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Original article:

Utility of D2-40 Immunostaining in assessment of lymphatic invasion in breast carcinoma

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Abstract

Background and Objectives: Breast cancer is one of the leading causes of deaths in females worldwide. It is the most common cancer in women worldwide and accounts for 20% of all cancers in women. The presence of vascular invasion in patients with carcinoma breast has been found to be a poor prognostic factor and is of two types- lymphatic vascular invasion and blood vascular invasion. In the present study we aim to detect lymphatic invasion in primary invasive breast cancer using D2-40 IHC marker and also to compare the incidence of lymphatic invasion by conventional H&E staining and immunohistochemical staining.

Materials and Methods: Study was an observational cross-sectional study in a Tertiary health care centre for a duration from July 2020 to June 2022 with a sample size of 40. Study population were modified radical mastectomy specimen. LVI was assessed by using H&E sections and D2-40 immunostaining, by two surgical pathologists.

Results: LVI was detected in 29 (72.5%) cases by H&E and in 21 (52.5%) cases by D2-40 IHC stain. Nineteen out of 29 cases with positive LVI in H&E were confirmed by D2-40 and 2 out of 11 cases with negative LVI in H&E. Kappa coefficient between H and E and D2-40 staining was 0.386 and p-value was 0.0074

Conclusion: The use of the D2-40 IHC marker is helpful in the diagnosis and confirmation of lymphovascular invasion in invasive carcinoma of the breast. D2-40 is a specific marker for lymphatic type of vascular invasion.

Introduction:

Breast carcinoma is the most common malignancy in females worldwide and is one of the leading cause of cancer death in developed countries.[1-4] Early detection of breast cancer due to availability of advanced screening modalities increases the incidence of in developed countries[5]. However, recurrence of the carcinoma and distant metastasis, rather than a primary tumour, are the prominent causes of death in patients with

carcinoma breast. Increasing number of patients are presenting with early stage, lymph node negative disease. However not all such patients have good prognosis.(6)

The presence of vascular invasion in patients with carcinoma breast has been found to be a poor prognostic factor and is of two types- lymphatic vascular invasion and blood vascular invasion. Vascular invasion in breast cancer is predominantly of lymph vessels 6 and is powerful independent prognostic factor and is associated with increased risk of recurrence and death from the disease.(7)

Lymph node metastasis is the main independent prognostic factor, and the lymphatic system is the major route for tumor dissemination. The term vascular invasion is used whenever blood or lymphatic vessels are invaded by tumoral cells. In fact, it has been proved that tumoral cells can trigger the growth of tumor-associated lymphatic vessels and eventually enter them.(8)

Vascular invasion is detected microscopically, and is defined as the presence of tumour cells within the blood or the lymphatic vessels using hematoxylin and eosin (H&E) staining. However, H & E stain slides have high rate of false interpretation between lymphovascular invasion and artifactual clearing around tumour. Immunohistochemistry is one of the most reliable indicators of lymphovascular invasion of cancer cells.(9)

D2-40 is a monoclonal antibody on lymphatic endothelial cells but not on blood endothelial cells. While CD31 and CD34 are markers present on blood endothelial cells. (10) It is a monoclonal antibody that detects podoplanin, mucin-type transmembrane glycoprotein's on the endothelium of lymphatic vessels.(11) D2-40 plays an important role in detecting lymphovascular invasion of tumors. Immunostaining with D2-40 significantly increased the accuracy of detection of lymphatic invasion compared to routine H&E staining in early breast cancer.(12)

Hence, in the present study we aim to detect lymphatic invasion in primary invasive breast cancer using D2-40 IHC marker and also to compare the incidence of lymphatic invasion by conventional H&E staining and immunohistochemical staining.

Aims and objectives:

- To assess utility of D2-40 IHC staining in identification of lymphatic invasion in primary invasive breast cancer.
- To compare the incidence of lymphatic invasion by conventional H&E staining and immunohistochemical staining in primary invasive breast cancer.

Materials and methods:

This study is an observational cross-sectional study that was conducted in a tertiary health care centre. Study duration was from July 2020 to June 2022. Study population was of total of 40 cases of histologically confirmed surgical specimen of primary breast carcinoma. This study was conducted after getting ethical approval from the Ethical Committee.

The inclusion criteria were as follows: Histopathologically confirmed cases of primary invasive breast carcinoma, lumpectomy or mastectomy specimens received along with lymph nodes. Exclusion criteria were as follows: Biopsy samples, Simple mastectomy specimen without lymph node resection, Recurrence cases will be excluded. Clinical details such as age, sex, menstrual status, chief complaints, personal and family history, obstetric history will be obtained from histopathological requisition form.

Tissue samples from carcinoma specimen were sliced at 4-5mm thickness and fixed in 10% neutral buffered formalin for 24 hours. Gross examination was done in detail. Lymph nodes dissection was done wherever

possible. Several sections were taken after proper gross examination of the specimen- from tumour mass, from nipple areola complex, from adjacent breast tissue, from posterior margin, and all dissected lymph nodes were submitted for histopathological examination. Formalin fixed paraffin embedded sections were taken. A 4-5µm-thick section were taken and stained with H & E and evaluated by two independent pathologists. Histopathological study of H&E sections was done to assess histological grade, histological subtype, axillary lymph nodal status and lymphovascular invasion.

For immunohistochemistry, the material required was purchased from Biogenex Life Sciences. The panel of antibody included were against ER, PR, HER2 and D2-40. The antibodies provided are already diluted, ready to use. Known positive controls were used with each batch of IHC performed. Tissue section taken on APES/Poly L-lysine coated slides. Baking in incubator overnight at 60^0C. Deparaffinize sections in xylene, 2-5 minutes. Rehydrated with 100% ethanol, 2-3 min. Rehydrated with 95% ethanol, 1 min and rinsed in distilled water. Antigen retrieval using EZ-Retriever system was done. 10 minutes at 95^0C in citrate buffer, pH 6 followed by PBS wash, 3 times. Then Peroxide block 10 minutes at room temperature and slide kept in humid chamber. PBS wash, 3 times. Draw a hydrophobic barrier around tissue using PAP Pen. PBS wash, 3 times. Power block (100µL). Primary Antibody (100µL) PBS wash, 3 times Super EnhancerTM (100µL) then 20 minutes at room temperature (20-25^0C) PBS wash, 3 minutes Polymer-HRP (100µL) kept for 30 minutes at room temperature (20-25^0C) PBS wash, 3 times. 1 drop of DAB chromogen in 1 mL stable DAB buffer this is added to the tissue and incubate for 5 minutes at room temperature. Washed under running tap water. Haematoxylin counter stain (100µL). Then mounted.

The diagnostics, typing, and grading of breast pathology were performed according to the World Health Organization Classification of Tumours (5^{th} edition) [13]

The lymphatic vessel invasion was considered positive if atleast one tumour cell cluster was clearly visible inside lymph vessel identified by D2-40 IHC.[14-17]

Statistical analysis: The data was collected and entered in a systematic format in Microsoft excel 2013. It was checked for any duplicate or incomplete entries. All the parameters in the data were analysed for mean, frequency, percentage. The statistical analysis for correlation among these parameters was determined using Kappa test and Chi-square test. The Statistical Package for Social Sciences (SPSS) 22.0; IBM Analytics, New York, U.S.A was used for the analysis. All p values < 0.05 was considered to be statistically significant. The data collected was coded and entered in Microsoft excel 2013.

Result and observations

In our study, there were a total of 40 cases of modified radical mastectomy. The Mean age of the study participants in our study was 55.3 years. LVI was detected in 29/40 (72.5%) cases by H and E stain and in 21/40 (52.5%) cases by D2-40 IHC staining method. The clinicopathologic characteristics of the tumour are shown in Table. 1

Table No 1: Clinicopathological characteristics of the cases

Tumour characteristic	Number of Cases (Percentage%)
Grade 1	13(32.5)
Grade 2	21(52.5)
Grade 3	6(15)
LVI present in H&E	29(72.5)
LVI absent in H&E	11(27.5)
LVI present in D2-40	21(52.5)
LVI absent in D2-40	19(47.5)

Table No 2: Lymphovascular invasion detection by H&E and D2-40 IHC methods

	LVI present in H&E	LVI absent in H&E	Total	p value
LVI present in D2-40	19	2	21	
LVI absent in D2-40	10	9	19	**0.0074**
Total	**29**	**11**	**40**	

*Abbreviations: LVI, Lymphovascular invasion; H&E, hematoxylin and eosin; IHC, Immunohistochemistry

The above table shows that 10 cases with identification of LVI in H&E slides were not confirmed by D2-40, indicating rather mis/over-interpretation of the H&E method mainly caused by retraction artefact. Two cases negative for LVI in H&E group were positive with D2-40 stained sections.

The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were 65.51%, 81.81%, 90.47% and 47.36% respectively.

Kappa coefficient factor for H and E and D2-40 IHC staining was 0.386 (Fair agreement) and the difference between these two methods was significant (p=0.0074). The findings in our study were in concordance with the study done by Vosough et al[18] and Abbasi et al[19]

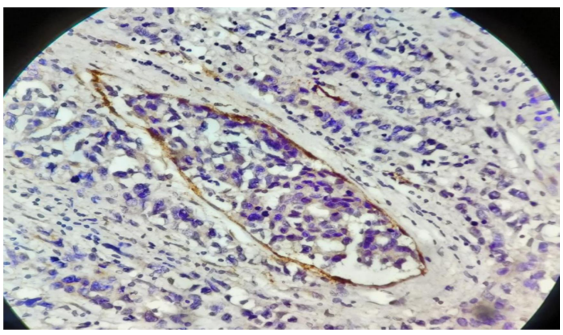

Fig. 1: Lymphovascular invasion on D2-40 IHC stain

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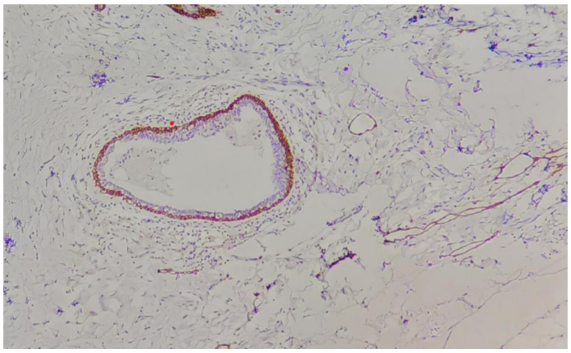

Fig. 2: Myoepithelial cell layer positivity and lymphatic vessel positivity (Clearly differentiable) on D2-40 IHC stain

Table No 03: Correlation between clinicopathological characteristics and D2-40 staining results

Clinicopathological Characteristic		**D2-40 positive No. of patients(%)**	**D2-40 Negative No. of patients(%)**	**P-value**
Age (Years)	≤40	1 (25%)	3(75%)	**0.246**
	>40	20 (55.55%)	16(44.45)	
Tumour size (cm)	≤2	1(25%)	3(75%)	**0.246**
	>2	20(55.55%)	16(44.45%)	
Tumour grade	Grade I	5(38.46%)	8(61.54%)	**0.409**
	Grade II	13(61.90%)	8(38.09%)	
	GradeIII	3(50%)	3(50%)	

DISCUSSION

Breast cancer is the most common malignancy in women around the world. LVI refers to invasion of lymphatic or blood vessels by tumour cells. According to the study done by Mohammed RAA et al (20), 97% of vascular invasion are of lymphovascular type and only 2-3% are of blood vascular invasion.

In our study, we used H&E and D2-40 IHC methods for the assessment of lymphovascular invasion in breast carcinoma. There was a noteworthy difference between H&E and D2-40 IHC method in the results. Twenty nine cases (out of 40) were interpreted as LVI present on H&E-stained sections and 21 cases were established positive by D2-40 IHC staining method. This difference in the result can be explained with retraction artifact caused during fixation (18). Retraction artifacts that isolate tumour cell aggregates due to shrinkage of tissue during fixation are sometimes jumbled with the true tumour emboli in lymphatic vessels.

D2-40 IHC method confirmed LVI in 19 cases out of 29 cases which were reported as LVI positive on H&E method. The difference in the results can also be attributable due to interfering role of blood vessel invasion with Lymphatic vessel invasion. An important limitation of our study was that we did not used CD31 and CD34 IHC stains that could have helped us in distinguishing blood vascular invasion from lymphovascular invasion. The difference in result could also be due to the tumor embolus, which completely filled the lumen of the lymphatic vessel. In addition to above reasons, it is difficult to identify vascular invasion, either lymphatic or

blood, as it can't be decided whether it is a lymph 93 vessel or a blood vessel on H&E slide method. For this reason, D2-40 was used which selectively stains the endothelium of lymphatic vessels.

Another issue mentioned in the literature regarding D2-40 is positivity in myoepithelial cells. [21][22] The positive staining of the myoepithelial cell layer in D2-40 IHC staining method can cause false positivity in cases of in situ carcinoma. In a study done by Rabban JT et al suggested simultaneous use of myoepithelial markers, such as p63 and D2-40, to differentiate between tumor emboli from in situ carcinoma.[23][24] Mohammed RAA et al [20] detected reactivity in myoepithelial cells in breast tissue that can lead to false-positivity assessment of LVI. However, in our study we could easily distinguish between myoepithelial cell reactivity and endothelial staining of lymph vessels. This finding was in concordance with study done by Mohammed et al [20] who also mentioned easy differentiation between myoepithelial reactivity and endothelial staining of the vessels by D2-40.

According to Mohammed RAA et al, [20] increasing number of patients are presenting with lymph node negative disease; but all those patients do not do well.[25][26] Hence, the presence or absence of vascular invasion is an important parameter that play an important role in the outcome of carcinoma breast. Therefore, it is important to highlight the detection of vascular invasion, including small and single ones.

CONCLUSION

In conclusion, the use of the D2-40 IHC marker is helpful in the diagnosis and confirmation of lymphovascular invasion in invasive carcinoma of the breast. D2-40 is a specific marker for lymphatic type of vascular invasion.

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