"IDENTIFICATION OF LYMPHATIC INVASIONIN BREAST CARCINOMA PATIENTS USINGIMMUNO-MARKER D2-40–A ONE YEAR CROSS SECTIONAL STUDY INA TERTIARY CARE CENTRE OF BELAGAVI."

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LIST OF ABBREVIATIONS USED

BC	-	Breast carcinoma
DCIS	-	Ductal Carcinoma in-situ.
H&E	-	Haematoxylin and eosin
IDC	-	Infiltrating ductal carcinoma
IHC	-	Immunohistochemistry
LE	-	Lymphatic endothelium
LEC	-	Lymphatic endothelial cell
LI	-	Lymphatic invasion
LN	-	Lymph node
LNM	-	Lymph node metastasis.
LVI	-	Lymphovascular invasion
MEC	-	Myoepithelial cell.
NPI	-	Nottingham Prognostic Index
RBC	-	Red blood cell
SBR	-	Modified Scarff-Bloom-Richardson
TDLU	-	Terminal duct lobular unit
TNBC	-	Triple negativebreast cancer
WHO	-	World Health Organisation

ABSTRACT

"IDENTIFICATION OF LYMPHATIC INVASION IN BREAST CARCINOMA PATIENTS USING IMMUNO-MARKER D2-40–A ONE YEAR CROSS SECTIONAL STUDY IN A TERTIARY CARE CENTRE OF BELAGAVI."

BACKGROUND: Metastasis is the leading cause of mortality in patients of breast cancer and lymphatic invasion is an independent predictor of lymph node metastasis. The present study aims at analysing D2-40 as a specific lymphatic endothelial marker for identification of lymphatic invasion in comparison to routine Haematoxylin & Eosin stain.

OBJECTIVES: 1.To assess lymphatic invasion in breast carcinoma patients using D2-40 immunohistochemical marker. 2.To correlate lymphatic invasion with the lymph node status.

METHODOLOGY: Mastectomy specimens of 35 breast carcinoma patients received in the Department of Pathology, Jawaharlal Nehru Medical College, Belagavi, were stained with Haematoxylin and Eosin and D2-40. Each case was evaluated for presence of lymphatic invasion.

RESULTS: Increased frequency of lymphatic invasion detection was observed with the use of D2-40 (68.57%) when compared to H&E (25.71%). Frequency of nodal metastasis was found to be higher (87.5%) in D2-40 positive lymphatic invasion group compared to those without D2-40 lymphatic invasion (63.64%).

CONCLUSION: D2-40 is far superior in detection of lymphatic invasion compared to routine H&E staining.

KEY WORDS: D2-40; Breast Carcinoma; Lymphatic invasion; Lymph node metastasis.

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INTRODUCTION

With more than one million cases occurring worldwide annually, breast carcinoma (BC) is the most common malignant tumour and the leading cause of cancerous death in women.[1] The climbing incidence of this disease both in the developed and developing countries is a cause of serious concern.[2]

In India, BC now occupies the top rank among cancers in women owing to the gradual change in lifestyle.[3] In India most of the patients present with palpable lump and even with lymph node metastasis (LNM) at the time of their first visit even though that the neoplasm arises in an exposed organ readily accessible to self-examination and clinical consult.[4]

BC is no longer seen as a single disease but rather a multidimensional disease comprised of distinct biological subtypes with manifold natural history, presenting a diverse spectrum of clinical, pathological and molecular features with different prognostic and therapeutic implications. With the development of more effective treatments there have been outstanding advances in BC management over the last few decades resulting in significant decline in BC deaths and improved life-quality.[5]

The leading cause of mortality in patients of BC is metastasis. It has recently been shown that assessing lymphovascular invasion (LVI) is related with other features of aggressiveness of BC, such as high proliferation index and low hormonal receptor status.[6] The term LVI encompasses both lymphatic invasion (LI) and angioinvasion. LI by tumor cells is imperative for dissemination of breast cancer via lymphatic channels. LI is an autonomous predictor of LNM in BC.[7]

Identification of LI with hematoxylin and eosin (H&E) stain becomes difficult if tumor embolus completely plugs the lumen. Moreover, tissue shrinkage during fixation leads to retraction artefacts that isolate tumor cells and are sometimes confused with LI.[7] This reflects the need for a marker specific to lymphatic endothelium (LE).

Today Immunohistochemistry (IHC) holds the limelight in the demonstration of antigens. D2-40, a novel IHC marker, is an IgG2a monoclonal antibody to the oncofetal antigen M2A, which is usually expressed in germ cell tumors and fetal testis.[8] It stains LE but not that of blood vessels. Although markers like Prox1, LYVE1, VEGF3 and others have been tried, D2-40 has now gained deliberation for its specificity and sensitivity against LE. Its use in infiltrating ductal carcinoma (IDC) aids in prognostication of disease process and can potentially alter the treatment modality of the patient.[9]

OBJECTIVES

1. To assess lymphatic invasion in breast carcinoma patients using D2-40 immunohistochemical marker.
2. To correlate lymphatic invasion with the lymph node status.

REVIEW OF LITERATURE

Historical aspects

Carcinoma of the breast may be one of the most archaic forms of cancerous tumors in humans. The earliest illustration of cancer was discovered in Egypt and traces back to 1600 B.C. The Edwin Smith papyrus accounts for 8 cases of tumors or ulcers of the mammary gland that were healed by cauterization.[10]

Hippocrates interpreted that tumors in the breast grew in firmness, without any pus formation. They in time spread to other parts of the body, associated with shooting pain radiating from the breast to the neck and shoulder blades. The demise was certain with the inception of thirst and emaciation. He suggested not to treat the masqueraded cancers.[10]

The codex "De Medicina" by Celsus, portrayed four stages of BC. The first stage was cacoethes (inflammation), followed by carcinoma without skin ulceration and later carcinoma with ulceration. The end stage was advanced exophytic and sometimes bleeding lesion, called the "thymium" which resembled the flowers of thyme. Celsus suggested excision for cacoethes and no treatment for other stages. In moments of uncertainty, the tumour was first treated with caustics and if symptoms ameliorated, then it was cacoethes. If they aggravated, then it was a carcinoma. Some lesions for which treatment was beneficial could have been fibroadenomas, phyllodestumors or even tuberculosis.[10]

The French surgeon Jean Louis Petit (1674-1750) and later the Scottish surgeon Benjamin Bell (1749-1806) were the first to resect LNs, breast tissue and

underlying chest musculature. Their accomplishment was inherited by William Stewart Halsted who began conducting mastectomies in 1882.[11]

Skey in 1851 approved breast aspiration of cysts. Sir James Paget in his discourse on Surgical Pathology (1853) advocated aspiration biopsy. In 1863 Prichard used a grooved needle for breast biopsy and furnished an exquisite description of cytological details of fat necrosis.[12]

EMBRYOLOGY

The mammary glands are derived from the thickening of the epidermis known as mammary ridges (milk lines) on the ventral surface of the fetus. They appear in the 5th week of gestation and extend from the axilla to the upper medial region of the thigh. In humans, the ridge vanishes with development except in the anterior pectoral region. Nipple formation starts at day 56. Then the primitive ducts known as mammary sprouts form on 84^{th} days and get canalized at around day 150. Nipple development occurs postpartum by the mesenchymal proliferation. The normal breast development is halted until adolescence.[13]

Adolescent Breast Development

Thelarche starts with the onset of cyclical estrogen and progesterone secretion at puberty. In females after achieving adolescence, the ducts become branched under the influence of estrogen. Accumulation of lipids in the parenchymal tissue by the adipocytes lead to postpubertal enlargement of breast.[13]

Gross Anatomy of the Adult Breast:

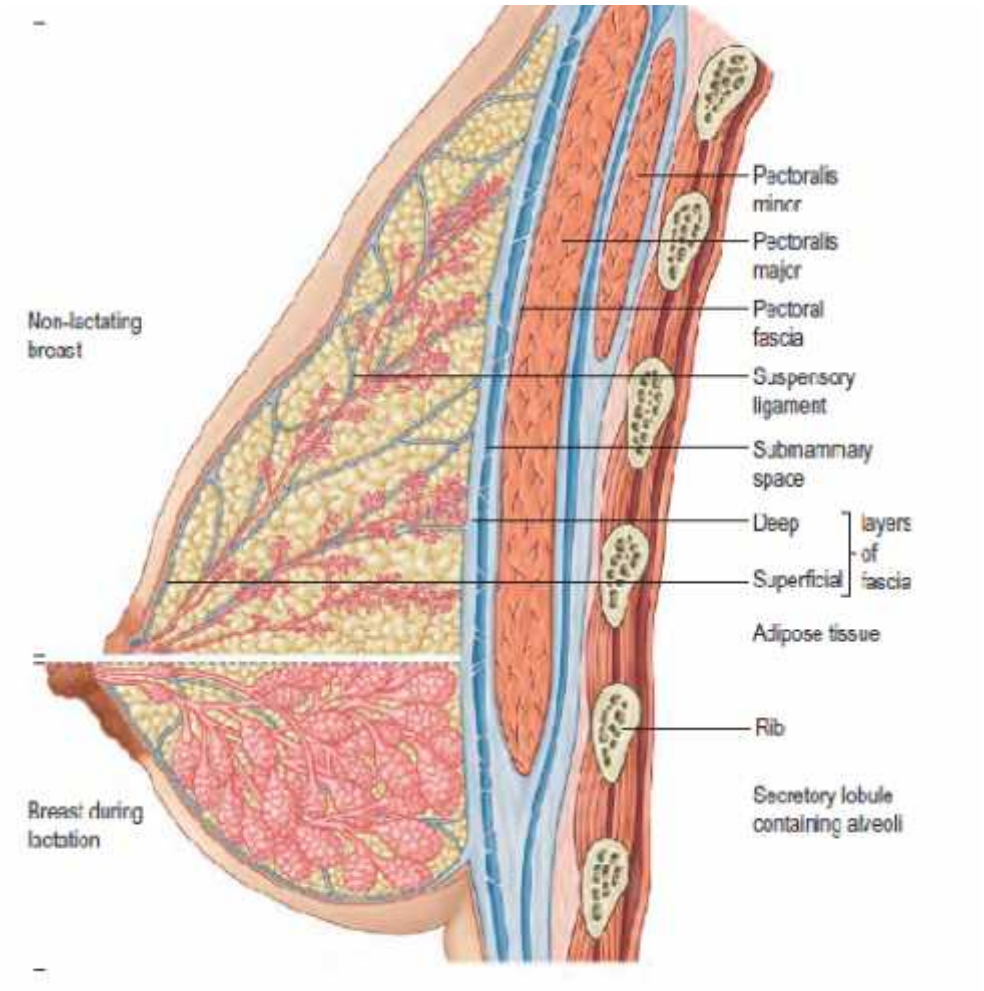


Figure 1: Structural anatomy of breast.[13]

The adult female breast has an eccentric arrangement with long axis largely upon the pectoralis major muscle. It extends into the axilla as the tail of Spence. The entire breast is sheathed by superficial fascia. Superiorly, this layer is in continuity with the cervical fascia, and inferiorly with the superficial abdominal fascia of Cooper. The pectoralis major fascia forms the suspensory ligament of the axilla. The loose areolar tissue lies within the retromammary or sub-mammary space. It separates the deep membranous layer of the superficial fascia from the pectoralis major fascia and serratus anterior muscles.[13]

The terminal duct lobular unit (TDLU) is considered the morphological unit of breasts. It is the lobes which contains glands, branching ducts and terminal secretory lobule embedded in a fibro-collagenous stroma. The TDLU has a lobular architecture and is hormone responsive. TDLU is the functional milk secretory component of the breast. Often it is the site for primary malignant lesions and several benign lesions within the breast.[13]

The skin overlying the nipple areola complex accommodates numerous sweat glands which open into the skin surface. These glands become evident in gravid women, arrangedcircumferentially as small elevations called as the Montgomery's tubercles.[13]

Arterial Supply and Venous System

The breast is perfused by the axillary, internal thoracic and intercostal arteries. In many, the internal mammary artery, branch of internal thoracic artery is the prime source of arterial supply. Veins tends to follow the arteries.[13]

Lymphatic Drainage

In 1786, Cruikshank called lymphatic vessels as "absorbents" and provided elaborate description about lymphatics. He identified the crucial routes of lymphatic flow from the breast as being alongside the path of the tributaries of the external thoracic and internal thoracic veins into the axilla and internal mammary regions, respectively. Few decades later, Sappey injected mercury to reveal the lymphatic flow of the lactating breast. He noticed drainage from the parenchyma into the subareolar plexus of vessels now reckoned to as the subareolar plexus of Sappey. This plexus

purports as a trail for cutaneous lymphatic drainage to the interlobular connective tissue of the breast and subsequently to the parenchymal lymphatic flow.[14]

Three significant routes for lymphatic drainage of the breast have been studied. Almost 75% of lymphatic drainage is into the axillary LNs, either directly or via retroareolar lymphatic plexus. The internal lymphatics conduct around 25% of lymphatic load. A third route is through the posterior intercostal lymphatic vessels to the posterior intercostal LNs in the chest. Ancillary minor lymphatics drain into supraclavicular, infraclavicular and intramammary LNs.[14]

The metastatic seeding of the BC occurs primarily via the lymphatics. Lymphatics from the right breast drain into the right subclavian vein. A fraction of the medial side of the right breast drains to the internal thoracic chain of LNs. The internal thoracic group may drain inferiorly via the superior and inferior epigastric lymphatic vessels to the groin. On the left, the lymphatics conclude in the thoracic duct which ultimately drains into the left subclavian vein.[13]

Lymphatic flow to the opposite axilla is via the lymphatics across the midline. There are 20-40 LNs which are grouped as pectoral (anterior), subscapular (posterior), central and apical. Surgically, the LNs are defined in relation to pectoralis minor. Level I LNs (low axillary) lie below pectoralis minor; level II (mid axillary) behind the muscle and level III lie amidst the upper border of pectoralis minor and the lower border of the clavicle (upper or apical nodes). There might be one or two other interpectoral chain of LNs, called Rotter's nodes.[13]

Lymphatic drainage in breast cancer and role of sentinel lymph node biopsy

A significant process in the staging of patients with early BC used to be lymphatic mapping with sentinel LN biopsy. The sentinel LN is the first draining node of the axilla and is resected for meticulous histopathological examination to identify metastases. Radical mastectomy can be avoided if sentinel LN is negative for metastasis. This decreases the associated morbidity with axillary LN dissection. Axillary dissection or radiotherapy is employed in those with axillary node metastasis.[13]

Innervation

The nerve supply of the breast is by anterior and lateral branches of the fourth to sixth intercostal nerves. The lateral cutaneous branch of T4 innervates the nipple.[13]

Histology

Each mammary gland contains 15-20 lobes of tubuloalveolar glands intervened by dense fibro-collagenous stroma and adipocytes. Each lobe per se is a discrete gland with its own duct system opening separately into the nipple. Within a lobe, each duct ramifies to form multiple terminal ducts which opens into a lobule. This functional and anatomical unit constitutes the terminal-duct-lobular-unit (TDLU). [13]

The lining of these ducts and acini is formed by double layer of cells; the inner luminal cells and the outer myoepithelial cells (MEC). The inner epithelium is comprised of cuboidal cells in smaller ducts and acini whereas tall columnar cells in the larger ducts. Overlying these cells are a layer of often discontinuous, stellate

shaped MECs with pale cytoplasm. During the reproductive ages,the luminal cells undergo slight cyclical transformations under the ovarian hormonal axis.[15]

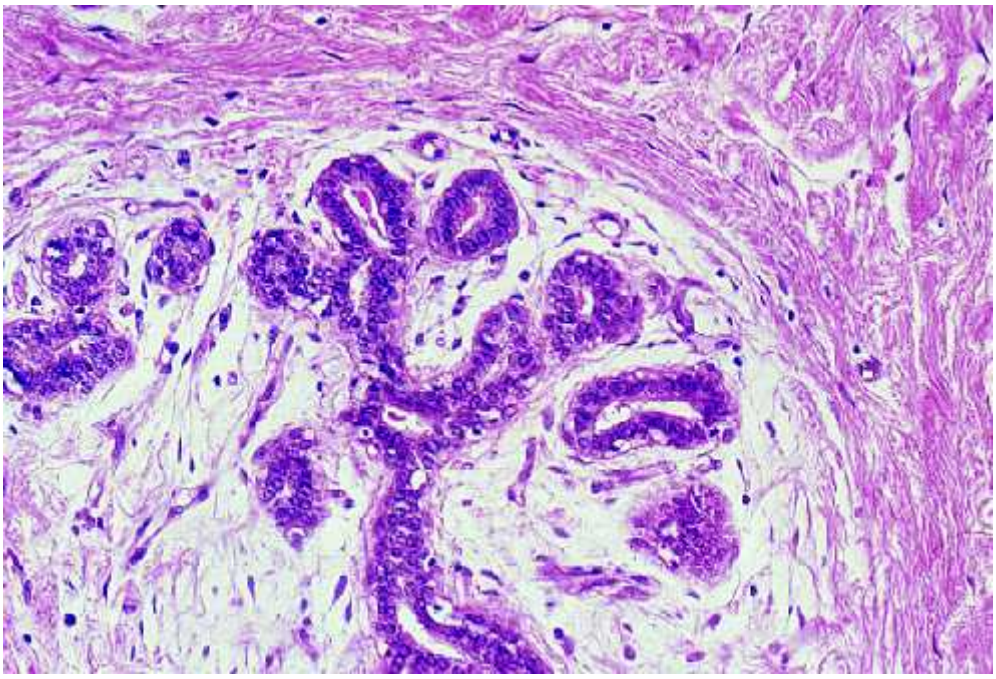


Fig. 2: Terminal Duct Lobular Unit [15]

BREAST CARCINOMA

EPIDEMIOLOGY

Annually, around 1.7 million BC cases are diagnosed. About 627,000 people expired due to BC in 2018. It is the most common cause of female cancer-death worldwide. Amongst Indian women, BC has replaced carcinoma of the cervix as the most common form of cancer. In the year 2018, 1,62,468 women were newly detected with BC in India alone. Also, BC accounted for 27.7% of all newly detected cancers in women. It means that, roughly, one in every four newly detected cancer in Indian women was BC.[16] In Karnataka annual age standardized incidence rate of BC as of 2012 rates was as high as 36.6 per 100,000 women.[17] In the year 2018, 87,090 Indian

women succumbed to BC. BC accounted for about 23.5% of all cancer related deaths in women in India.[16]

Evolution of a prognostic index in breast carcinoma

Von Hansemann is credited for commencing many of the histological grading systems currently used. He gauged the extent of nuclear anaplasia in tumors and observed that, the higher the degree of nuclear atypia, the higher is the probability of metastasis. [18]

Greenough later devised a grading method for BC that classified tumours into three histological grades depending on histological and cytological details. The attributes taken into consideration were the extent of tubule formation, the secretory activity of cells, the overall size of cells and nuclei, alteration in the size of both cells and nuclei; nuclear hyperchromasia, and mitotic activity. Greenough's method was simplified by Patey and Scarff that examined only three variables namely the tubule formation, variation in nuclear size and shape and nuclear hyperchromatism. [18]

In 1957, Bloom and Richardson instigated a numerical scoring system to the system defined by Patey and Scarff. It merged the attributes of cell morphology (nuclear pleomorphism) with an estimation of differentiation (tubule formation) and proliferation (mitotic frequency).[19]

A huge study of 2219 cases were methodically studied by EmadRakha et al in 1957 and deduced that histological grade, as evaluated by the Nottingham modification of Bloom Richardson histological grading system, provides a firm predictor of outcome in patients with invasive BC and should be integrated in BC staging systems.[20]

A multitude of prognostic factors in BC have been explained, but some when put in multivariate analysis attain unconventional relevance. Prognosis assessment is multifactorial and the best differentiation is attained by combining individually relevant parameters. An exhaustively used system of integration would be Nottingham Prognostic Index (NPI), first mentioned in 1982. It is the sole index to have both intra- and inter-centre prospective authorisation. [21]

The prior studies separated patients into three NPI groups but Blamey et al (2007) acknowledged six groups: an Excellent Prognostic Group (EPG) with an observed NPI range of 2.08–2.4, Good Prognostic Group (GPG) 2.42 to 63.4; Moderate Prognostic Group I (MPG I) 3.42 to 64.4, Moderate Prognostic Group II (MPG II) 4.42 to 65.4, Poor Prognostic Group (PPG) 5.42 to 66.4 and Very poor Prognostic Group (VPG) > 6.517. [21]The WHO has embraced the NPI comprising of the tumour size, tumour grade and LN status.[22]

Recent prognostic parameters in breast carcinoma

Prognostic factors, albeit uncertain predictors of response to a therapy, is useful for selection of suitable treatment to patients with cancers. Patients with an exceedingly good prognosis after tumour resection may not require toxic adjuvant therapies which themselves bear substantial morbidity. Contrarily, patients with a poor prognosis may improve from an aggressive adjuvant therapy.[23] Thus, recognition of the prognostic indicators of IDC is crucial as the disease has a remarkably diverse course. [24] A subset of patients with treatable carcinomas who do not sustain significant benefit from adjuvant treatment can be tagged, while others will yield rapidly to the disease on a comparative scale.[25] By virtue of this diverse

clinical outcome prognostic parameters in BC are perhaps among the most exhaustively studied.[23,26]

Several novel research on probable prognostic factors in primary BC have taken into consideration new parameters, either morphologically, immunohistochemically or biochemically, which at least tentatively are linked with invasion, metastasis, differentiation or proliferation of the tumor. [23,26]The prognosis of BC is correlated with several clinical and pathologic parameters. These are as follows:

- **Tumor size**: Small tumors, without micro-metastasis have better prognosis. Larger tumors which disseminate to axillary LNs are linked with subclinical systemic spread and may manifest as advanced disease.[27]
- **Tumor type**: Tubular, mucinous, and medullary subtypes of BC have better prognosis. [28,29]
- **Lymph node status**: Presence or absence of palpable axillary LNs is a significant prognostic predictor especially in patients with early breast cancer.[30] Increasing number of involved axillary LNs is associated with an increased chance of recurrence and mortality.[31] Axillary LN status continues to be the single most significant predictor of prognosis in BC despite identification of novel tumor markers.[32]
- **Lymphovascular invasion**-presence of LVI bears worse prognosis.[33]
- **Scarff-Bloom-Richardson (SBR) classification**- It is the most widely accepted grading methodology. Higher grade is associated with poor prognosis.[34]

- **HER2/*neu*-** Her 2 enriched subtype has an amplification of oncogene HER2/*neu*. HER 2/*neu*positive subtype has a worse outcome.[35] Such tumors tend to be resistant to Tamoxifen therapy and are more amenable to therapies containing Adriamycin. [36]
- **Triple Negative breast cancer** (TNBC) lack the expression of ER, PR and do not overexpress HER2/*neu.* Most TNBCs are of molecular subtype. They have an aggressive course and have a predilection for women of younger age group. Tumors are often large and a have a higher histologic grade when compared to breast cancers with ER/PR positivity.[31]
- **Proliferation Markers** includeS-phase fraction (SPF), thymidine labelling index, mitotic index, and IHC analyses using antibodies directed against proliferation antigens such as Ki-67 and proliferating-cell nuclear antigen. Higher recurrence was associated with higher proliferation index.[37-40]
- **E-cadherin** - Negative expression of E-cadherin is linked with higher grade and development of distant metastasis. [31]

Histological classification of Breast Carcinoma

Several classifications of BC have come up in the previous two decades. Depending on the histopathology, BC can be divided into carcinoma in situ and invasive carcinoma. Carcinoma in situ can be ductal or lobular; depending on the growth patterns and cytological features. Ductal carcinoma in situ (DCIS) exhibits various morphological patterns: Cribriform, Micropapillary, Comedo, Papillary and Solid. DCIS is commoner than lobular carcinoma in situ (LCIS).[41,42]The molecular classification was introduced by Perou and Sorlie in the year 2000. It has the advantage of predicting response to chemotherapy. They are classified into five

distinct subtypes namely basal like, HER2/neu positive, Luminal A type, Luminal B type and normal breast like. [43-47]

The commonest type of BC is IDC of no special type which accounts for 47%-80% cases. It is a class of BC which cannot be put into a particular group by virtue of its heterogeneity. Morphologically, the tumor size varies from 0.5-10cm in diameter, firm to hard in consistency with irregular or moderately ill-defined borders. On microscopic examination, the cells are arranged in tubular formations or cords or are diffusely infiltrative based on the degree of differentiation. The individual cells have abundant eosinophilic cytoplasm with hyperchromatic pleomorphic nuclei and prominent nucleoli. Atypical mitotic figures may also be evident. Associated DCIS component may be present alongside. Stroma is hypercellular and often desmoplastic. No MEC lining is seen.[48]

WHO CLASSIFICATION OF TUMORS OF THE BREAST.

The 2012 WHO classification was the first edition to separate breast tumors from tumors of female genital organs. It takes into account the newer developments in the understanding of specific lesions. It also includes staging recommendations in more contentious situations i.e. pT-stage encapsulated papillary carcinoma as Tis as opposed to size. There is updated information on molecular pathology, expression profiling and molecular classification of breast tumors; however, the focus remains on the morphologic classification of breast carcinomas. [49,50] The latest update was dispatched in its 5th edition (2019). [50]

- **Epithelial tumors**
  - Microinvasive carcinoma
- **Invasive breast carcinoma**
  - Invasive carcinoma of no special type (NST)
    - Pleomorphic carcinoma
    - Carcinoma with osteoclast like stromal giant cells
    - Carcinoma with choriocarcinomatous features
    - Carcinoma with melanotic features
  - Invasive lobular carcinoma
    - Classic lobular carcinoma
    - Solid lobular carcinoma
    - Alveolar lobular carcinoma
    - Pleomorphic lobular carcinoma
    - Tubulolobular carcinoma
    - Mixed lobular carcinoma
  - Tubular carcinoma
  - Cribiform carcinoma
  - Mucinous carcinoma
  - Carcinoma with medullary features
    - Medullary carcinoma
    - Atypical medullary carcinoma
    - Invasive carcinoma NST with medullary features
  - Carcinoma with apocrine differentiation
  - Carcinoma with signet ring differentiation
  - Invasive micropapillary carcinoma
  - Metaplastic carcinoma of no special type
    - Low-grade adenosquamous carcinoma
    - Fibromatosis like metaplastic carcinoma
    - Squamous cells carcinoma
    - Spindle cell carcinoma
    - Metaplastic carcinoma with mesenchymal differentiation
      - Chondroid differentiation
      - Osseous differentiation
      - Other types of mesenchymal differentiation
    - Mixed metaplastic carcinoma
    - Myoepithelial carcinoma
  - Rare types
    - Carcinoma with neuroendocrine features
      - Neuroendocrine tumor, well differentiated
      - Neuroendocrine carcinoma, poorly differentiated (small cell carcinoma)
      - Carcinoma with neuroendocrine differentiation
    - Secretory carcinoma
    - Invasive papillary carcinoma
    - Acinic cell carcinoma
    - Mucoepidermoid carcinoma
    - Polymorphous carcinoma
    - Oncocytic carcinoma
    - Lipid rich carcinoma
    - Glycogen rich clear cell carcinoma
    - Sebaceous carcinoma
    - Salivary gland / skin adnexal type tumors
      - Cylindroma
      - Clear cell hidradenoma

- **Epithelial-myoepithelial tumors**
  - Pleomorphic adenoma
  - Adenomyoepithelioma
    - Adenomyoepithelioma with carcinoma
  - Adenoid cystic carcinoma

- **Precursor lesions**
  - Ductal carcinoma in situ
  - Lobular neoplasia
    - Lobular carcinoma in situ

        - Classic lobular carcinoma in situ
        - Pleomorphic lobular carcinoma in situ
    - Atypical lobular hyperplasia

- **Intraductal proliferative lesions**
    - Usual ductal hyperplasia
    - Columnar cell lesions including flat epithelial atypia
    - Atypical ductal hyperplasia

- **Papillary lesions**
    - Intraductal papilloma
        - Intraductal papilloma with atypical hyperplasia
        - Intraductal papilloma with ductal carcinoma in sit
        - Intraductal papilloma with lobular carcinoma in situ
    - Intraductal papillary carcinoma
    - Encapsulated papillary carcinoma
        - Encapsulated papillary carcinoma with invasion
    - Solid papillary carcinoma
        - In situ
        - Invasive

- **Benign epithelial proliferations**
    - Sclerosing adenosis
    - Apocrine adenosis
    - Microglandular adenosis
    - Radial scar / complex sclerosing lesion
    - Adenomas
        - Tubular adenoma
        - Lactating adenoma
        - Apocrine adenoma
        - Ductal adenoma

- **Mesenchymal tumors**
    - Nodular fasciitis
    - Myofibroblastoma
    - Desmoids type fibromatosis
    - Inflammatory myofibroblastic tumor
    - Benign vascular lesions
        - Haemangioma
        - Angiomatosis
        - Atypical vascular lesions
    - Pseudoangiomatous stromal hyperplasia
    - Granular cell tumor
    - Benign peripheral nerve sheath tumors
        - Neurofibroma
        - Schwannoma
    - Lipoma
        - Angiolipoma
    - Liposarcoma
    - Angiosarcoma
    - Rhabdomyosarcoma
    - Osteosarcoma
    - Leiomyoma
    - Leiomyosarcoma

- **Fibroepithelial tumors**
    - Fibroadenoma
    - Phyllodes tumor
        - Benign
        - Borderline
        - Malignant
        - Periductal stromal tumor, low grade
    - Hamartoma

- **Tumors of the nipple**
    - Nipple adenoma
    - Syringomatous adenoma
    - Paget disease of the nipple

- **Malignant lymphoma**
    - Diffuse large B cell lymphoma
    - Burkitt lymphoma
    - T cell lymphoma
        - Anaplastic large cell lymphoma, ALK negative

- Extranodal marginal-zone B cell lymphoma of MALT-type
- Follicular lymphoma

- **Metastatic tumors**
- **Tumors of the male breast**
  - Gynaecomastia
  - Carcinoma
    - Invasive carcinoma
      - In situ carcinoma

- **Clinical patterns**
  - Inflammatory carcinoma
  - Bilateral breast carcinoma

TNM STAGING

The American Joint Committee for Cancer (AJCC) introduced the TNM (primary tumor [T], regional lymph nodes [N], distant metastases [M]) staging in 1959.

With the advancement in imaging techniques, treatment and prognosis, periodic revisions are made. Currently, the TNM staging includes tumor size, lymph node involvement, and presence of metastatic disease. In the eighth edition, which is effective as of January 2018, incorporates biologic biomarker which would improve the prognostic discrimination.

TNM staging is the most widely accepted staging system which is used in clinical practice.

It undergoes occasional updating after significant breakthroughs in this field.[51,52]

Biological markers in breast carcinoma

IHC markers comprising ER, PR, Ki-67, Her2/neu and p53 are often employed by the histopathologist. Of these, ER, PR and Her2 biomarkers are considered as standard requirements in reporting of BC cases. The MEC markers commercially available are smooth muscle myosin - heavy chain (SMM-HC), P63, α-smooth muscle actin (SMA), P-cadherin, CK-5, CK-14, CK-17, caldesmon and mapsin. Markers for myoepithelium will be negative in IDC. [1,4,53-58]

LI and markers

The demonstration of lymphatic vessels and lymphatic endothelium has always been challenging. Initially, lymphography was performed by intradermal injection of Evans blue and later by oil-contrast lymphangiography. Later, intradermal injection of radiotracer and whole-body lymphangioscintigraphy emerged as the imaging technique of choice. [60]

Lymphatic endothelial cells (LEC) are quite indiscernible using H&E stain alone. Identification of LI with H&E stain becomes difficult if tumor embolus completely fills the lumen. Moreover, retraction artefacts that isolate tumor aggregates due to tissue shrinkage during fixation are sometimes confused with LI.[7,8,60] LEC specific markers such as 5'-nucleotidase have been introduced, but they are difficult to handle and dependent on quantitative rather than qualitative differences between LECs and blood vascular endothelial cells. [60] Recently, certain newer markers have been described.

- **LYVE-1**

LYVE-1 is a CD44 homologous transmembrane glycoprotein which acts as a major receptor for extracellular matrix hyaluronan. It is expressed in the syncytiotrophoblast, sinusoidal endothelial cells, and LECs with varying levels of intensity. Like CD44, the LYVE-1 molecule binds both soluble and immobilized hyaluronan.[61]

Albeit, unlike CD44, the LYVE-1 molecule tags hyaluronan only on the luminal surface of the lymphatic vessels and is completely absent from blood vessels. The highest concentration of LYVE-1 expression was observed in submucosallymphatics in the colon, and the lacteal vessels of intestinal villi that transport dietary lipid absorbed from the small intestine.[61] Nevertheless, some investigators have questioned the specificity of LYVE-1as it is also expressed in some blood vessels of the lung and in hepatic sinusoids.[62] Moreover, antibodies against it generally are commercially unavailable.[63]

- **Prox1 and VEGFR3**

Prox1 a homeobox-containing transcription factor, is the mammalian homologue of the Drosophila gene prospero. Hence the name Prospero-related homeobox gene–1 (Prox-1). It plays a crucial role in the initial transdifferentiation of endothelial cell by assigning to them the lymphatic fate and thus is of significance in lymphangiogenesis. It is reckoned as a master gene that controls the development of lymphatic progenitors from embryonic veins.[63]

VEGFR-3 is a glycosylated tyrosine kinase cell surface receptor expressed specifically by LECs.[62] In 19-wk-old fetuses, Prox1 and VEGFR-3 are co-expressed in LECs of lymphatic trunks and lymphatic capillaries.VEGFR-3 acts as a receptor of the lymphangiogenic growth factor VEGFC and is initially expressed in embryonic blood vessels but is subsequently confined to lymphatic vessels. [63-66]

Prox1 stains the nucleus while VEGFR3 stains the cell membrane and cytoplasm of LECs.[67] However, due to widespread expression of VEGFR-3 in intratumoral blood vessels, it suffers from loss of lymphatic specificity in case of tumors. In case of Prox1, its use in the IHC analysis of human neoplasms has been limited.[62]

- **D2 40**

D2-40 is an IgG2a mouse monoclonal antibody that was initially generated against the oncofetal membrane antigen M2A identified in ovarian carcinoma cell lines and germ-cell tumors and present in normal testis. Studies have shown that D2-40 reacts with an O-linked sialoglycoprotein, podoplanin found on LECs, fetal testis and on the surface of testicular germ-cell tumors.[67] Podoplanin is a 43‑kDa membrane glycoprotein initially identified in glomerular podocytes of rats and is a target gene of Prox1. Expression of podoplanin in LECs was first identified in-vivo by Wetterwald and colleagues. They named it the E11 antigen. [63]

D2-40 has been shown to be a very sensitive and specific marker for LECs in most tissues and especially in BC. As D2‑40 is strongly expressed in the cytoplasm and membrane of LECs, it has been widely used as a specific marker in detecting LI

by tumor emboli.[67] Whilst capillary LECs express both LYVE-1 and podoplanin, LECs of collecting lymphatic vessels exhibit only podoplanin, not LYVE-1. [8,62]

Podoplanin is also expressed in a broad range of other cell types including choroid plexus cells, lung type I alveolar cells, ciliary epithelial cells of the eye, osteocytes, MECs in breast, follicular dendritic cells of lymphoid organs and basal keratinocytes.[8,62] In current practice, D2-40 is used in malignant mesothelioma and lymphovascular tumors. D2-40 stains lymphangiomas, Kaposi's sarcoma and Dabska tumor, but intriguingly does not stain endothelial lining of blood vessels, hemangiomas, glomus tumors, pyogenic granulomas, vascular malformations and angiolipomas. [67]

Vanessa fortes et al studied 123 invasive BC patients who had undergone surgical treatment with axillary LN dissection. They selected 41 axillary LN negative cases, 41 cases having micrometastasis and 41 cases with macrometastsis. Neoplastic cell clusters measuring between 0.2 - 2 mm were considered as micrometastasis and that > 2 mm were taken as macrometastsis. LI was found in H&E stained slides in 17/123 cases. Blood vessel invasion was found in 5/123 cases. By IHC with D2-40 and CD 31, LI was found in 35/123 cases and blood vessel invasion in 19/123 cases. There was positive correlation between LI and metastasis especially in cases with macrometastsis. Positive correlation between blood vessel invasion and tumour size was seen mostly in tumors>2 cm. They did not find any positive relation between LI and tumour size. LI had a positive correlation with high grade tumors. No correlation was found between blood vessel invasion and tumour type. [7]

Another study held in Seoul by Jung Ah Lee et al examined 80 specimens of invasive BC retrospectively for D240 expression [age ranged from 28 to 76 years

(mean, 49.4±10.727)]. A significant correlation was found between D2-40 LI positivity and LNM [p=0.022]. Recurrence was associated with positive LI detected by D2-40 and H&E stain (p=0.014 and p=0.037respectively) when patients were followed up for a mean period of 35±11.026 months. Axillary LNM was also associated with recurrence (OR 2.332; p=0.319), although in the multivariate analysis, D2-40 LI positivity was the only significant factor. D2-40 LI positivity was found to be more important prognostic predictor and associated with poor disease-free survival (OR, 6.855; p=0.007) on multivariate analysis. [68]

Schoppman et al, in Austria, investigated 374 specimens of invasive BC patients (mean age -57 years) by immunostaining for podoplanin. They found that LI is an independent predictor of LNM in breast cancer. LI was found to be significantly associated with a higher risk of developing LNM (p=0.004).[69]

Kenji Tezuka et al had got 67 of 132 BC cases studied,having positive LI on H&E stained sections. But with D2-40 they got positivity only in 55 cases. 14 cases had a false negativity and 26 cases had a false positivity on H&E. Certain cases had lumen completely filled with tumour emboli, which was left out on H&E and was made obvious only by D2-40 staining. They noted a significant correlation between LI and LNM. D2-40 LI was found to be associated with disease free survival, younger age and premenopausal status. No correlation was observed with estrogen receptor status and tumour size. [70]

The present study aims at analyzing D2-40 as a novel lymphatic endothelial marker for identification of LI in breast cancer and whether LI could be a predictor of LNM.

MATERIALS AND METHODS

Study design: Cross sectional study

Study population and data collection: Mastectomy specimens of breast carcinoma received in the histopathology laboratory at KLE DR. PRABHAKAR KORE HOSPITAL from 1st January 2019 to 31st December 2019. Archival data and paraffin blocks of the previous year (2018) was retrieved.

Sample size: 35 (Universal sample)

Selection criteria:

Inclusion criteria:

1. Primary invasive breast carcinomas.

Exclusion criteria:

1. Metastatic lesions of breast.
2. Non epithelial malignancies of the breast (eg.sarcoma, lymphoma etc.)

Ethical clearance

The ethical clearance was obtained from the Institutional Ethics Committee, JNMC, Belagavi prior to commencement of study.

Method of data collection

Procedure: The tumor size and number of lymph node (LN) received were noted on gross examination. The specimen was fixed in 10% formalin and after processing and

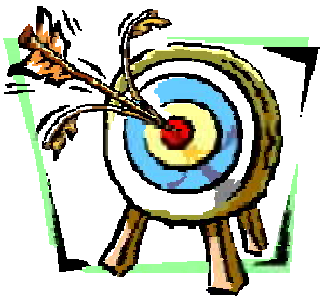
embedding in paraffin, sections of 3–4 μm thickness were cut with a microtome and stained with H&E.

These stained sections from tumor were examined microscopically and allocated tumor grade as per the modified Scarff-Bloom-Richardson (SBR) grading system for BCs. SBR grade was assigned by assessing the tumor for the extent of tubule formation, nuclear pleomorphism, and mitotic activity. The same sections were assessed for the presence of LI and tumor architecture. Sections from all the LNs were screened for the existence of carcinomatous deposits. LI was taken as carcinoma cells lying within a definite, endothelium-lined cavity. Tumour cell emboli which were observed in a lumen with the appearance of a vessel but without a definite endothelial lining were considered as negtive for LI . Channels lined by monolayer of endothelial cells, occasionally containing lymphocytes, lymphatic fluid and without any red blood cells (RBC) were taken as lymphatics on H&E.

Sections from the same tissue blocks were subjected to IHC with D2‑40 using routine immunohistochemical procedure. 5 micrometer thick sections were made on poly L Lysine coated slides. For incubation, these slides were kept at 37 degree Celsius overnight and at 60 degree Celsius for 1hour next day followed by antigen retrieval and staining. Antibody used was D2-40 which specifically stains LECs. Tumour deposits seen within D2-40 stained vessels were considered positive for LI.

Data analysis: Data was entered in Microsoft Excel software. The association between variables were attained using Chi-square test using standard statistical software. A probability value (p value) of ≤0.05 at 95% CI were considered statistically significant.

Introduction



Objectives

Review of Literature

Methodology

Results

Discussion

Conclusion

Summary

Bibliography

Annexure-I

Annexure-II

Annexure-III

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Annexure-V

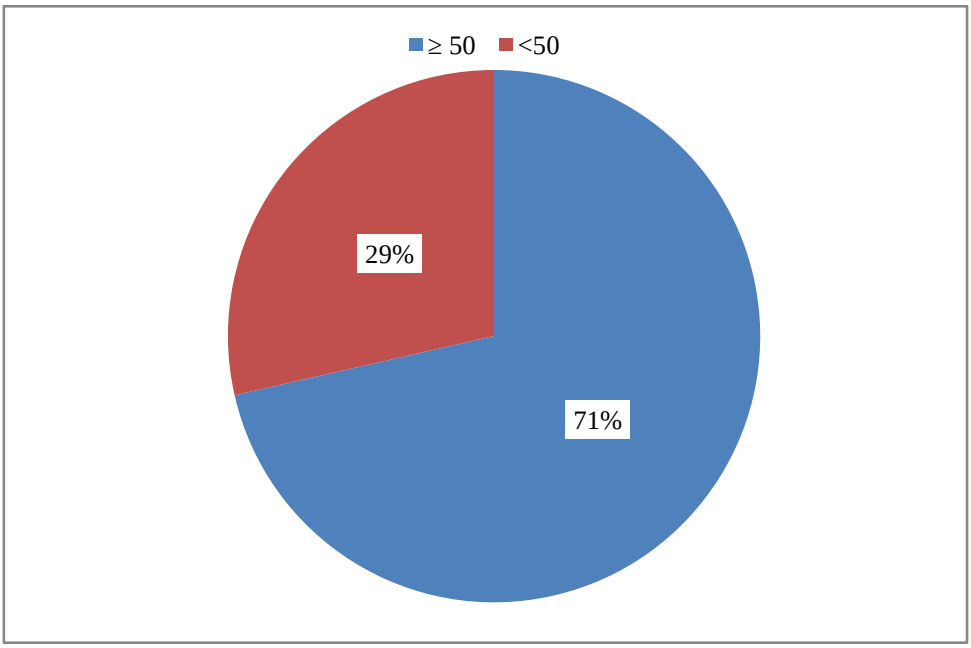
RESULTS

Data was collected from 35 mastectomy specimens of IDC and LI was assessed by H&E and D2-40 stains.

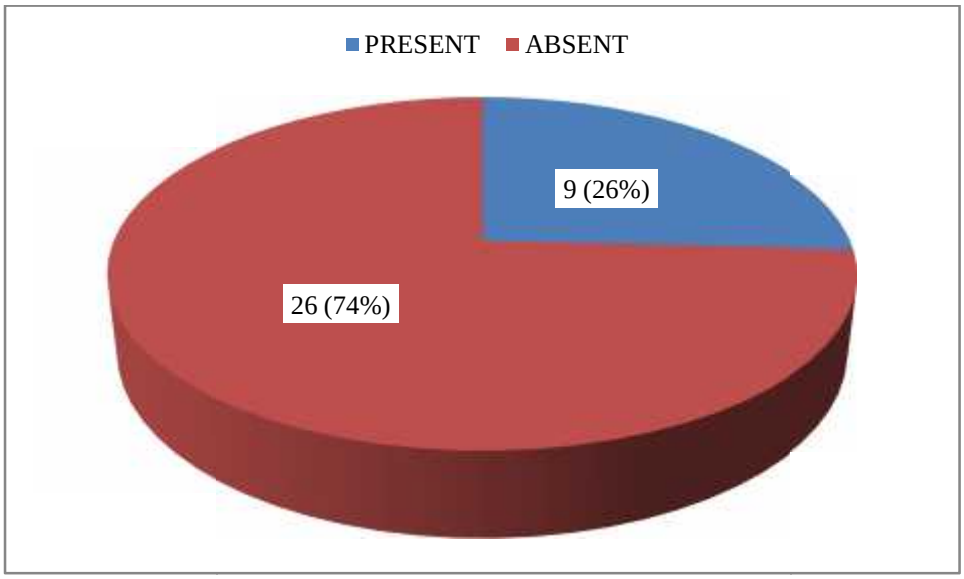
Data obtained from this study was compiled; tabulated and statistically analysed.

The patient's ageat the time of surgeryranged from 30years to 87 years (mean = 56.29 years). 25 cases were 50 years of age and above, and the remaining 10 were below 50 years. All the patients were female.

Graph 1: Age distributionof cases(in years)

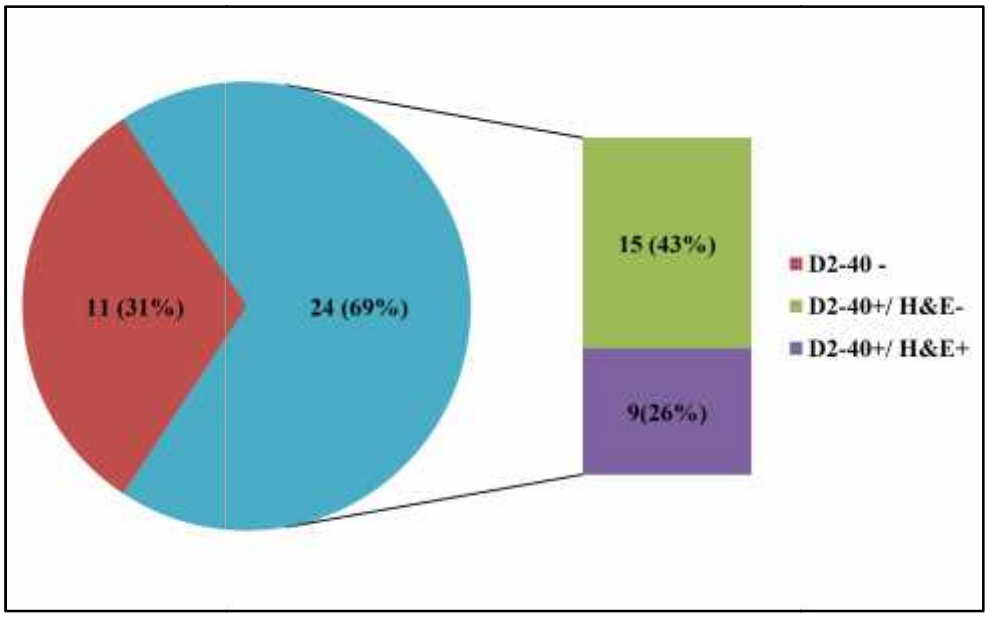


Graph 2: Frequency of Lymphatic invasion on H&E stain



LI was detected by H&E in 9 out of 35 cases (26%)

Graph 3: Frequency of Lymphatic invasion detected by D2-40 staining.



LI was detected by D2-40 in 24 of the 35 cases (68.57%). Of these, 15 (62.5%) cases were detected exclusively by D2-40 which were missed on H&E. Lymphatic vessels were identified in all the sections with D2-40 staining.

Table 1: Sensitivity and specificity of D2-40

			LI on H&E		
			Present	Absent	Total
D2-40	LI present	Count	9	15	24
		% within D2-40	37.5%	62.5%	100.0%
	LI absent	Count	0	11	11
		% within D2-40	00%	100%	100.0%
Total		Count	9	26	35
		% within D2-40	25.71%	74.29%	100.0%

Thus, sensitivity of D2- 40 in detecting LI was found to be 100%.

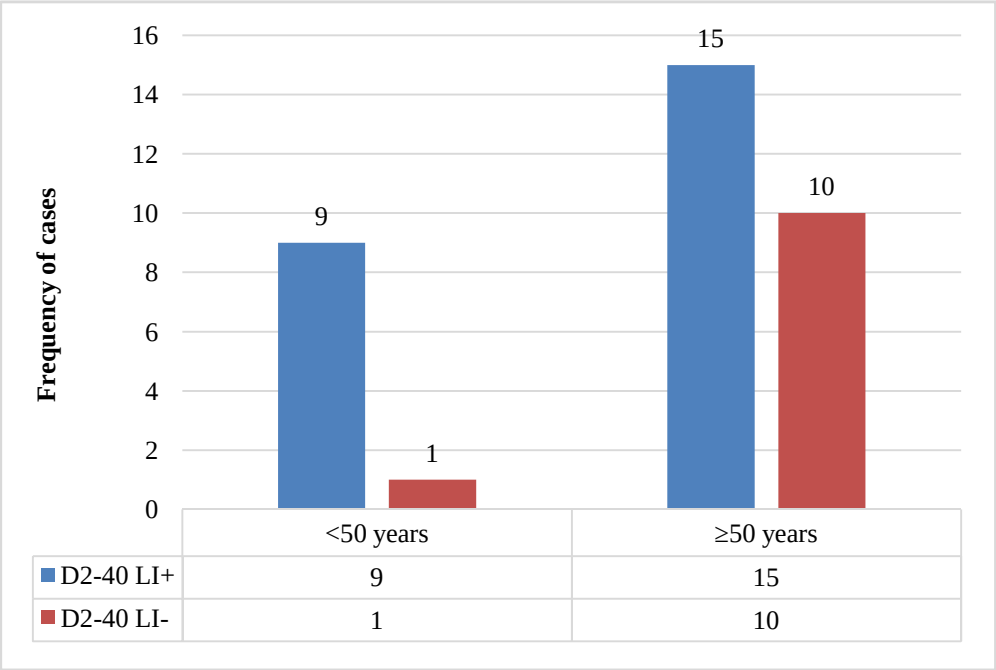
Specificity: 42.31 %

Positive Predictive Value: 37.50%

Negative Predictive Value: 100.00%

Kappa statistics =0.27392, indicating fair agreement.

Graph 4: Frequency distribution of LI on D2-40 with age

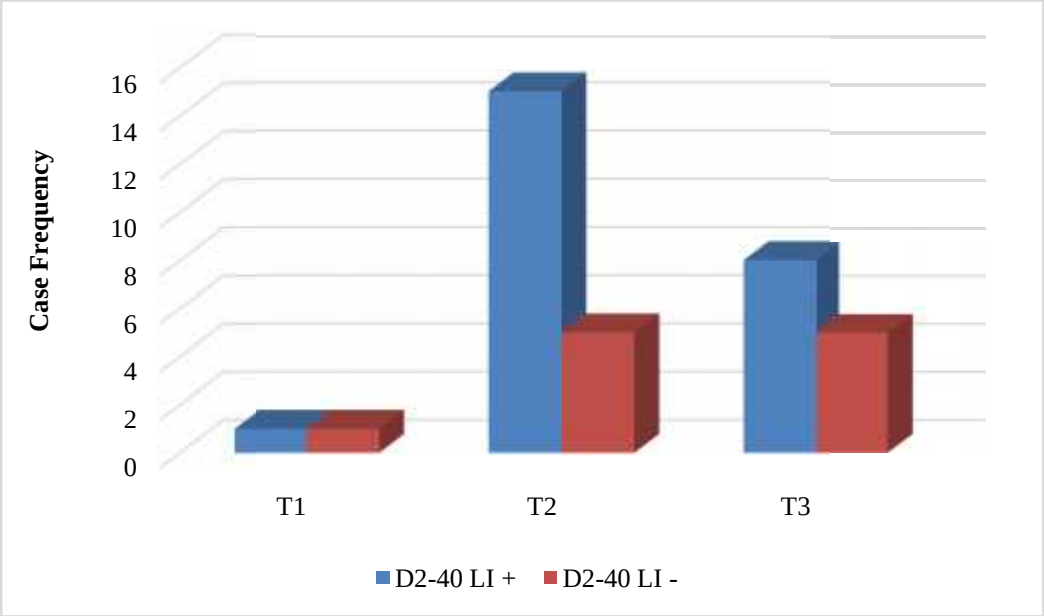


15 among the 24 LI positive cases (62.5%) were 50 years and above. However, there was no association between age of tumour presentation and D2-40 LI. (p value= 0.0841)

Table 2: Relationship of LI on D2-40 with tumour size

			Tumour size			
			T1	T2	T3	Total
D2-40	LI present	Count	1	15	8	24
		% within D2-40	4.2%	62.5%	33.3%	100.0%
	LI absent	Count	1	5	5	11
		% within D2-40	9.01%	45.45%	45.45%	100.0%
Total		Count	2	20	13	35
		%	5.71%	57.14%	37.15%	100.0%

Graph 5: Distribution of LI on D2-40 as per tumor size



The mean size of the tumour was 4.67cm in the greatest dimension. Most of the tumors belonged to T2 stage (57.14%). No statistically significant association was found between LI and size of tumour (p = 0.61)

Graph 6: Frequency distribution of tumor grades in cases

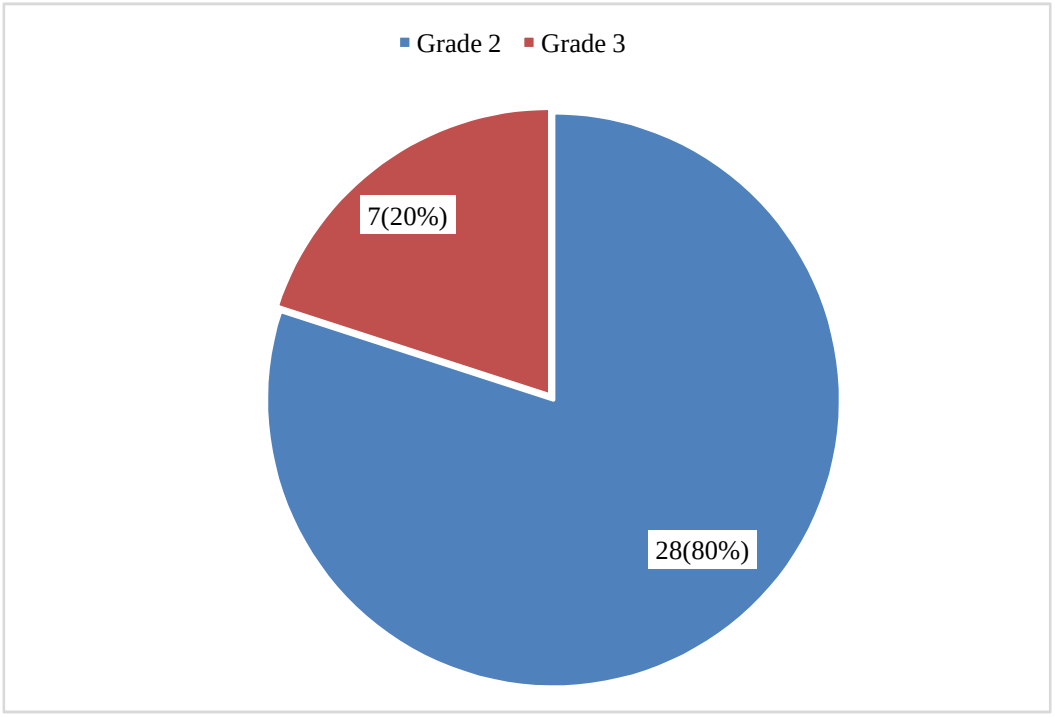
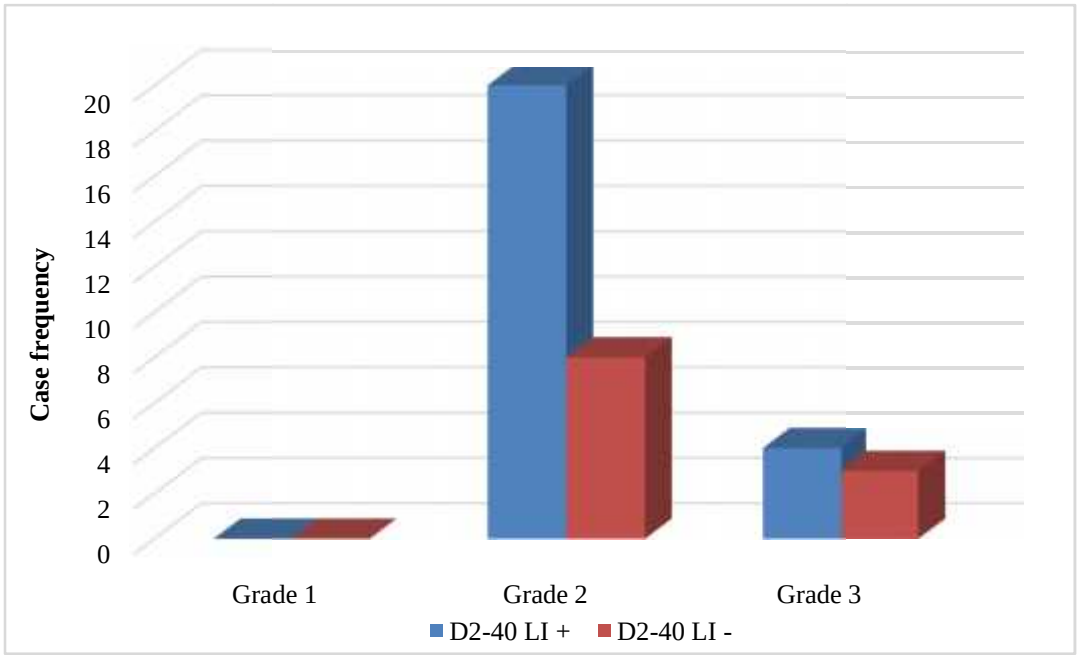


TABLE 3:Frequency of LI in various tumour grades.

	LI with D2-40 stain		Total
Grade	Present	Absent	
2	20	8	28
3	4	3	7
Total	24	11	35

Graph 7: Relationship of with LI on D2-40 with tumor grades



There were no grade 1 cases in this study. Most of the cases were of grade 2 (80%).

LI with D2-40 was also maximum in grade 2 tumors (83.33%). However, there was no association between tumour grade and D2-40 LI positivity (p=0.337).

Graph 8: Frequency of axillary lymph node metastasis in cases

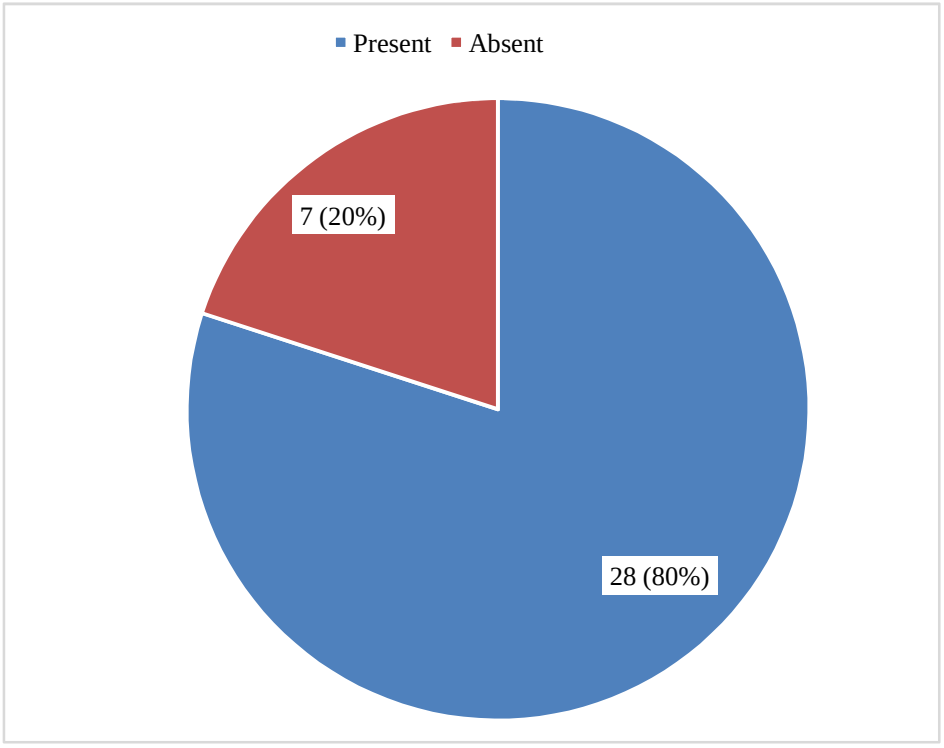


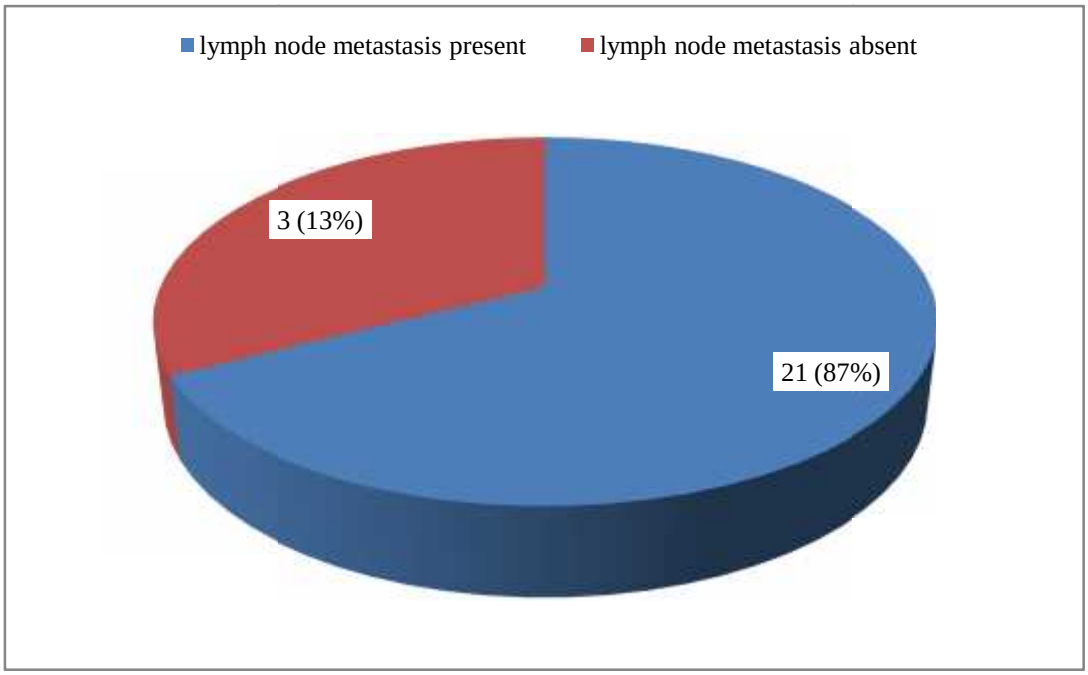
TABLE 4: Distribution of LNM with LI

	LI with D2-40 stain		Total
Lymph node metastasis	Present	Absent	
Present	21	7	28
Absent	3	4	7
Total	24	11	35

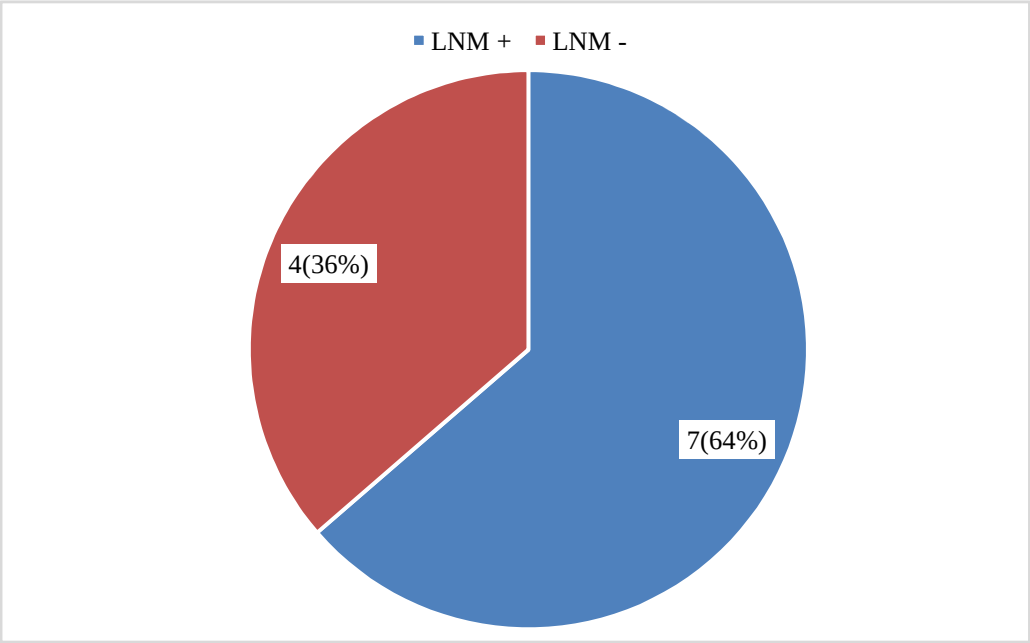
28 out of the 35 (80%) cases showed LNM and 21 of them (75%) showed LI with D2-40.

No statistically significant association was observed between LI and LNM (p=0.1013).

Graph 9: Frequency of LNM in D2-40 LI positive cases.



Graph 10: Frequency of lymph node metastasis in D2-40 LI negative cases.



Out of the 35 cases of IDC, 4 cases were such that they also exhibited a component of DCIS. These cases had a mean age of 55.5 years and they showed a mean tumour size of 5.75 cm.

DISCUSSION

Being the commonest malignancy in females worldwide, BC accounts for 24% of entire female cancers and is the most common cause of female cancerrelated death globally. With its burden of 11.6% cases in both sexes combined, it is now the second most common cancer overall. In recent decades, IDC has been climbing in incidence in most low and middle-income nations.

The leading cause of mortality in patients of BC is metastasis.[71] LI by tumour cells is imperative for dissemination of BC via lymphatic system. LI is an independent predictor of LNM in BC. The presence of tumor emboli in lymphatics is associated with poor survival rates. [7,67,69] It has recently been shown that assessing LI is related with other features of aggressiveness of BC, such as high proliferation index and low hormonal receptor status. [72]

The screening for LI was previously done using H&E in which LI could not be distinguished from blood vessel invasion.[68] Identification of LI with H&E stain becomes difficult if tumor embolus completely fills the lumen. Moreover, tissue shrinkage during fixation leads to retraction artefacts that isolate tumor aggregates and are sometimes confused with LI. [73] This reflects the need for a marker specific to LE.

D2-40, a novel IHC marker, is an IgG2a monoclonal antibody to the oncofetal M2A antigen, which is usually expressed in germ cell tumors and fetal testis.[8] It stains endothelium of LE but not that of blood vessels. Although biomarkers like Prox1, LYVE1, VEGF3 and others have been tried, D2-40 is now considered as one of the most specific and sensitive markers of LE commercially available.[9]

The present study aims at analysing D2-40 as a novel LE marker for identification of LI in BC and whether LI could be a predictor of LNM.

Our study analysed 35 women with IDC. Concurrent component of DCIS was present in 4 out of the 35 cases.LI was identified on H&E section. Then sections were stained with D2-40. Tumour cells lying in spaces lined by D2-40 positive cells were taken as LI as it stains only LE.

LI was detected by H&E only in 9 out of 35 cases (25.7%) whilst D2-40 detected LI in 24 of 35 cases (69%). LI was missed in 15 cases (43%) on H&E. Some of these cases had tumour emboli occupying the lymphovascular lumen entirely which became obvious only on IHC staining. Retraction artefact which was indiscernible from LI with H&E was easily differentiated by D2-40. Clearly, D2-40 increased the detection rate of LI (by 15 cases; 42.86%). Our study reaffirmed that D2-40 stains endothelium of lymphatics alone and not that of blood vessels.

Other studies reported LI detection rate ranging from 28.5% to 72% using D2-40.[20,73-75] Sensitivity of D2-40 stained LI was excellent in our study (100%) and specificity was 42.3% whichcould have been a chance event attributable to low sample size. Kappa value for diagnostic agreement was fair (0.2739).

These findings are comparable with the study conducted by Dileep AP et al which observed sensitivity of 83.3% and specificity of 44.4% (kappa=0.25).[75] Nevertheless, D2-40 stained lymphatic vessel in all the cases in our study making it highly specific for LE. Thus, D2-40 was found to be a suitable immunomarker to discern out LI from angiovascular tumour emboli in our study.

LNM is one of the most significant predictors of patient survival, especially in clinical stage I and II BCs.[76]We observed that 28 (80%) of our cases had LNM at the time of clinical presentation. Concordant with other studies frequency of LNM was higher (75%) in our cases with LI compared to that of LI negative group (63.63%). [68,74,75] Nevertheless, statistically no significant association was observed between LI and LNM (p=0.1013).

The ninth St Gallen Conference held in Switzerland in 2005 dispensed new consensus principles on endocrine treatment of early BCs. The expert panel recommended consideration of peritumoral LVI (particularly LI) in the adverse prognostic factors, in the guiding principles for adjuvant therapy of early BCs. Assessment of LI with H&E alone might lead to higher false negativity thus affecting quality patient treatment in such subset.[45,62,67,77]Rakha *et al*. observed in LN negative cases and noted that its prognostic worth was identical to that furnished by 1–3 LNs positive cases (pN1) without showing LVI in the tumour.[20] Multiple studies have endorsed LN‑negative cases with LI to be contenders for adjuvant systemic therapy.[62,78-80] Three cases in our study had lymphatic tumor emboli without any carcinomatous deposits in the LNs..

Jung Ah lee et al in their experimental study observed a positive correlation of D2-40 positive LI with LNM and recurrence.[68] According to a study by Emad A Rakha et al it was observed that LI correlated positively with large tumour size, negative estrogen receptor status, and high Ki-67 index and tumour grades. So, responsiveness to chemotherapy was less in cases with LI.[20] In a meta-analytical study of invasive breast cancers by Shen et al in 2015, tumors with LI showed higher Her 2 status which in turn was associated with poor prognosis. In such cases with

metastasis, tumor dissemination often occurred to the central nervous system.[81] This highlights the significance of discerning LI from angio-invasion.

In 2008, Marinho et al observed that tumour size was not associated with LI. They suggested that lymphatics were not a necessity for the thriving tumour cells as lymphatic system formed a part of the draining vascular compartment.[73] Similar trend was seen in our study with no statistically significant association between D2-40 LI and tumour size (p value-0.61). The mean tumour size in our study was 4.67cm in the greatest dimension. Most of the tumors belonged to T2 stage (57.14%).

RAA Mohammed et al studied prognostic significance of LI in LN-positive BC. They observed higher frequency of LI in patients younger than 50 years of age.[82] We found no statistically significant association between D2-40 LI and age of patients (p value- 0.0841) albeit frequency of LI was found to be more in cases ≥50 years of age (62.5%) compared to those less than 50 years (59.3%).

In the study by Marinho et al, they reported that LI correlated with higher tumor grade. It seemed possible that hastily multiplying malignant cells manufactured growth factors to meet its growing demands. By virtue of this, larger assortment of clonal malignant cells capable of LI were possible.[73] There were no grade 1 cases in our study. Most of the cases were of grade 2 (80%). LI with D2-40 was also maximum in grade 2 tumors (83.33%). However, there was no association between tumour grade and D2-40 LI positivity (p=0.337).

All the cases in this study presented with histomorphology of IDC- no special type (NST). Hence, a correlation between histological type and LI could not be attained.

In our study, cross reactivity of D2-40 with MECs was encountered in three cases in the normal peritumoral breast parenchyma. Nevertheless, this was not a cause of concern in this study because almost consistently, the foci of IDC are devoid of MECs but present only in benign breast lesions and at the margin of in-situ carcinomas.[83] Moreover, we observed that the staining pattern of MEC was different from that of LECs. The cytoplasmic staining with MECs varied from moderate to less intense, was irregular and sometimes discontinuous in comparison to that of the LECs. Further, MECs in the normal larger ducts were apparently plump than LECs (fig.11,12).

These findings are concordant with multiple studies which have reported cross reactivity of D2-40 with MECs in benign breast tissue and in-situ carcinomas. These studies have elaborated that MECs in normal breast tissue and in the periphery of in-situ carcinomatous nidus exhibited mild to moderately intense, feebler and sporadic (especially in small ducts) D2-40 positivity.[62,67,83,84] Studies by Abdel-Dayem HM and Ibraheim AT, and A Arnaout-Alkarain et al reaffirm that this cross-reactivity can easily be distinguished morphologically.[62,67] However, studies by Rabban and Chen, and RAA Mohammed et al elaborate that this should be dealt with caution in cases of in-situ carcinomas. They even recommend using myoepithelial markers like p63 and smooth muscle myosin to avoid pitfalls in diagnosis.[82,84]

The limitation in our study is the small sample size by virtue of which statistically significant results could not be furnished. Similar studies with larger sample size are required.

CONCLUSION

The findings of this study highlight the significance of a marker specific to LE. In our study D2-40 has been far superior in detection of LI compared to basic H&E staining.

It has consistently been one among the most sensitive and specific LEC markers, as corroborated by our study.

Our results emphasize that identification of LI with H&E is insufficient and that D2-40 can be utilised for detection of LI in BC patients.

D2-40 detected LI is associated with higher frequency of LNM and therefore it can predict tumour dissemination into regional LNs earlier in node negative BC cases. Therefore, D2-40 should be considered in selective cases of BC for better prognostication of disease.

SUMMARY

This cross-sectional study included 35 cases of IDC who underwent mastectomy in KLE'S DR. PRABHAKAR KORE HOSPITAL AND MRC, Belagavi. LI was identified using D2-40 immunomarker. D2-40 is a relatively new marker considered highly specific and sensitive for LECs.

The significant observations and inferences from our study are summarized underneath:

- The patient's age at the time of surgery ranged from 30 years to 87 years (mean = 56.2).
- Frequency of D2-40 LI was found to be more in patients of age ≥ 50 years (71.43%) compared to those less than 50 years (28.57%).
- Increased frequency of LI detection was observed with the use of D2-40 (68.57%) when compared to H&E (25.71%).
- Sensitivity of D2- 40 in detecting LI was 100% and specificity was found to be 42.3%.
- Positive predictive value of D2-40 was 37.5% and negative predictive value was 100%.
- Kappa for diagnostic agreement was fair (0.2739)

- Frequency of LNM was found to be higher (87.5%) in D2-40 positive LI group compared to those without D2-40 LI (63.64%). 3 cases with LI had no LNM.

- Most of the cases were of tumor grade 2 (80%). LI with D2-40 was also highest in grade 2 tumors (83.33%).

- No statistically significant association was observed between LI and LNM (p=0.1013), LI and tumor size (p-0.61), and LI and grade of tumour (p=0.337).

- There was no association between age of presentation of BC and D2-40 LI. (p=0.0841)

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ANNEXURE-I-ETHICAL CLEARANCE LETTER

K.L.E. ACADEMY OF HIGHER EDUCATION AND RESEARCH
(Deemed – to- be- University)
Accredited 'A' Grade by NAAC (2nd Cycle) Placed in Category 'A' by MHRD (GoI)
JAWAHARLAL NEHRU MEDICAL COLLEGE,
NEHRU NAGAR, BELAGAVI-590010 (KARNATAKA-INDIA)
Website: http//www.jnmc.edu
E-Mail : dome@jnmc.edu
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Principal: 2471701
Fax No. +91 (0)831 – 2470759

Ref: MDC/DOME/21 **Date: 24/11/2018**

To,

REG NO: BN0118001

Sub: Institutional Ethical Clearance for the study.

With reference to the above, we wish to inform you that your proposed research project titled **"IDENTIFICATION OF LYMPHATIC INVASION IN BREAST CARCINOMA PATIENTS USING IMMUNE-MARKER D2-40-A ONE YEAR CROSS SECTIONAL STUDY IN A TERTIARY CARE CENTRE OF BELAGAVI"**, is ethical and justifiable. The proposed research project has been cleared by the JNMC Institutional Ethics Committee on Human Subjects Research.

(Dr. Arathi Darshan)
Member Secretary
JNMC Institutional Ethics Committee
on Human Subjects Research,
J.N.Medical College, Belagavi.

(Dr. Roopa M Bellad)
Chairman,
JNMC Institutional Ethics Committee
on Human Subjects Research,
J.N.Medical College, Belagavi.

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ANNEXURE II – CONSENT FORM

INFORMED CONSENT

IDENTIFICATION OF LYMPHATIC INVASION IN BREAST CARCINOMA PATIENTS USING IMMUNO-MARKER D2-40– A ONE YEAR CROSS SECTIONAL STUDY IN A TERTIARY CARE CENTRE OF BELAGAVI.

Purpose of the study: The purpose of this study is to determine the role of D2-40 in identifying lymphatic invasion in invasive breast cancer. This study will help in determining a better diagnostic tool for invasiveness of cancers. Either those who have underwent mastectomy for breast cancer, or those who are about to undergo mastectomy, can volunteer to take part in this study.

Procedure: During this study, you will only be asked questions regarding your age, I.P number and any treatments undergone if any. If you agree to enroll yourself in this study, the breast tissue sample received in the hospital will be examined for the presence of lymphatic invasion by cancer cells.

Risks and benefits: There are no risks involved in taking part in this study and benefit is we will be able to know a better way to diagnose invasive cancers which is essential for providing appropriate treatment.

Alternatives: Taking part in this study is voluntary. You may choose not to take part in this study or if you decide to take part now, you still possess the right to withdraw from the study later on, in case you change your mind. The investigator may terminate your participation in this study anytime for any valid reason.

Privacy and confidentiality: All information collected about you during the course of this study will be kept confidential to the extent permitted by law. The code numbers will identify you in this research record. Information from this study will be published but your identity will be confidential in any publication. No information about you or information provided by you during research will be disclosed to other without your written permission except:

1. In emergency to protect your rights and welfare.
2. If required by law.

Financial incentives for participation: You will not be paid / offered any gift /incentives for participating in this study.

Authorization to publish results: The results of this study would be forwarded to the KAHER, Belagavi as a part of requirement towards the completion of MD degree, review and publishing.

Questions: In case you have any questions related to the study in future you can contact:

1. If you have any queries about your rights as a study subject, you may call Dr. RoopaBellad, Professor, Department of Paediatrics, Chairman of J.N. Medical College Institutional Ethical Committee of Human Subjects Research, Ph No-9448113403, at J.N. Medical College, Belagavi

CONSENT STATEMENT

I voluntarily agree to take part in this study by signing below. I may withdraw at any time. I am not giving up any legal rights by signing this form. My signature below indicates that I have read, or it has been read to me, this entire consent form and have had all my questions answered.

In case of the queries during the study or in future you may contact following person.

Principal Investigator: _____

Guide :_____

If you have any queries about your rights as a study subject, you may call Dr. RoopaBellad, Professor, Department of Pediatrics, Chairman of J.N. Medical College Institutional Ethical Committee of Human Subjects Research, Ph No- 9448113403, at J.N. Medical College, Belagavi

Name of the participant:

(signature/thumbprint)

Name of the witness : (signature)

Name of the investigator: (signature)

Date:

Address:

Phone no:

ANNEXURE-III

PROFORMA

Sl No.

PATIENT HISTORY

Name: Biopsy no.:

Age :

Tumour Size :

HISTOPATHOLOGICAL DIAGNOSIS:

GRADE:

AXILLARY LYMPH NODE STATUS:

Treatment history if any:

	H&E	D2-40
LI	Positive/negative	Positive/negative

Other prognostic features if any:

ANNEXURE-IV

HEMATOXYLIN AND EOSIN STAINING PROTOCOL

Bancroft D, Laylon C. The haematoxylin and eosin, In: Kim SS Ed, Bancroft's Theory and practice of histopathological techniques. &th Ed., China, Churchill Livingstone; 2013: p173-187.

1. Deparaffinize in Xylene I and II and III changes. (III change use warmed xylene) (5 minutes in each)
2. Rehydrate using
    a. Absolute ethanol 100% (5 minutes)
    b. Absolute Ethanol 100% (5 minutes)
3. Rinse in distilled water (5 minutes)
4. Rinse in running tap water (5 minutes)
5. Stain in Harris's haematoxylin by progressive method (2 minutes) Fresh and filtered
6. Rinse in running tap water (20 minutes)
7. Decolorize in 1% acid alcohol (1 second)
8. Rinse well in tap water (5 minutes)
9. Immerse in hot water bath, 55˚C for blueing (3 seconds)
10. Rinse in tap water (5 minutes)
11. Counterstain in Eosin (15 seconds)
12. Dehydrate with absolute alcohol 100% (2-4 dips)
13. Clear in xylene I and II (5 minutes)
14. Mount with DPX.

Stock solution – Eosin:

Stock – 1% aqueous Eosin – Y

Stock – 1% aqueous Phloxin B

Working Solution – Eosin:

100ml stock Eosin

10 ml stock Phloxin B

780 ml 95% Ethanol

4 ml glacial acetic acid

Working Solution – Hematoxylin

Harris Hematoxylin, 1 litre

Working solution – 0.25% Acid alcohol

95% Ethanol, 2578 ml

dH2O, 950 ml

HCl, 9ml

Result: Nuclei – blue, cytoplasm – pink, RBCs – red.

PROCEDURE FOR IHC STAINING FOR D2-40 ANTIBODY

1 Cut the sections at approximately 4 µm thickness in poly L Lysine coated slides.

2 Float on to the positive charged slides.

3 Slides were air dried for 2 hours at 58 ºC.

4 Two changes of xylene of 10 minutes each for deparaffinization.

5 Hydration.

- Absolute alcohol – 2dips
- 80% alcohol -- 2dips
- 70% alcohol -- 2dips
- Distilled water -- 2 changes 5 minutes each.

6 Antigen retrieval by heat, using microwave using TRIS EDTA Buffer.

7 Cooling of sections to room temperature.

8 Rinse in distilled water for 3 minutes.

9 Wash in TBS buffer two times for 3 minutes each.

10 Treatment with peroxide block for 10 minutes to block endogenous peroxidase.

11 Wash in TBS buffer two times for 3 minutes each.

12 Treatment with primary antibody (Dako D2-40) for 60 minutes

13 Wash in TBS buffer two times for 3 minutes each

14 Treatment with Target binder for 10 minutes

15 Wash in TBS buffer two times for 3 minutes each

16 Treatment with HRP Polymer for 10 minutes

17 Wash in TBS buffer two times for 3 minutes each

18 Treatment with DAB (secondary antibody) for 3-5 minutes to give brown colour to antigens

19 Wash in distilled water for 3 minutes

20 Counter stain with Harris haematoxylin for 30 seconds to 1 minute

21 Wash in tap water for 3 minutes to remove excess stain

22 Two changes of absolute alcohol for 2 minute each for dehydration

23 Clearing with xylene for two minutes. Dry the slides and mount with DPX

Preparation of reagents

1. Antigen retrieval Buffer

TRIS EDTA Buffer- pH: 8.5 to 9.0

Preparation:

TRIS Base- 1.21 gram

EDTA (atomic number:372)- 0.37 gram

Dissolve in 1000ml of water

2. Wash buffer

TRIS BUFFERED SALINE (TBS)-pH: 7.2 to 7.6

Preparation:

TRIS Base- 8.6 gram

NaCl- 9.6 gram

Dissolve in 1000ml of water.

Adjust pH by using concentrated HCl

ANNEXURE V– PICTOMICROGRAPHS

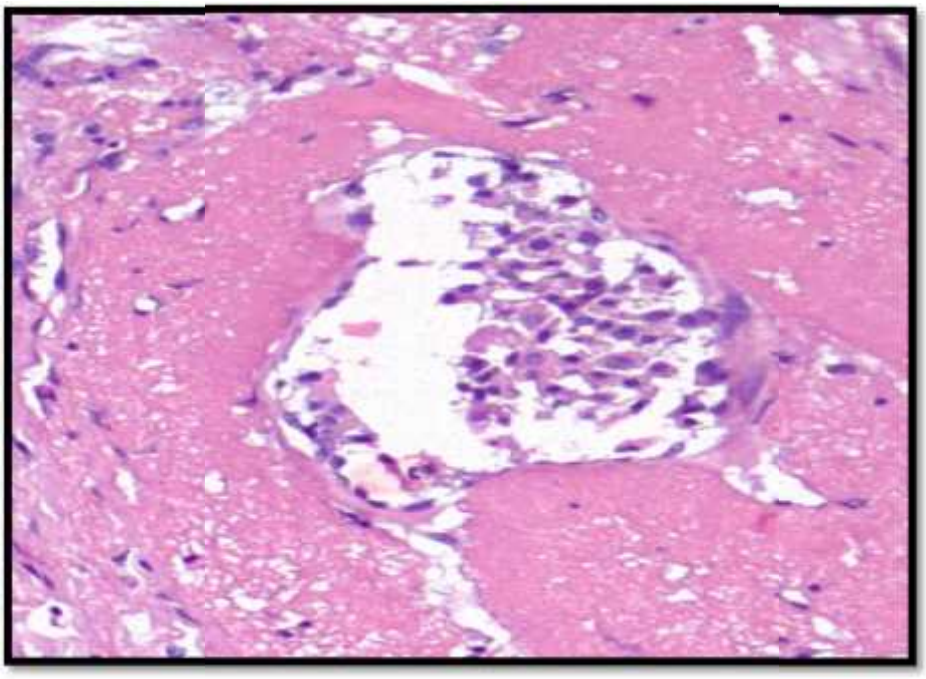


Figure A: Lymphatic emboli on H&E staining (20X). The lymphatic vessel is lined by single layer of flattened endothelium without any RBCs in its lumen.

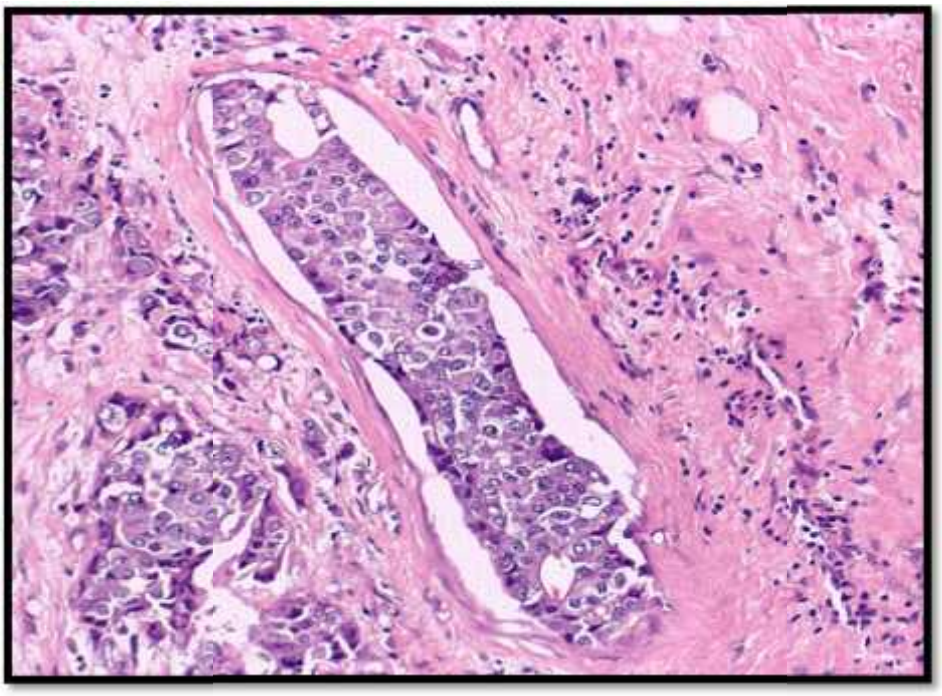


Figure B: Blood vessel with thick tunica media showing tumour emboli. (H&E-20X)

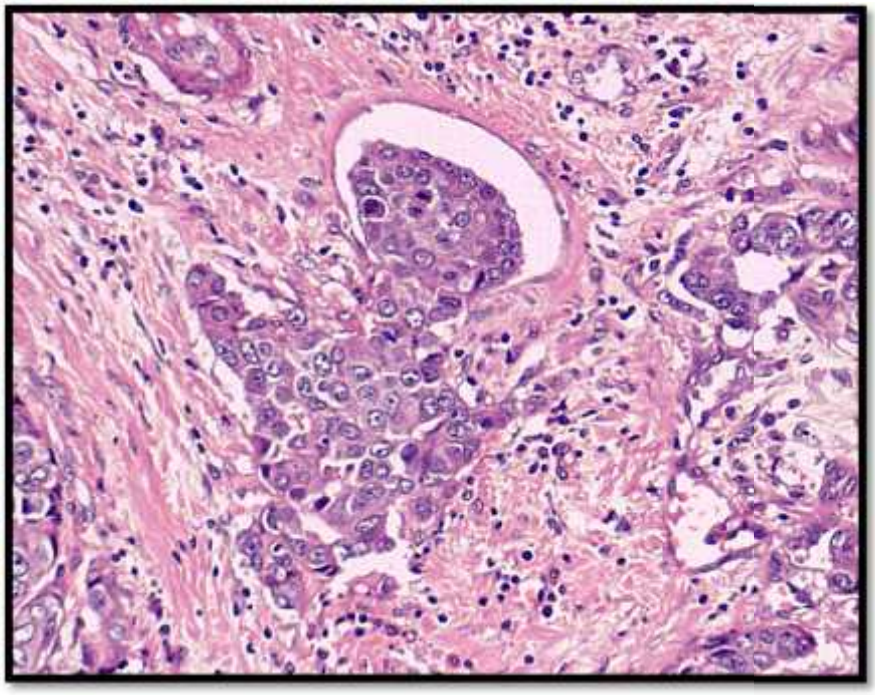


Figure C: Retraction artefact which can be misinterpreted as tumour emboli on H&E staining. (20X)

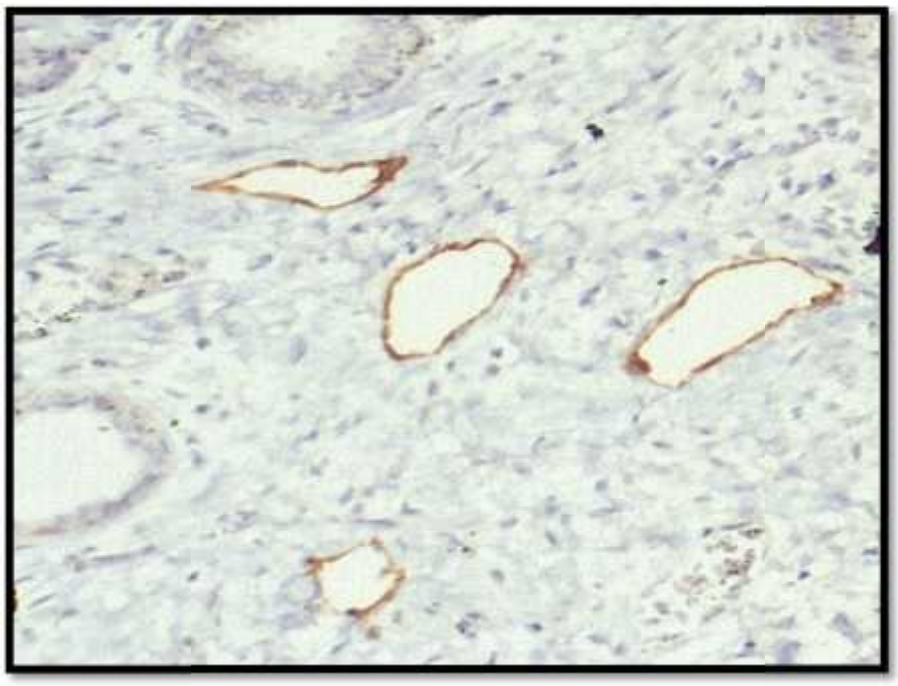


Figure D: Positive immunostaining of lymph vessels for D2-40. No tumor emboli are noted within the lumen of these lymph vessels (10X)

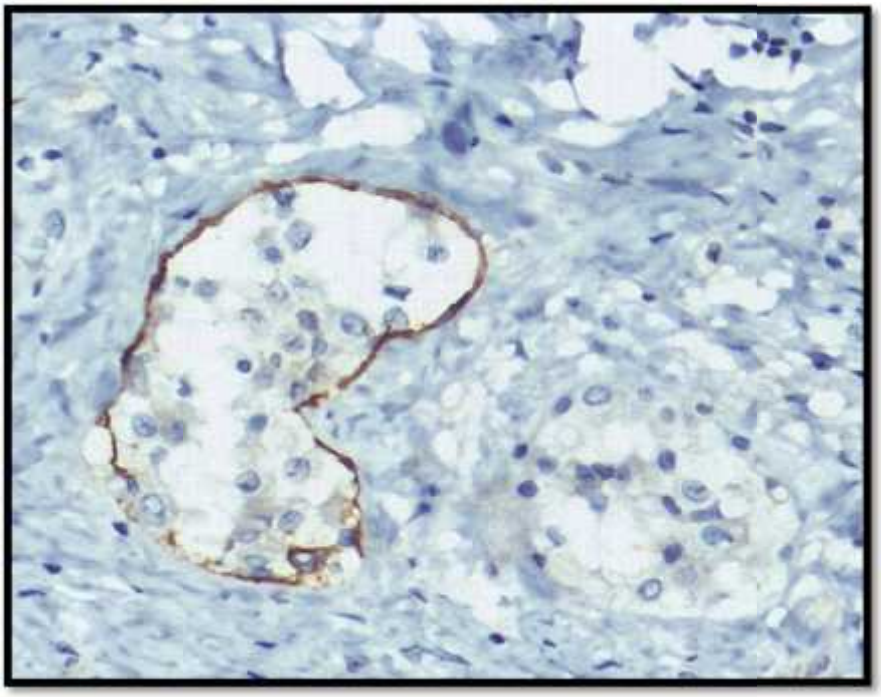


Figure E: D2-40 staining the lymphatic endothelium with good intensity. The lumen shows tumor cells. (20X)

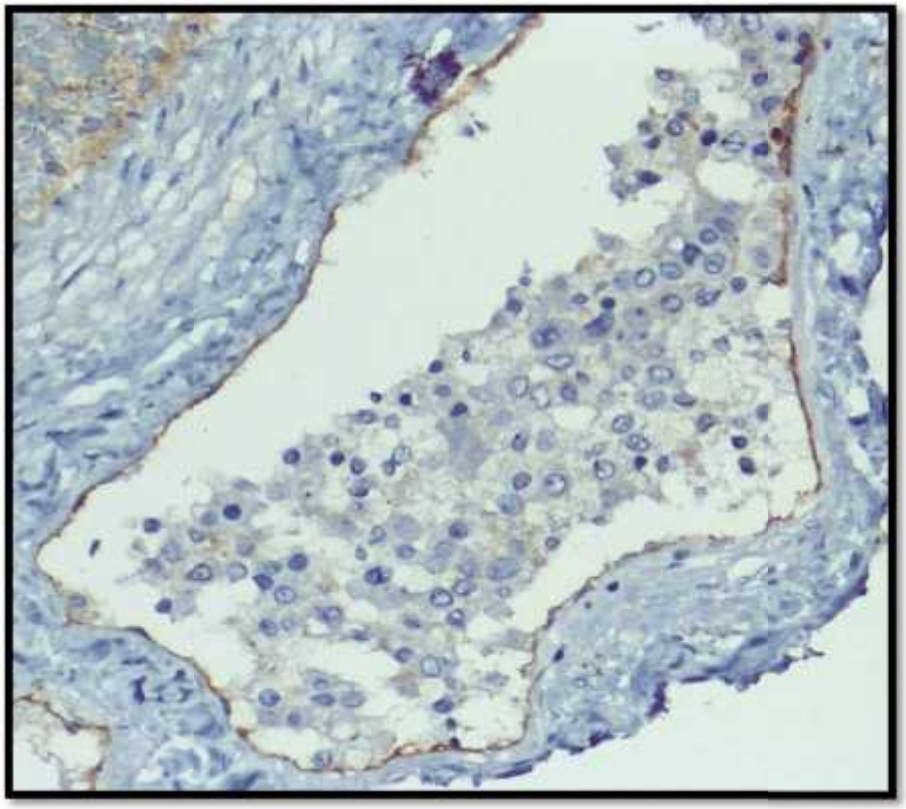


Figure F: A large tumor emboli within a D2-40 stained lymphatic vessel. (20X)

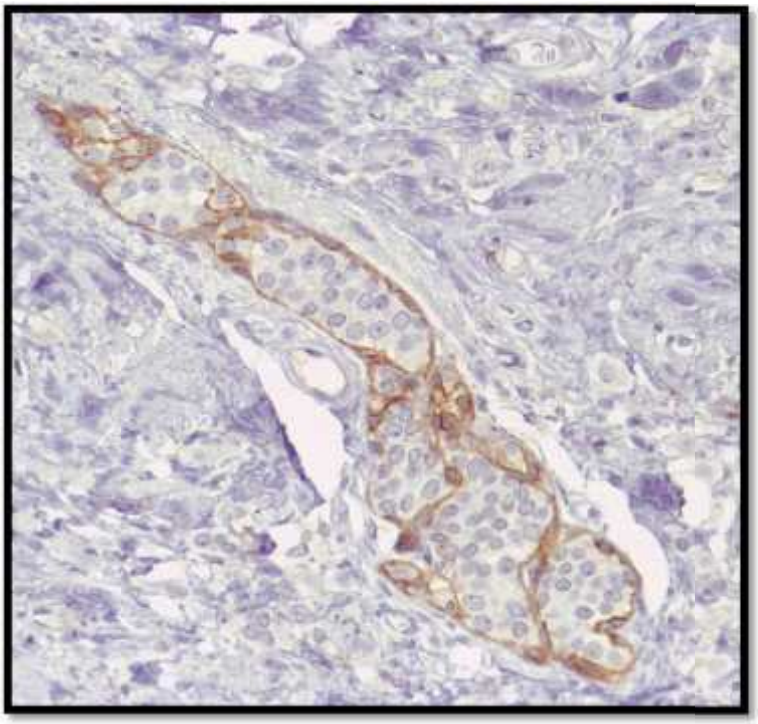


Figure G:Positive staining of lymphatic endothelium with D2-40 outlining the tumor emboli (20X). Tumor emboli was not visualized on the H&E section as it completely obliterated the lumen.

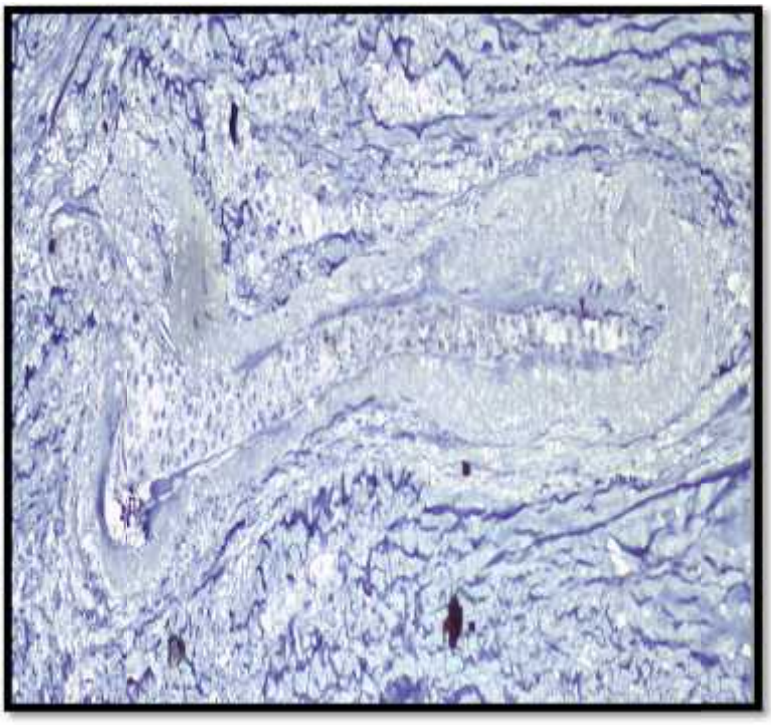


Figure H: Angio-vascular invasion by tumor cells (10X). D2-40 does not stain the blood vessel endothelium.

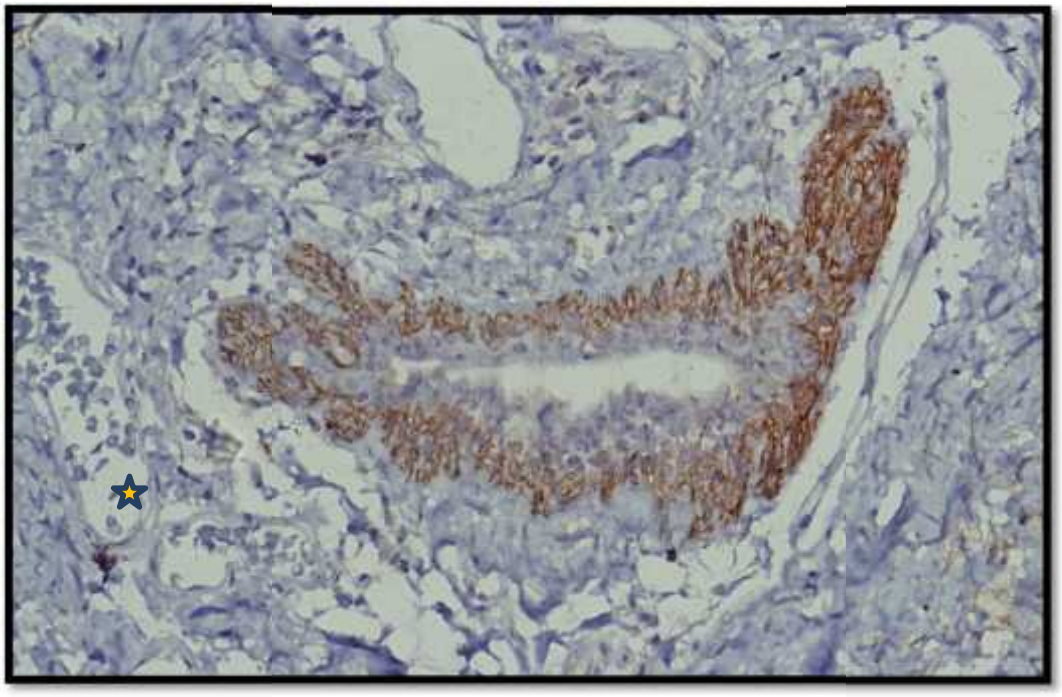


Figure I: Cross reactivity of D2-40 with myoepthelium in the peritumoral normal foci of breast tissue (20X). The adjoining focus shows a blood vessel negatively stained with D2-40 containing RBCs in the lumen.

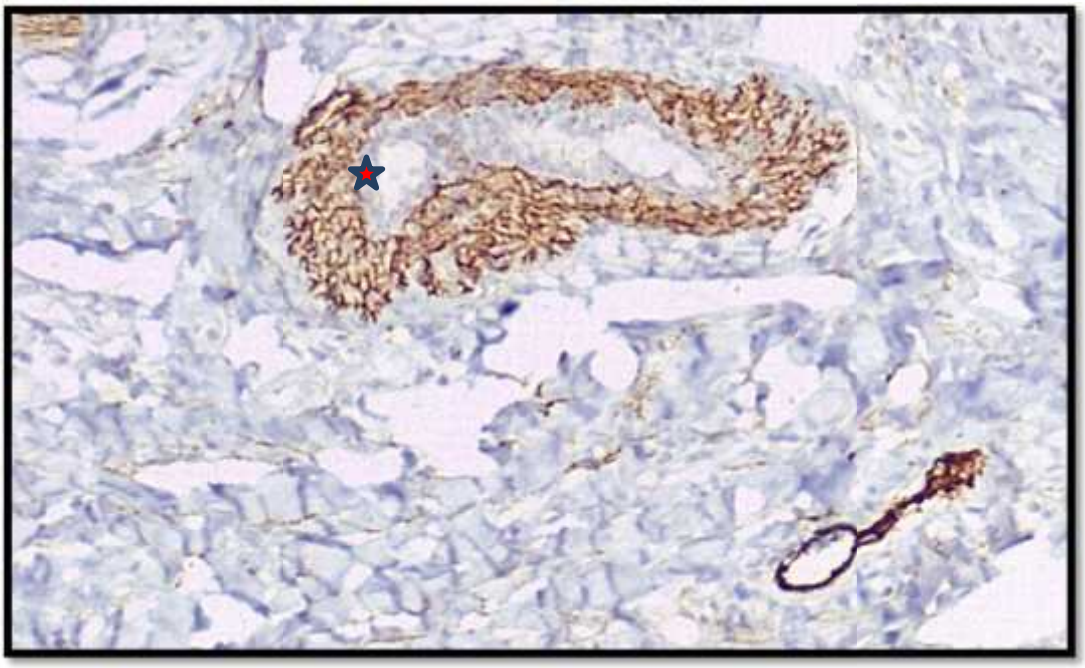


Figure J: D2-40 showing difference in staining pattern and intensity between myoepithelium and lymphatic endothelium (10X).

ANNEXURES VI - MASTER CHART

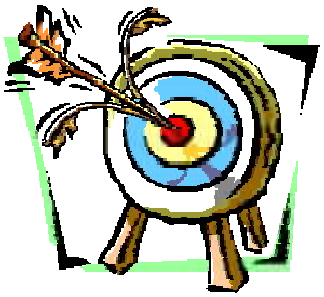
Sl. No	Age (years)	HPR Diagnosis	Tumor size (cm)	ALNM	Tumor Grade	LI (H&E)	LI (D2-40)
1	55	IDC; DCIS	7	+	2	-	+
2	50	IDC	2	-	2	-	-
3	41	IDC	4.5	-	2	-	+
4	49	IDC	4	+	2	-	+
5	57	IDC	13	+	2	+	+
6	83	IDC; DCIS	5	+	2	-	-
7	63	IDC	3	-	3	-	-
8	53	IDC	2.5	+	3	-	+
9	50	IDC	2	+	2	-	+
10	36	IDC	2.5	+	2	+	+
11	65	IDC	3	+	3	+	+
12	30	IDC	3	-	2	-	+
13	68	IDC	4	+	2	-	+
14	60	IDC	6	-	3	-	-
15	86	IDC	3	+	2	+	+
16	61	IDC	4.5	-	2	-	-
17	68	IDC	6	+	2	+	+
18	65	IDC	5.5	+	2	-	+
19	59	IDC	6	+	2	+	+
20	54	IDC	3	+	2	+	+
21	62	IDC	3	+	2	+	+
22	58	IDC	4.5	+	2	-	-
23	45	IDC	7.5	+	3	-	+
24	58	IDC	3	+	3	-	+
25	46	IDC	2.5	+	2	-	+
26	40	IDC	4	+	2	-	+
27	50	IDC	6	+	2	-	-
28	74	IDC	4	+	2	-	-
29	63	IDC	6.5	+	2	-	-
30	87	IDC	6.5	-	2	-	+
31	43	IDC; DCIS	4	+	2	+	+
32	64	IDC	3.5	+	2	-	+
33	41	IDC; DCIS	7	+	3	-	-
34	35	IDC	5.8	+	2	-	+
35	51	IDC	6	+	2	-	-

ANNEXURE-VII

KEY TO MASTER CHART

cm	–	Centimeter
DCIS	–	Ductal Carcinoma In-Situ
HPR	–	Histopathology
IDC	–	Invasive Ductal Carcinoma
ALNM	–	Axillary Lymph node metastasis
LI	–	Lymphatic invasion.
+	–	Present
-	–	Absent

Introduction



Objectives

Review of Literature

Methodology

Results

Discussion

Conclusion

Summary

Bibliography

Annexure-I

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Annexure-II

Annexure-III

Annexure-IV

Annexure-V

Annexure-VI

Annexure-VII