully establishes that IHC is essential to evaluate LVI as a diagnostic pitfall of this entity is the presence of retraction artifacts. This is the first study to comprehensively evaluate LVI and assess its correlation with multiple prognostic parameters. We observed that LVI is associated with other markers of poor prognosis and can predict the tumor progression.

D2-40 proved to be an extremely useful marker for identification of lymph vessel invasion, peritumoral, and intratumoral lymphangiogenesis and was well complemented by CD34 as an endothelial marker to distinguish blood vessels confidently.

We recommend and support the inclusion of LVI as a marker of poor prognosis and confirmation and distinction with IHC, wherever the resources permit so.

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Conflicts of interest

There are no conflicts of interest.

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