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Detection of Lymphovascular Invasion in Invasive Ductal Carcinoma of Breast: Comparison of Results of Histochemical Stains and Immunohistochemistry

Shaikh Alamgir Hossain[1], Mohammed Kamal[2], A K M Maruf Raza[3] , Nasima Akter[4], Tarana Tabashum[5], Tamanna-E-Nur[6], Zaman Ahmed[7], Fairuz Faiza[8]

[1]*Senior Consultant (Pathology), Bangladesh Secretariat Clinic, Dhaka, Bangladesh .*
[2]*Professor, Department of Pathology, Bangabandhu Sheikh Mujib Medical University, Dhaka, Bangladesh.*
[3]*Associate Professor of Pathology, Jahurul Islam Medical College, Kishoregonj, Bangladesh.*
[4,5]*Associate Professor of Pathology, East West Medical College, Dhaka, Bangladesh.*
[6]*Associate Professor of Pathology, Army Medical College, Jashore, Bangladesh.*
[7]*Assistant Professor of Pathology, Abdul Malek Ukil Medical College, Noakhali., Bangladesh.*
[8] *MBBS, Jahurul Islam Medical College Hospital, Kishoregonj, Bangladesh.*
Corresponding Author: Dr. Shaikh Alamgir Hossain

Abstract: *Breast cancer is the most commonly occurring cancer in women worldwide and it is the third leading cancer in Bangladeshi women. Lymphovascular invasion (LVI) in breast cancer is established as an important prognostic factor and is defined as tumor emboli present within a definite endothelium-lined space in the breast surrounding invasive carcinoma. The existence of LVI help identify who is at increased risk for axillary lymph node and distant metastasis. The aim of this study was to compare the LVI detection using routine H&E stain,PAS stain and immunohistochemistry. It was a cross sectional analytic study where fifty cases of histologically diagnosed invasive ductal carcinoma of breast were included. Microscopic detection of vessels and lymphovascular invasion and comparison between findings of H&E and PAS stained sections with that of VWF & CD34 stained sections were carried out. This study proved that using only histochemical or immunohistochemichal method individually was not that much beneficial for the patients of invasive ductal carcinoma to find out LVI. It would also be noted that limitations like small sample size and absence of prognostic data did exist in this study.*

Keywords: *Invasive ductal carcinoma, lymphovascular invasion, endothelial cells, H&E stain, PAS stain, CD34 stain, Von Willebrand factor.*

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I. Introduction

Breast cancer is the most commonly occurring cancer in women, comprising almost one third of all malignancies in females. A woman who lives to the age of 90 has a one in eight chance of developing breast cancer [1]. According to World Health Organization (WHO) cancer report in 2005, it is the third leading cancer in Bangladeshi women[2]. Breast carcinomas are notorious for their invasive and metastasizing potential. These tumours have several prognostic and predictive factors. The prognostic factors can be used to predict the natural history of breast cancer, so they are included in the decision to apply adjuvant systemic chemotherapy for patients with breast cancer [3].

Lymphovascular invasion (LVI) is defined as tumor emboli present within a definite endothelium-lined space in the breast surrounding invasive carcinoma[4]. Cells that have acquired invasive potential must invade the host stromal tissue before local invasion. This is followed by sequestration through endothelium-lined lymphovascular spaces. Theoretically, once lymphovascular invasion (LVI) has occurred, tumour cells have the potential to metastasize to regional lymph nodes or distant sites. So the existence of LVI may help identify who is at increased risk for axillary lymph node and distant metastasis[5]. Currently, the standard method for assessing LVI is light microscopic examination of haematoxylin and eosin (H&E) stained sections. Microscopic detection of LVI depends on identifying tumour cells within a confined space lined by endothelial cells and has three important limitations. First, tumour cells within lymphovascular spaces may evade detection on H&E staining if they are present in very small numbers, surrounded by a greater number of circulating cells, or have been trapped within a fibrin clot. Second, it is often difficult to differentiate tumour cells that completely fill up a lymphovascular space from stromal invasion. Finally, artifactual tissue retraction around tumour islands complicates the identification of true LVI. The limited ability of H&E staining to identify isolated tumour cells

has been overcome by using immunohistochemistry (IHC) for various endothelial cell markers such as Von Willebrand Factor (VWF), CD34[6]. VWF is synthesized in the endothelial cells of blood vessels and is widely used as a marker of endothelial cell differentiation [4]. Periodic acid-Schiff (PAS) stain is used to demonstrate tissue carbohydrate. Basement membrane contains a mucoprotein which is a PAS-positive carbohydrate[7]. Vascular channels are well delineated by PAS rections due to its property to stain basement membrane collagen. So vascular channels containing tumour emboli can be identified and it will act as an adjunct with H&E stain to increase the likelyhood of having more LVI in tumour sections. Nevertheless it is cost effective and easy to perform.

Though very important, a few studies have been done in this field. Till date inBangladesh no such study has been conducted so far known. So the aim of this study was to compare the LVI detection using routine H&E stain and immunohistochemistry.

II. Material and Methods

This prospective cross sectional analytical study was carried out at the Department of Pathology, BSMMU , Dhaka, Bangladesh from January 2011 to March 2013. Total 50 cases of Surgically resected formalin fixed mastectomy and lumpectomy specimens having invasive ductal carcinoma of breast were included in the study.

Inclusion Criteria:

Histologically diagnosed lymph node negative and lymph node positive cases of invasive ductal carcinoma of breast.

Exclusion criteria:

i) Tumour with extensive areas of necrosis
ii) Ductal carcinoma in-situ (DCIS)

Statistical analysis:

Statistical analyses of the results were obtained by window based computer software devised with Statistical Packages for Social Sciences (SPSS-17). The results were calculated by using statistical formulas and were presented in tables and figures.

Laboratory methods:

Sample selection:

During the period from January 2011 to March 2013, 199 cases of mastectomy and lumpectomy specimens were received in the Department of Pathology, BSMMU. Histologically 190 cases were diagnosed as invasive ductal carcinoma, NOS from which 50 cases were included in the study. So most cases belonged to this group. On the basis of suspicion of LVI which were mostly confused with retraction artifacts, immunohistochemistry (IHC) was perfomed to isolate them. As this procedure was much costly, only 50 cases were selected.

Grossing technique:

3 mm thick tissue blocks were cut including some peritumoural area.(Appendix-II)

Tissue processing:

Tissues were processed according to routine histopathology processing protocol of BSMMU.

Microtome sectioning:

4 micrometer thick, four consecutive sections were cut from each block and four slides were made.

Staining of slides:

The slides were stained with H&E stain, (Appendix-IV). From the cases diagnosed as invasive ductal carcinoma, NOS by H&E stain, 50 cases were selected for PAS stain (histochemical stain) and CD34 and VWF stain (immunostains) on the basis of suspicion of LVI in H&E slides.

Methods used for immunohistochemistry:

DAKO EnVision™ +/HRP (Horseradish peroxidase) system which was based on advance Labeled Strept Avidin-biotin (LSAB) method was used for visualizing the section. This method was carried out manually.

Antibody and control used in Immunohistochemistry

Monoclonal antibody CD34 was used to see the reactivity for CD34 antigen which is a single chain transmembrane protein expressed on the cell surface membrane of vascular endothelial cells. It also reacts with class II epitope. Monoclonal Mouse Anti-human CD34 Class II, clone QBEnd-10, Code M7165 was used for this purpose. In demonstrating cytoplasmic immunorectivity for endothelial cells, Polyclonal Rabbit Anti-Human Von Willebrand factor, Code No. A0082 was used. In this study, sections of normal vermiform appendix was taken as positive control.

Specimen preparation for CD34 and VWF immunostaining:

Formalin fixed paraffin embedded tissue sections from invasive ductal carcinoma of breast (test) and from appendix (control) were pretreated by heat-induced method with DAKO Target retrieval solution code S1699 at high pH 6. Care was taken to prevent drying out tissue sections.

Microscopic examination of H&E stained sections:

1. At first tumours were histologically graded according to the Nottingham modification of the Bloom Richardson histological grading system.
2. Number of vessels (blood vessel and lymph vessel) were counted per 4 mm^2 area
3. Number of vessels containing tumour emboli were counted per 4 mm^2 area.

Microscopic examination of PAS stained sections:

1. Basement membrane of the blood vessel was noted.
2. Number of vessels (blood vessel and lymph vessel) were counted per 4 mm^2 area.
3. Number of vessels containing tumour emboli were counted per 4 mm^2 area.

Microscopic examination of immunohistochemically stained sections of CD34 :

1. Endothelial cells were identified by staining pattern of CD34 (cell surface membrane).
2. Numbers of vessels (blood vessel and lymph vessel) were counted per 4 mm^2 area.
3. Numbers of vessels containing tumour emboli were counted per 4 mm^2 area.

Microscopic examination of immunohistochemically stained sections of VWF:

1. Endothelial cells were identified by staining pattern of VWF (diffuse or sometimes slightly granular staining in the cytoplasm).
2. Numbers of vessels (blood vessel and lymph vessel) were counted per 4 mm^2 area.
3. Numbers of vessels containing tumour emboli were counted per 4 mm^2 area.

All findings were noted on data sheet. Individual and combination results for H&E, PAS, CD34 & VWF were recorded and comparison between the results were done. Statistical analysis was performed to find out any significant difference.

III. Result

Total 50 cases of invasive ductal carcinoma of the breast were included in the study. Each section was stained with H&E, PAS, CD34 and VWF stain. Number of tumour emboli detected by each method and detected by combination of the staining methods were recorded by examining the whole of the marked area (approximately 4mm^2) on the slide containing tumour including peritumoral area. This area of section was measured by performing ocular micrometry. Number of blood vessels and lymphatics were counted in each section of H&E, PAS, CD34 and VWF stain. Similarly the vessels containing tumour emboli were counted and findings were compared between histochemical and immunostained sections (Figure 1,2).

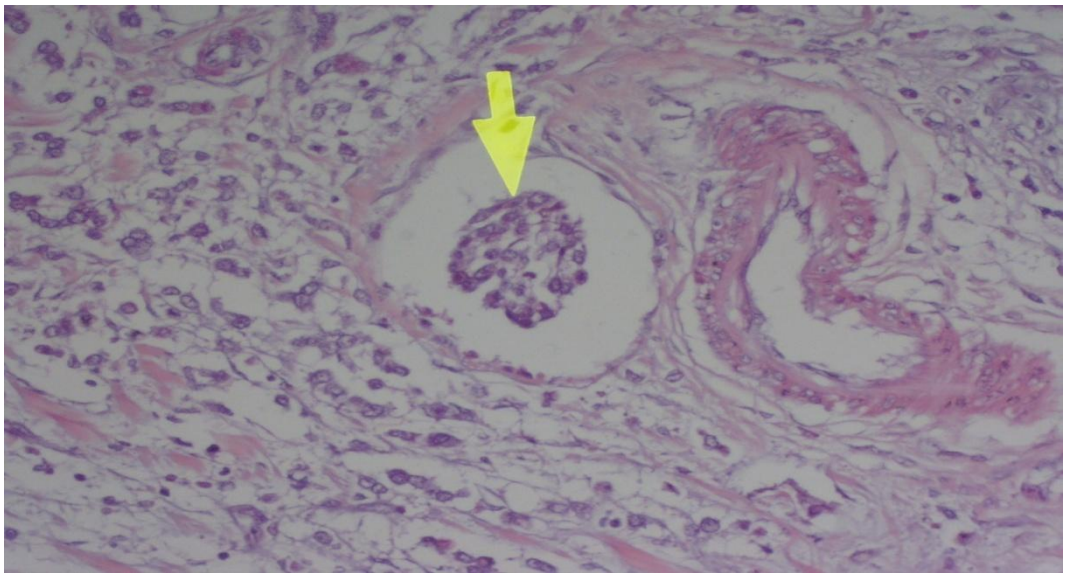
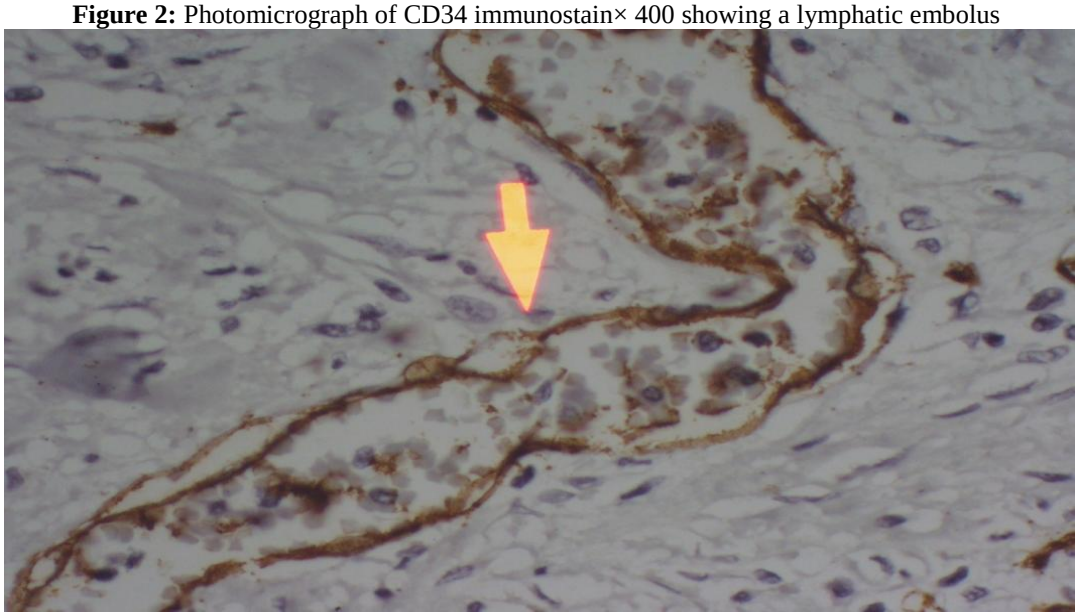


Figure 1: H & stain **× 200,** Photomicrograph of an invasive ductal carcinoma of breast showing a venous embolus.

Figure 2: Photomicrograph of CD34 immunostain× 400 showing a lymphatic embolus



The age range was 22 to 110 years with a mean age of 46 years including a woman who was at the extreme age of 110 years. Family history among the first degree relatives was present in five (10%) cases. Thirty (60%) cases were at postmenopausal age group 20 (40%) cases were premenopausal. Four (8%) cases had history of tumour recurrence. Only 3 (6%) cases did not give any history of breast feeding. Twenty nine (58%) cases took oral contraceptive pills. In 8 (16%) cases there was history of nipple discharge (Table 1).

Table 1: Demographic status of 50 patients

Total number of patients (n=50)	Age distribution	Family history	Menstrual history	History of previous surgery	History of breast feeding	History of contraception	Nipple discharge
Total 50 cases of invasive ductal carcinoma, NOS	Age range = 22-110 years Mean age = 46 years	Present = 5 cases(10%) Absent= 45 cases (90%)	Premenopausal = 20 cases (40%) Postmenopausal = 30 cases (60 %)	Present = 4 cases (8%) Absent= 46 cases (92%)	Present = (47 cases (94%) Absent= 3 cases (6%)	Present = 29 cases (58%) Absent= 21 cases (42%)	Present = 8 cases (16%) Absent= 42 cases (84%)

Forty five cases were radical mastectomy specimens. Twenty four cases had left sided breast tumour accompanied by axillary lymphnode sampling. Eight cases were found in N1 stage, 12 in N2 and 9 in N3 stage group. Histomorphological analysis revealed 2 cases with tumour size ≤ 2 cm. In 26 patients the tumour size range was between 2-5 cm (the largest group) and 12 cases belonged to tumour size of more than 5 cm. One patient had multicentric tumour (3 tumours). Microscopically grade II tumours were found in most cases (37 cases). Grade I and grade II tumours were found in 4 and 9 cases respectively (Table 2).

Table 2: Tumour characteristics and nodal status

Specimen type	Laterality of the lump	Lymph node Sampling	Tumour size	Tumour grade
Mestectomy = 45 cases (90%) Lumptectomy = 5 cases (10%)	Right sided= 19 cases (38%) Left sided=24 cases (48%) Not mentioned=7 cases (14%)	NX=11 cases (22%) NO =10 cases (20%) NI= 8 cases (16%) N2=12 cases (24%) N3=9 cases (18%)	T_1=2 cases (4%) T2=26 cases (52%) T3=12 cases (24%) T4=10 cases (20%)	Grade I =4 cases (8%) Grade II = 37 cases (74%) Grade III = 9 cases (18%)

The intratumoural and peritumoural lymphovascular spaces (total number of vessels and total number of emboli) were counted separately in H&E, PAS, CD34 and VWF stained sections of the defined 4 mm^2 area (Table 3).

Table 3: Total number of vessels and total number of emboli in 50 cases

Staining methods	Total number of vessels in 4 mm^2 area	Total number of emboli in 4 mm^2 area
H & E	202	30
PAS	173	35
CD34	226	39
VWF	253	36

Detection of LVI according to different staining methods in all 50 cases and their statistical analysis was done. Kruskal-Wallis test was performed to find out any significant difference between LVI detection by H&E, PAS, CD34 and VWF stain when considered as single. Result of histochemical stain showed significant difference (P=0.027) with the result of immunohistohcemical stain when they were performed alone (Table 4).

Table 4: Detection of LVI according to different staining methods

Staining Methods	No. of cases positive for LVI	No. of cases negative for LVI	P value
H&E	26 (52%)	24 (48%)	0.027
PAS	23 (46%)	27 (54%)	
CD34	37(74%)	13 (26%)	
VWF	28 (56%)	22 (44%)	

Mann-Whitney U test was done to find out any significant difference between LVI detection by histochemical stain and immunohistochemical stain. Result of histochemical stain showed significant difference (P=0.028) with the result of immunohistohcemical stain when they were performed alone or in combination (Table 5).

Table 5 : Comparison between detection of lymphovascular invasion (LVI) by histochemical method (H&E & PAS) and immunohistochemical method (CD34 &VWF)

Staining Method	No. of cases positive for LVI (%)	No. of cases negative for LVI (%)	P value
H&E/PAS	26 (52%)	24(48%)	0.028
CD34/VWF	40 (80%)	10(20%)	

Mann-Whitney U test was done to find out any significant difference between LVI detection of H&E stain and combination of all staining methods (H&E, PAS, CD34 & VWF). Result of H&E stain showed significant difference (P= 0.018) with the result of immunohistohcemical stain when they were performed alone or in combination (Table 6).

Table 6 : Comparison between detection of lymphovascular invasion (LVI) by H&E and by combination of all methods (H&E/ PAS/ CD34 & VWF)

Staining Method	No. of cases positive for LVI (%)	No. of cases negative for LVI (%)	P value
H&E	26 (52%)	24 (48%)	0.018
H&E/PAS/CD34/VWF	41(82%)	9(18%)	

IV. Discussion

The present study detected 52% LVI in H&E sections. This result was higher than the result found in a study by Bettelheim[8]. He found that by haematoxylin and eosin preparations 11 out of the 30 lesions (36.6%) showed clumps of malignant cells within endothelium-lined spaces, regarded six cases as lymphatic and five as both lymphatic and vascular. In one similar study, LVI was detected in H&E stained sections in 17/123 cases (13.8%)[9]. This difference might be due to inclusion of more lymph node positive cases in this present study. In this study 58% cases were lymph node positive. Another reason might be the retraction artifacts which were regarded as lymphovascular spaces. On a pathological specimen of breast cancer, retraction artifact during histological processing mimics true lymphovascular invasion. A positive stain for CD34, VWF or CD31 differentiates true LVI from retaction artifact. Variable degree of retraction artifact may be seen in 16% to 60% cases of invasive breast carcinoma[10].

When PAS stain was used to detect LVI in this study, 46% cases were found positive. PAS stain when accompanied by H&E stain the detection of LVI increased to some extent. This was due to the reason that some LVI that were missed in H&E were present in subsequent sections. In the present study, H&E stain was positive for 26 cases and PAS stain for 23 cases. Twenty three cases were common for both H&E and PAS stain. So combination method showed 26 cases being positive for LVI.

CD34 stain was positive in 37 cases and VWF stain was positive in 28 cases. Twenty five cases were common for both CD34 and VWF stain. Three cases showed positivity for VWF stain (case numbers: 20, 27 and 44). Twelve cases showed positivity for CD34 stain (case numbers: 1, 2, 6, 8, 16, 18, 19, 21, 22, 33, 43 and 47). So combination method (CD34/VWF) showed 40 cases being positive. But when staining was performed alone or in combination i.e. (H&E/PAS/CD34/VWF) 41 cases became positive. This increment was due to the positivity of LVI in the H&E stained section (case number 35). Marinho et al[9] demonstrated that lymphatic and blood vessel emboli were found more frequently in the immunostained sections than in the H&E stained sections. This study found similar results as combined use of CD34 and VWF led to the detection of LVI in 80% cases. Significant differences (P=0.028) were found by performing Mann-Whitney U test. When all four stains used detection of LVI was upto 82% which showed significant difference (P=0.018) carried out from the same Mann-Whitney U test.

The topography of lymphatics in malignant tumours, particularly in breast cancer, has, for some time, been a hotly debated issue in the literature. Initial studies investigated lymphatics in xenografted sarcomas and reported that vessels were only found in peritumoural areas and absent from the intratumoural region[11]. With advances in imaging techniques, intratumoural lymphatics were detected and found to be functional, with tumour cells detected flowing within the vessels[12]. Controversy exists regarding lymphatics in human breast cancer, some studies reported absence of intratumoural lymphatics and others reported their presence[13].

This short histological survey has confirmed the pathological value of an immunohistochemical approach to the detection of lymphovascular invasion in breast carcinomas. This study allows us to conclude that thin haematoxylin and eosin stained sections of primary mammary carcinomas with a generous stromal edge are adequate material for the histological diagnosis of vascular invasion.

V. Conclusion

LVI positivity was one of the most important prognostic factors of invasive ductal carcinoma of breast as it is directly correlated with the presence or absence of metastatic disease. Detection of LVI with routine histopathological H&E stain may sometimes be difficult and inaccurate due to retraction artifact. So here comes the role of special stain and immunohistochemical stain. From this study we came to know that the use of all the stains together (H&E, PAS, CD34 and VWF) significantly increased the detection of LVI compared to conventional H&E stain. This study also proved that using only histochemical and immunohistochemichal method individually was not that much beneficial for the patients of invasive ductal carcinoma to find out LVI. It would also be noted that limitations like small sample size and absence of prognostic data did exist in this study.

VI. Recommendation

Further study can be conducted in future to rule out the limitations of the current study by using newer vascular markers such as D2-40 which is specific for lymphatic endothelium. Others are Friend leukaemia-1 (FLI-1) protein, Ulex europaeus-1 lectin, CD31[4].

Ethical compliance:

For ethical clearance, the protocol was placed before the Institutional Review Board (IRB) of BSMMU and was approved. Informed consent was obtained for each subject for being included in this study.

Conflict of interest:

The authors declare that they have no conflict of interest.

References

[1]. Lester SC(ed). The breast in Kumar V, Abbas AK, Fausto N, Aster JC.Robbins and Cotran Pathologic basis of disease, Eighth edition, 2010 ,Published by Elsevier, pp,1065-1094.

[2]. Bangladesh Directorate General of Health Services, Ministry of Health and Family Welfare. The national cancer control strategy and plan of action 2009-2015, Dhaka: non communicable diseases and public health intervention. 2008:1-32.

[3]. Young SJ, Sun SHJCS, Min PH, Jung YH, Young JJ (2011). The Role of Lymphovascular Invasion as a Prognostic Factor in Patients with Lymph Node-Positive Operable Invasive Breast Cancer. J Breast Cancer 2011; 14:198-203.

[4]. Rosai J (ed.). Breast, Rosai and Ackerman's Surgical Pathology, 10th edition , 2011, vol-2 , Mosby , London, pp-1682-2106.

[5]. Davis B, Gelber R, Goldhirsch A, Hartmann W, Hollaway L, Russell I (1985). Prognostic significance of peritumoral vessel invasion in clinical trials of adjuvant therapy for breast cancer with axillary lymph node metastasis. Human Pathology 1985; 16:1212-1218.

[6]. Van den Eynden GG, Van der Auwera I, Van Laere SJ, Colpaert CG, van Dam P, Dirix LYet al. (2006). Distinguishing blood and lymph vessel invasion in breast cancer: A prospective immunohistochemical study. Br J Cancer. 2006; 94(11):1643-1649.

[7]. Bancroft JD, Gamble M (ed.). Theory and practice of histological techniques, 5th edition, 2002. Churchill Livingstone, London, pp-177.

[8]. Bettelheim R, Mitchell D, Gusterson BA (1984). Immunocytochemistry in the identification of vascular invasion in breast cancer. J Clin Pathol. 1984; 37:364-366.

[9]. Marinho V, Metze K, Sanches F, Rocha G, Gobbi H. Lymphovascular invasion in invasive mammary carcinomas identified by the endothelial lymphatic marker D2-40 is associated with other indicators of poor prognosis . BMC Cancer. 2008; 8:64.1-9.

[10]. Zaorsky NG, Patil N, Freedman GM, Tuluc M. Differentiating Lymphovascular Invasion from Retraction artifact on Histologic. Specimen of breast Carcinoma and their implication on Prognosis . J Breast Cancer. 2012; 15:478-480.

[11]. Leu AJ, Berk DA, Lymboussaki A, Alitalo K, Jain RK. Absence of functional lymphatics within a murine sarcoma: a molecular and functional evaluation, Cancer Res. 2000; 60: 4324-4327.

[12]. Pathak AP, Bhujwalla ZM, Pepper MS. Visualizing function in the tumor-associated lymphatic system, Lymphat Res Biol. 2004; 2:165-172.

[13]. Bono P, Wasenius VM, HeikkilA P, Lundin J, Jackson DG Joensuu H (2004). High LYVE-1-positive lymphatic vessel numbers are associated with poor outcome in breast cancer, Clin Cancer Res. 2004; 10:7144-7149.

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