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Evaluation of Lympho-vascular Invasion in Breast Carcinoma Using Lymphatic Endothelial Marker (D2-40)

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Breast cancer has become the most frequently diagnosed cancer and the leading cause of cancer-related death among women worldwide, overtaking lung cancer. The Present study attempts to investigate the use of D2 -40 for the detection of lympho- vascular invasion in node negative breast cancer. Additionally the lympho-vascular invasion using D2 -40 and its association with distant metastasis and overall prognosis of the patient was also explored.

Keywords: Vascular invasion; microscopy; LVD; D2-40; prognosis; adenocarcinomas and tumours.

1. INTRODUCTION

The malignant tumour that has developed in the cells of the breast is called breast carcinoma or breast cancer. Breast carcinoma has been extensively studied and with the availability of vast evidence based data [1] and literature, various treatment modalities have been introduced to cure this disease. Breast cancer has become the most frequently diagnosed cancer and the leading cause of cancer-related death among women worldwide, overtaking lung cancer. Although incidence rates are higher in the West, the highest burden for breast cancer is

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in the developing countries [2]. Majority of the breast carcinomas are usually asymptomatic and the usual mode of presentation is an incidental palpable lump or pain and rarely, they present with nipple discharge and skin changes [3]. Breast carcinomas have varying levels of invasion and aggressiveness irrespective of the duration of presentation. Mammary glands, also known as breasts are modified sweat glands [4-6]. In utero, breast development starts in the first trimester of gestation with multiple bilateral thickenings of the ectoderm on the ventral aspect of the fetus [5-10]. This thickened ridge extends in a linear fashion from the axilla to the groin, forming the milk line [11]. The breasts begin to develop at puberty. This development is stimulated by the estrogen- both mammary glands plus the deposition of fat. During pregnancy, large quantities of estrogen is secreted by the placenta which cause the ductal system of the breasts to grow and branch [12].

For the breasts to develop into milk - secreting organs also requires progesterone. Once the ductal system has developed, progesterone starts acting synergistically with estrogen causing additional growth of the breast lobules along with budding of alveoli and development of secretory characteristics in the cells of the alveoli [13].They are a group of malignant epithelial tumours which are characterized by invasion of adjacent tissues and have an increased tendency to metastasize to various sites [14]. The majority of them are adenocarcinomas. They exhibit a wide range of morphological and specific histopathological subtypes and most of them have specific prognostic or clinical characteristics [15]. Breast cancer in women is a major health burden globally. There are about 2.1 million newly diagnosed female breast cancer cases in 2018, accounting for almost 1 in 4 cancer cases among women. The incidence rates are highest in Australia/New Zealand, Northern Europe, Western Europe with Belgium being the highest in global rates, and the Netherlands, and France, Southern Europe (Italy), and Northern America [16]. Myoepithelial cells appear to arise from basal cells between 23-28 weeks of gestation and they play an important role in the morphogenesis of the mammary gland through the synthesis of basement membrane constituents such as laminin, type IV collagen, and fibronectin, as well as metalloproteinases and growth factors. In the last 2 months of gestation, the epithelial columns branch, canalize, and transform into ducts (and eventually into lobules). A ‘pit’ in the epidermis forms at the convergence of the major lactiferous ducts, and thereafter, its eversion forms the protuberant nipple [11].

Even though extensive screening programs and clinical tests are available for early detection of this disease, there is higher morbidity and mortality rate of breast cancer in developing countries due to increased socioeconomic dependence and delayed diagnosis [4]. There is a lack of self- awareness of this life threatening malignancy in our country.

Triple assessment including clinical examination, imaging (mammography and ultrasound) and tissue sampling by either fine needle aspiration cytology or needle core biopsy helps in evaluating breast abnormalities [17]. A systematic clinical examination should be done taking into account the nature of the lump and, if present, any skin dimpling or change in contour of the breast and also assessment of the axilla is very important 4.

Lymph node metastasis is considered as one of the most important prognostic factors for breast carcinomas [3]. So, the recognition of lymphatic vessel invasion in histopathological sections is crucial. Histopathological correlation along with analysis by immunohistochemistry (IHC) has instigated various modalities of treatment. The standard norm of management for breast cancer includes surgery, chemotherapy and radiotherapy depending on the various parameters [5]. The breasts are supplied by branches of the axillary artery, the internal thoracic artery and some intercostal arteries. The second perforating artery, a branch of anterior intercostal artery is usually the largest, and supplies the upper region of the breast, nipple, areola and adjacent breast tissue [11,12].

Interest in D2-40 first instigated its use as a lymphatic endothelial marker, since it does not stain vascular endothelium. As such, D2 -40 immuno-stains have been used as lymphatic endothelial marker to study lymph-angiogenesis in breast carcinoma [18] and has been proved to be helpful in determining lymphatic invasion by the tumour [19].

2. MATERIALS AND METHODS

2.1 Source of Data

The present study was undertaken in the

Department of Pathology, Sree Balaji Medical College & Hospital, Chennai.

2.2 Type of Study

Cross sectional study.

2.2.1 Inclusion criteria

Only histopathologically confirmed cases of invasive carcinoma of breast will be included in the study

2.2.2 Exclusion criteria

- Benign and inflammatory lesions of the breast will be excluded.
- Patient who has received adjuvant chemotherapy will be excluded from the study

2.3 Statistical Methods

1. Descriptive statistics
2. Contingency table analysis using SPSS for Windows (version 24.0)
3. P <0.005 is considered as statistically significant.

2.4 Method of Collection of Data

Purposive sampling technique (26):

All mastectomy specimens of invasive breast carcinoma were studied noting the clinical details. The formalin fixed tissue were subjected to routine processing and sections stained with Haematoxylin & Eosin (H&E) studied extensively for all the prognostic factors.

The grading of breast carcinoma was done according to the Nottingham combined histologic grade (Elston Ellis modification of Scarff Bloom Richardson grading system) [20].

TNM staging was done according to AJCC classification (8th edition) [21].

Nottingham's Prognostic index (NPI) was calculated by the formula,
NPI = [0.2 x S] + N + G, where,

- S is the size of the index lesion in centimetres,
- N is the node status: 0 nodes = 1, 1 -4 nodes = 2, >4 nodes = 3,
- G is the grade of tumour: Grade I =1, Grade II =2, Grade III =3.

And patients were divided into three NPI groups [22].

Immunohistochemistry:

Sections from each block was subjected to IHC staining with D2-40 (Monoclonal mouse antihuman antibody, clone PM231, PathnSitu).

2.4.1 Procedure

- 3-4 µm thick sections from each block were placed on Poly- L-Lysine coated slides and air dried.
- The slides were baked at 60°C for 1 hour in hot air oven.
- Slides were deparaffinised and rehydrated.
- Trisodium citrate buffer was prepared - 1000ml, pH -6.0
- Trisodium citrate buffer was poured into the pressure cooker with slide carrier.
- Sections were left in the Tris buffer for about 7 -8 minutes or 2 whistles.
- Slides were then washed in tap water for 3 -5minutes followed by distilled water.
- Endogenous peroxidase was quenched by dipping slides into a fresh aqueous solution of 3% peroxide for 15 minutes
- Slides were then washed in tap water for 3 -5 minutes followed by distilled water.
- Then slides were rinsed with Tris buffer for each 5 minutes, three changes.

Detection of Antigens in Paraffin sections

- The primary antibody was added at a dilution selected usually for half an hour to 1hour.
- Sections were soaked in Tris buffer for 10 minutes (2x5 minute washes)
- Secondary antibody was then added and left for half an hour.
- Sections were again soaked in Tris buffer for 10 minutes (2x5 minute washes)
- A solution of chromogen, 3,3'-diaminobenzidine (DAB) at1mg/ml was

made in Tris and added.

- The sections were washed in tap water.
- The sections were washed with wash buffer and counterstained with haematoxylin and again rinsed in water for 5 minute.
- The slides are air dried and mounted.

General

- Absence of LVI in the context of proven lymph node metastasis may be due to sampling error.
- The prognostic significance of LVI, generally, is dependent on the tumour grade and lymph node status.
- Generally, LVI = Poor Prognosis

2.5 Lymphatic Vessel Density Assessment

Determination of LVD was performed according to Weidner et al. [23]. The immuno-stained sections were scanned by light-microscopy at low magnification (x40 & x100) and the areas of tissue with the greatest number of distinctly highlighted lymph vessels ('hot spots') were selected. LVD was then assessed by counting all immuno-stained vessels at a total magnification of x400 from three areas for each case.

Table 1. D2-40 stained lymphaticmicrovessel density score

Score	Range
0	0
1 (Mild)	>5
2 (Moderate)	5-6
3 (Increased)	>6

The mean number of lymph vessels in each case was counted. Both the scoring and counts were performed blindly without any prior clinical knowledge of the patients. LVI was considered evident if at least one tumour cell cluster could be clearly visualised inside the D2-40 positive lymphatic space.The LMD ranged from 0 -16 micro-vessels/hpf and the mean was calculated as 5.4. Anything above it was considered as increased microvessel density.

Rosen criteria of detecting LVI microscopically (in the breast) [24]:

- LVI should be outside tumour proper and usually very close -within 0.1 cm.
- Tumour nest countour should differ from the vascular countour - DCIS with retraction artefact may simulate LVI as it has a contour that matches its surrounding fibrous tissue.

3. RESULTS

This study was undertaken from September 2016 to October 2018 in the Department of Pathology, Sree Balaji Medical College and Hospital, Chennai. A total number of 30 breast carcinoma cases were evaluated.

3.1 Age at the Onset of Disease

Of 30 cases, the maximum number of cases 16/30 (53.3%) was in the age group of in the age group 30 to 40 years, followed by 6 cases (20%) in the age group 61 -70 years and 4 cases (13.3%) each in the age group 41 to 50 and 51 to 60 years. The youngest patient in our study was 36 years old and the eldest was 70 years old.

3.2 Site of Involvement

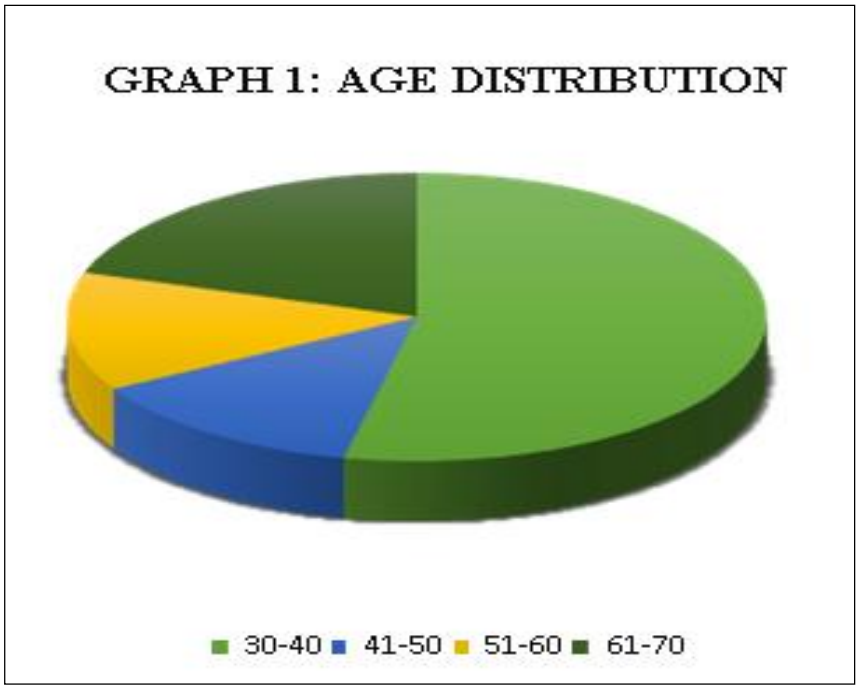
Of 30 cases, majority of them were located in upper outer quadrant (30%), followed by upper inner quadrant (26.7%), lower inner quadrant (20%), and lower outer quadrant (16.7%), one central and diffuse (3.3%) in the order of decreasing frequency.

3.3 Side of Involvement

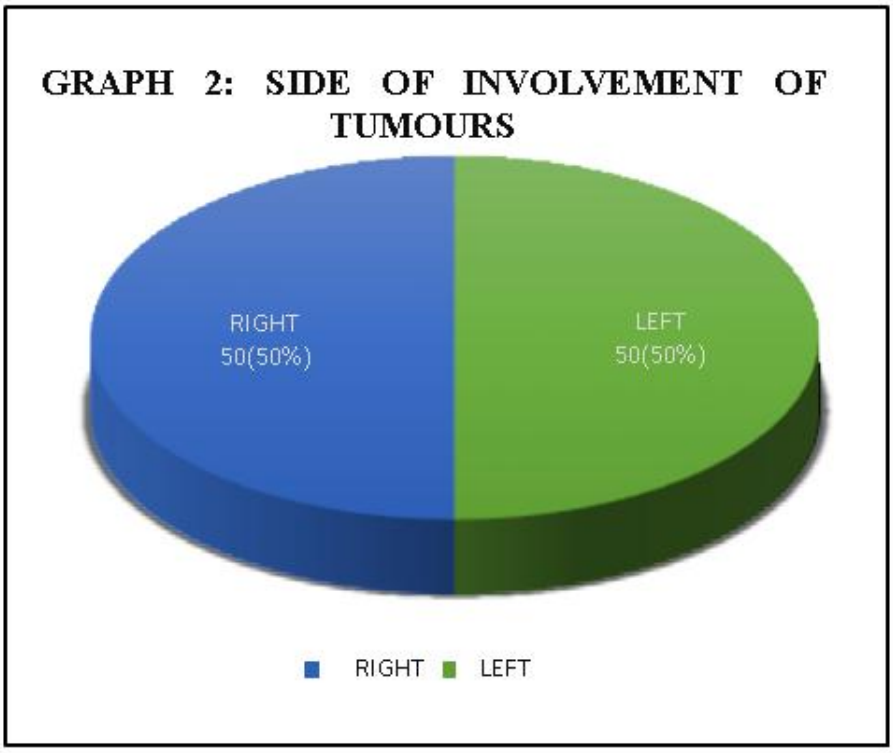
Out of 30 cases, 50% had tumour on left side and 50% had tumour on right side.

3.4 Histological Grade of the Tumour

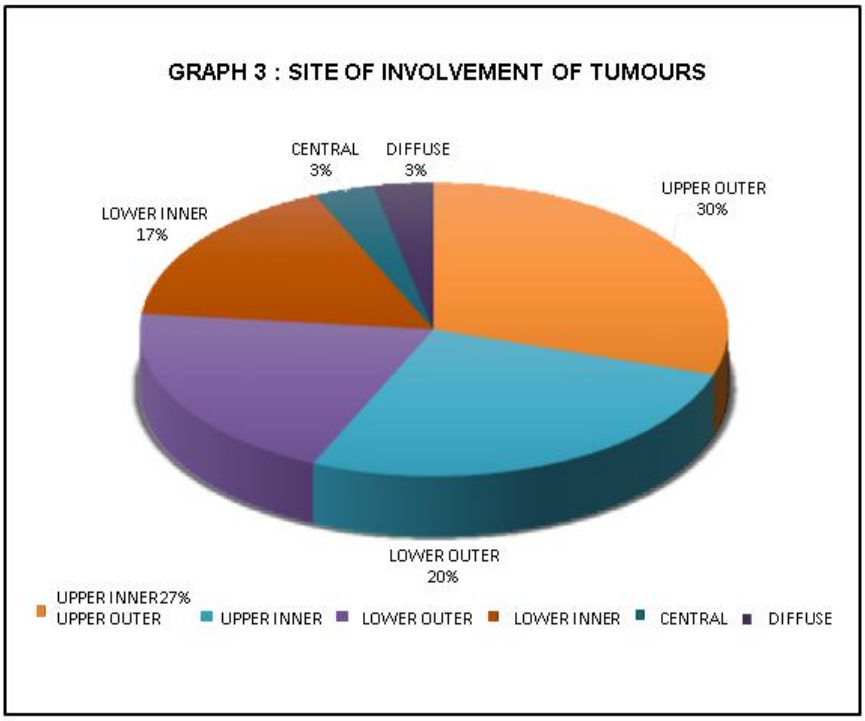
30 cases of mastectomy specimens when graded based on Nottingham modification of the Scarff-Bloom-Richardson grading system, and it showed a predominance of grade 3 tumours (66.6%) compared to the grade 2 tumours (26.7%) followed by grade 1 tumours (6.7%).



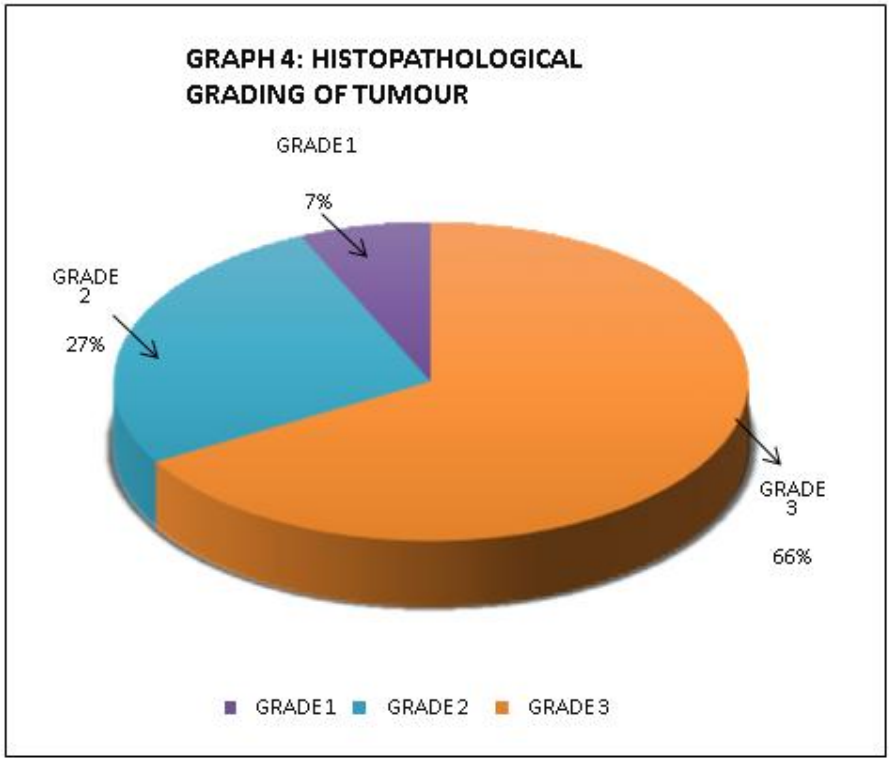
Graph 1. Age distribution



Graph 2. Side of involvement of tumours



Graph 3. Site of involvement of tumours



Graph 4. Histopathological grading of tumour

3.5 Presence of Lympho-vascular Invasion

Lympho-vascular invasion (Fig. 1) was observed in 62.9% of cases of the 30 cases. Out of 30 cases, 23 patients (76.7%) had lymph node metastasis of which 33.3% were detected by H & E and 66.7% were detected by D2 -40.

3.6 Associated Morphological Parameters

Lymphocytic infiltration (Fig. 2) was observed in 62.9% of cases while necrosis (Fig. 3) was seen in 58.1% of cases respectively.

3.7 TNM Staging of the Tumour

Staging of the tumours was done using AJCC system of classification based on tumour (T), node (N) and Metastasis (M).

Majority of the cases 21/30 (70%) were of pT2 with tumours being more than 2cm but less than 5cm in their greatest dimension. There were 8/30 (26.7%) of pT3 (tumour more than 5 cm in greatest dimension) and 1/30 (3.3%) of pT1 (tumours being < 2cm in their greatest dimension). There were no cases of pT4 (tumour of any size with direct extension to skin/chest wall).

100% cases having tumour size of 5cm were poorly differentiated tumours (Grade 3), 66.7% cases having tumour size of 2-5 cm were Grade 3 and 25% cases with tumour size 2 - 5cm were of Grade 2. There was positive correlation between increased tumour size and higher histological grade, though not statistically significant.

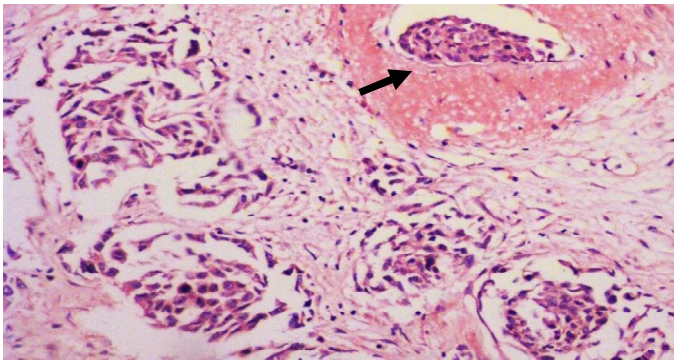
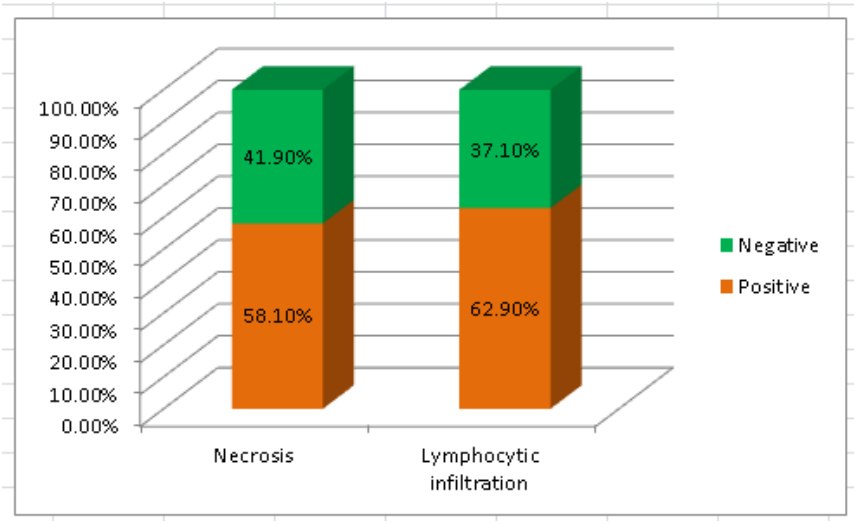


Fig. 1. Lympho-vascular invasion seen within both lymphatic and blood vessels (H&E, x100) [25]



Graph 5. Necrosis and Lymphocytic Infiltration

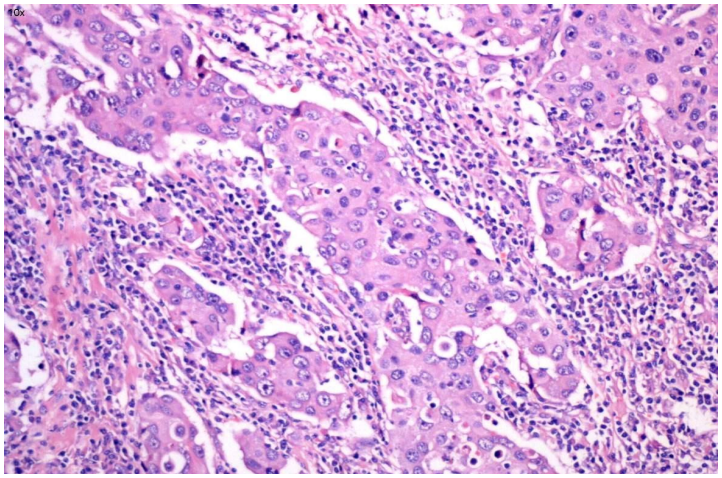


Fig. 2. Stromal reaction – section shows IBC with stroma showing extensive infiltration by lymphocytes (H&E, x100) [25]

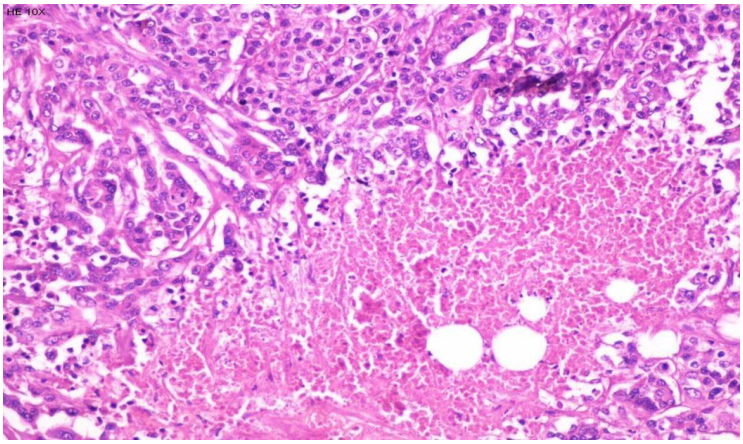


Fig. 3. Tumour necrosis – Section shows IBC with wide area of necrosis (H&E, x100) [25]

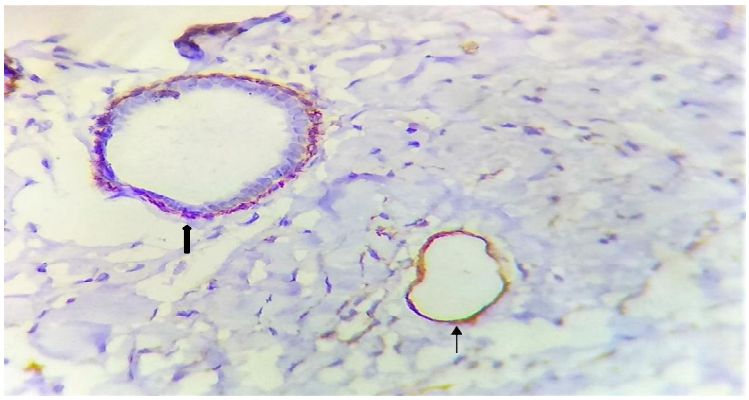
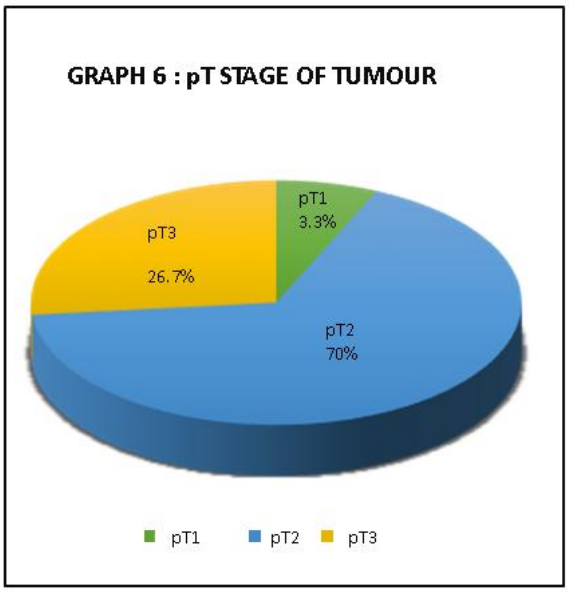


Fig. 4. D2-40 staining both lymphatic vessels (thin arrow) and myoepithelial cells (bold arrow) (x400)



Graph 6. pT stage of tumour

There was no lymph node involvement (N0) in 10/30 (33.3%) of cases, 7/30 (23.3%) of cases showed metastasis in 1-3 lymph nodes (N1), 6/30 (20%) of cases showed metastasis in 4-9 axillary lymph nodes (N2) and 7/30 (23.4%) of cases showed metastasis in ≥10 axillary lymph nodes (N3).

85.8% and 100% of cases showing increased number of lymph node metastasis i.e., N2 and N3, respectively were poorly differentiated tumours (Grade 3).

There was a significant association between tumours with increased number lymph node metastasis and higher histological grade.

3.8 Nottingham's Prognostic Index Groups

Patients were divided into three NPI groups based on Nottingham's Prognostic index (NPI) calculated by the formula,

NPI = [0.2 x S] + N + G, where,

S is the size of the index lesion in centimeters, N is the node status:1=0 nodes

2= 1-4 nodes 3= > 4 nodes

G is the grade of tumour: Grade I =1, Grade II =2, Grade III =3.

Majority of cases were coming under Poor Prognostic Group – PPG (63.3%), followed by Moderate Prognostic Group - MPG (30%), Good Prognostic Group- GPG (6.7%) [25].

3.8.1 D2-40 stained LVI and H & E stained LVI correlation

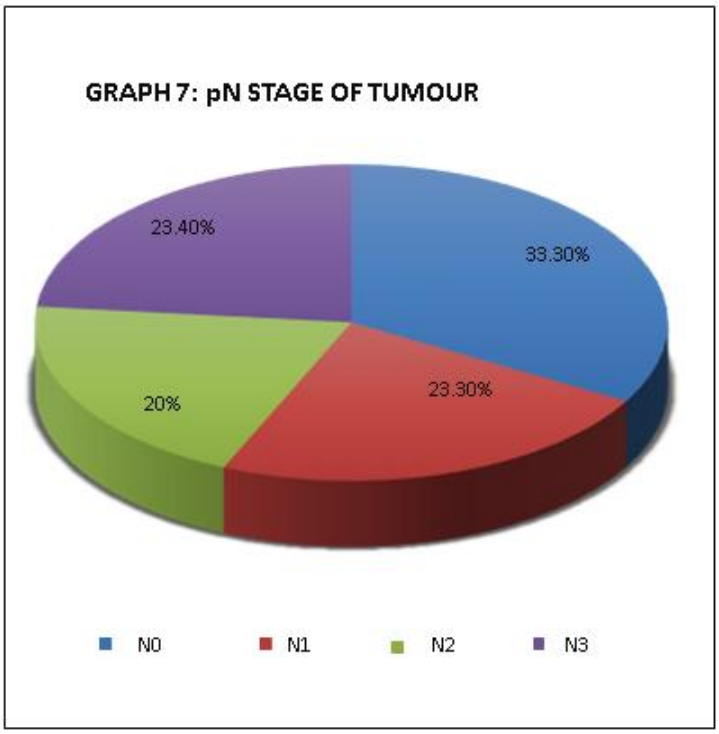
Out of 30 cases, 20 (66.6%) cases showed lympho- vascular invasion stained by D2 -40 whereas H&E stained only 10 cases (33.3%).

3.8.2 D2-40 Stained LVI with age

Out of 30 cases, 14 cases (46.7%) were > 40 years and 16 cases (53.3%) were ≤ 40 years. LVI was seen in 20 cases of which 7 cases (35%) were > 40 years and 13 cases (65%) were < 40 years.

3.8.3 D2-40 stained LVI with histo-pathological grade

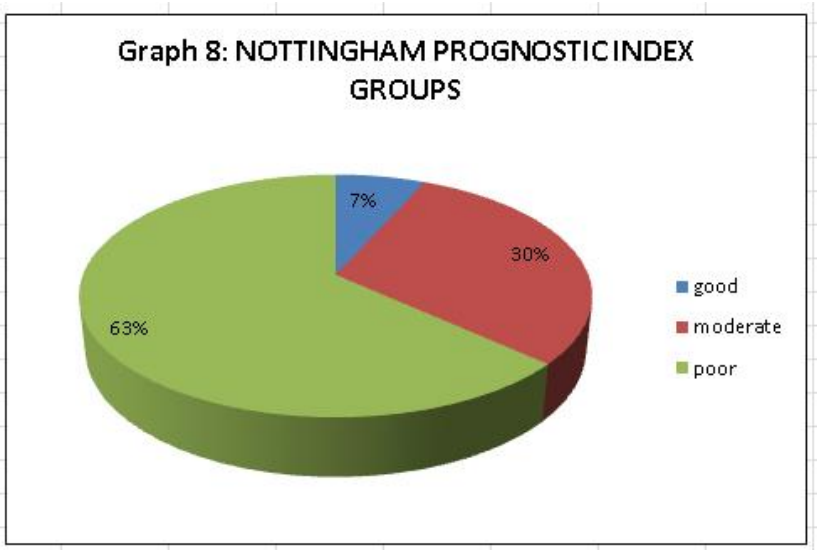
LVI was seen in 20 cases (66.7%), out of which D2 -40 stained LVI was seen in 2 cases (33.3%) of grade 2 tumours and 18 cases (81.8%) of grade 3 tumours, whereas 100% of grade 1 tumours were D2 -40 stained LVI negative. There was significant association of D2 -40 expression with higher grade tumours.



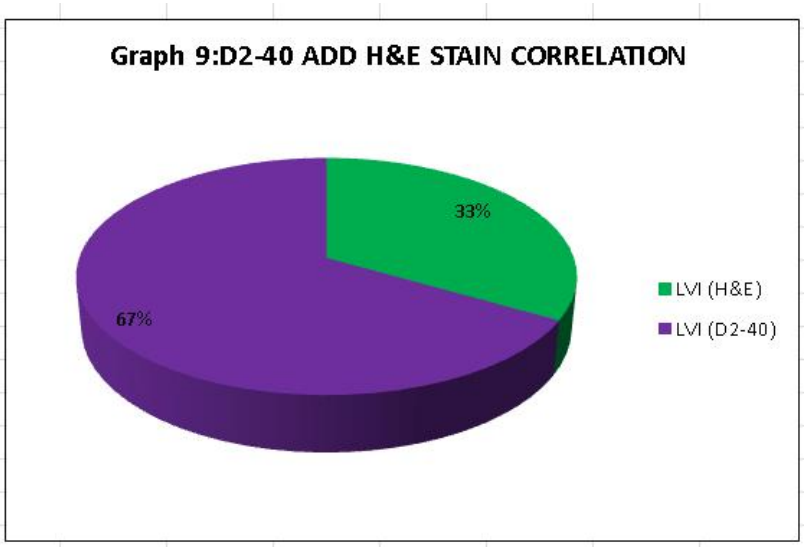
Graph 7. pN stage of tumour

Table 2. Nottingham prognostic index scoring[114]

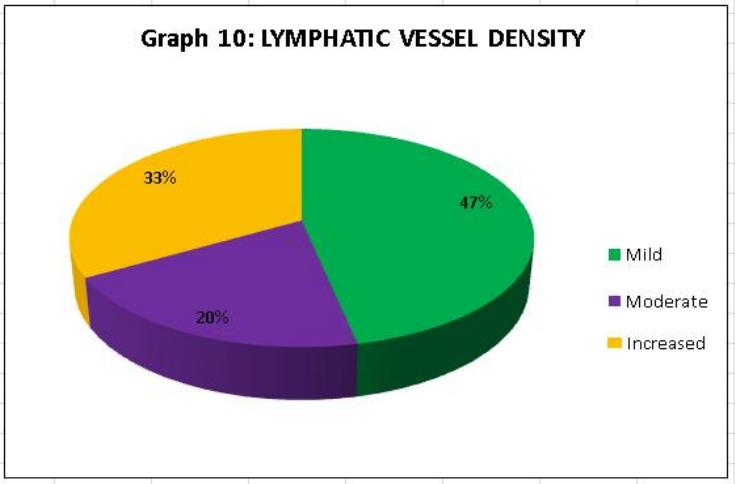
Score	5-year survival	Prognosis
<3.4	85%	Good
3.4 to 5.4	70%	Moderate
>5.4	50%	Poor



Graph 8. Nottingham prognostic index groups



Graph 9. D2-40 add H and E stain correlation



Graph 10. Lympathatic vessel density

3.8.4 D2-40 stained LVI associated with tumour size

All tumours of size >5 cm showed D2 -40 stained LVI (100%) and 14 cases (58.3%) of tumours of size 2 -5 cm also showed LVI, while 10 cases showed no LVI. There was significant association between D2 -40 expression and greater size of tumour.

3.8.5 D2-40 stained LVI associated with lymph node metastasis

LVI was seen in 30% (3/10) of tumours at N0 stage, 71.5% (5/7) of tumours at N1 stage, 83.3% (5/6) at N2 and 100% (7/7) at N3 stage. Out of 30 cases, lymph node metastasis was seen in 23 cases (76.7%) and LVI was seen in 20 cases (66.7%). Tumours with increased number of lymph node metastasis showed increased LVI.

4. DISCUSSION

Breast carcinoma is known since ages and first documented in history by the Egyptians in 3000 BC 116. Presently, it is considered as one of the commonest cause of cancer deaths in women [26].

One of the main causes of cancer death is distant metastasis, understanding the

mechanism of tumour metastasis is important. Metastasis can be explained by the metastatic cascade- , where the tumour cells undergo a process known as tumour cell dissociation. They separated from the primary and invade the extracellular matrix and enter the lymphatic/ vascular stream for transport to distant organs- a process known as intravasation. If tumour cells reach other organs and proliferate, it is known as extravasation [27,28]. Based on this theory, if the presence of cancer cells within the lymphatic channel can be predicted, the probability of metastasis could also be predicted.

As mentioned earlier, LVI was detected in the past using H&E stain in cases in which BVI could not be distinguished. However, new markers have been discovered along with advances in immune-histochemical technique. Using D2 -40 as a marker, several studies have concluded that LVI is a valuable prognostic factor in breast cancer [29,30].

4.1 Age Distribution

In the present study, the age range of the patients with invasive breast carcinoma varied from 36 years to 70 years with a mean age of 48.1 years. LVI was seen in 20 cases of which 7 cases (35%) were > 40 years and 13 cases (65%) were < 40 years.

Various studies have demonstrated that younger age is a risk factor for LVI and axillary lymph node metastasis [31,32]. This has been attributed to biologically more aggressive tumours in this younger age group [33].

Table 3. Correlation of age with D2-40 stained LVI

Sl. No.	Study	Age distribution	Significance
1.	Gill PG et al. [31]	40-49 years 50- 59 years 60-69 years >70 years	< 0.001
2.	Liu YL et al.[34]	Mean age- 52±1.6	0.72
3.	Jung Ah Lee et al.[35]	< 40 years > 40 years	0.038
4.	Tezuka K et al.[36]	<50 years >50 years	0.037
5.	Present Study	≤40 years > 40 years	0.73

Table 4. Correlation of tumour size with D2-40 stained LVI

Sl. No.	Study	Size of tumour	ALNM (LVI+)	Significance
1.	Rivadeneira DE et al. [33]	T1a T1b	19.4% 80.6%	0.01
2.	Liu YL et al. [34]	≤ 5 cm > 5cm	45% 55%	0.53
3.	Jung Ah Lee et al. [35]	≤ 2 cm > 2 cm	7.3% 12.8%	0.415
4.	Krishnamurthy J et al. [37]	≤ 2 cm 2-5 cm > 5 cm	60% 61.3% 72.2%	0.069
5.	Tezuka K et al [36]	≤ 2 cm >2 cm	38.6% 62.5%	NS
6.	Present Study	2-5 cm > 5 cm	58.3% 100%	0.090

4.2 Correlation of Tumour Size with D2 -40 Expression Andcomparison with Other Studies

Various studies have shown that the size of tumour is one of the most significant prognostic factors in breast carcinoma and there is increased incidence of axillary lymph node metastasis and decreased survival with increasing size of the tumour.

In our study, tumour size varied from 2 cm to 8 cm with a mean of 3.88 cm. Maximum number of tumours were in the range of 2cm to 5 cm (T2) (66.7%) followed by tumours of size >5 cm (T3) (26.6%) and of <2 cm (T1) (6.7%).

Studies have confirmed that LVI is significantly lower in tumours ≤2 cm than that in tumours>2 cm. These studies were in concordance with our findings.

4.3 Correlation of Histological Tumour Grade with LVI and Comparison with Other Studies

Histologic grading has become widely accepted as a powerful indicator of prognosis in breast carcinoma and its importance has been validated by multiple independent studies. High grade and fast growing tumour may produce more growth factors and offer a bigger clonal variety of tumour cells capable of invading lymphatic vessels compared with low grade and slow growing tumour [38].

In the present study, there is a significant association of LVI in grade 3 tumours (81.8%) when compared to grade 2 tumours (33.3%). This was in concordance with these following studies.

4.4 Correlation of Lymph Node Status with Lympho-vascular Invasion

In our study, D2-40 expression correlated significantly with node positive tumours. Axillary lymph nodes were positive for metastasis in 66.7% of cases. In cases with higher lymph node metastasis, i.e. N2 and N3 had increased LVI and LMVD stained by D2 -40 in 83.3% and 100% of cases, respectively.

Rakha EA et al. [26] reported that presence of LVI in LN negative tumours should be considered prognostically equivalent to those cases with 1–3 positive LNs (pN1) and should recommended to be candidates for neoadjuvant therapy.

Studies mentioned below observed a similar trend with a significant association between LVI stained by D2 -40 and axillary lymph node involvement.

4.5 Correlation of Lymphatic Microvessel Density with Lympho-vascular Invasion

An increased lymphangiogenesis is found to be associated with increased LN positivity and hence, increased lympho-vascular invasion. Increase in lymph mean vessel density significantly increases the potential

Table 5. Correlation of tumour grade with D2-40 stained LVI

Sl No.	Study (Cases)	Tumour grade	LVI + cases	Significance
1.	Gill PG et al. [37]	1	17.3%	< 0.001
		2	26.0%	
		3	34.5%	
2.	Gujam et al. [39]	1	20.8%	< 0.001
		2/3	29.5%	
3.	Krishnamurthy J et al. [37]	1	2.8%	Nil
		2	46.5%	
		3	50.7%	
4.	Jung Ah Lee et al. [35]	1	6.7%	0.022
		2	0%	
		3	20%	
5.	Present Study	1	0%	0.010
		2	33.3%	
		3	81.8%	

Table 6. Correlation OF D2 -40 stained LVI with axillary lymph node metastasis

Sl No.	Study (Cases)	ALNM	LVI + cases	P value
1.	Gujam FJ et al. [39]	154 cases	61 cases (39.6%)	< 0.001
2.	Lee JA et al. [35]	39 cases	7 cases (17.9%)	0.022
3.	Tezuka K et al. [36]	69 cases	34 cases (49.3%)	0.046
4.	Rivadeneira DE et al. [33]	165 cases	28 cases (16.9%)	<0.001
5.	Krishnamurthy J et al. [37]	53 cases	29 cases (34.1%)	0.016
6.	Agarwal et al. [40]	48 cases	10 cases (20%)	< 0.001
7.	Atiken et al. [32]	168 cases	58 cases	< 0.001
8.	Present Study	23 cases	20 cases	0.015

Table 7. Correlation of increased LMVD with LVI

Sl No.	Study	Cases	Mean LMVD (micro-vessels/field ± SD)	LVI	Significance
1.	Yasushi Nakamura et al. [43]	113	13.63 ± 7.82	57 (50.5%)	<0.001
2.	Mumtaz A. Ansari et al. [44]	35	22.85 ± 13.29	13 (37.1%)	= 0.001
3.	Sebastian F. Schoppmann et al. [45]	374	12 ± 4.2	105 (28.07%)	= 0.001
4.	Present Study	30	5.4	20 (66.7%)	0.004

for a tumour cell to invade lymphatic vessels and subsequent LN positivity [41]. In contrast to blood vessels, lymphatic vessels also sustain the spread of metastatic cells but not tumour cell proliferation and expansion of the tumour mass. There was a significant correlation of increased LVD (>5.4) with increased LVI and axillary lymph node metastasis in our study. This is in concordance with our study. Furthermore, Krishnamurthy et al. [37], Jun Wang et al. [42] also stated that increased LVD associated with the poor prognostic indicators like larger tumour size, higher grade, lymph node metastasis.

5. CONCLUSION

A total of 30 cases of invasive breast carcinomas were included in the study. Lympho-vascular invasion was analysed by D2-40 immuno-stain and was correlated with other prognostic factors. A significant trend was noted with D2 -40 stained lymphovascular invasion and poor prognostic factors including larger tumour size, lymph node metastasis, and higher histological grade. Vascular drainage upsurges the chance of visceral metastasis resulting in the circulating tumour cells having a greater potential of distal implantation. The association between lympho-vascular invasion and the distant metastasis signify that early detection of LVI can be used as an independent poor prognostic marker in breast carcinoma patients with lymph node-negativity. Also, this study reinforces the significance of pathologic analysis in the prognostic evaluation of breast cancer, especially those factors that cannot be analysed on molecular level, such as size, nodal status, and LVI positivity. Presence of LVI was consistently associated with reduced disease-free interval (DFS) and overall survival (OS), regardless of tumour and treatment characteristics in the high-risk group.In addition, moderate to marked LVI has to be an indicator of postoperative irradiation after conservative breast surgery in node negative patients.

D2-40 has been proven to be a valuable marker in identifying lympho-vascular invasion in various studies. So, we propose the consistent use of D2 -40 immunostain while evaluating the lymphatic invasion by tumour cells for further follow-up and overall prognosis. The effect of neoadjuvant therapy on LVI is out of the concept of this study, and in order to have a uniform patient group, we excluded the patients who had undergone neoadjuvant therapy.

CONSENT AND ETHICAL APPROVAL

As per international standard or university standard guideline patients consent and ethical approval has been collected and preserved by the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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