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Original Article

Pathology Section

Peritumoural and Tumour Infiltrating Lymphocytes in Breast Carcinoma and their Relation with Tumour Grade, Lymphovascular Emboli and Nodal Metastasis: A Cross-sectional Study

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ABSTRACT

Introduction: Inflammatory cell infiltrate in tumours may be involved in immunosurveillance or tumourigenesis. Tumour Infiltrating Lymphocytes (TILs; within the tumour) and peritumoural lymphocytes (at the invasive margin) are associated with improved prognosis and response to therapy.

Aim: To estimate the presence of peritumoural lymphocytes and TILs in breast carcinoma, identify their subsets by Immunohistochemistry (IHC) and assess the relationship between them and tumour grade, lymphovascular emboli and axillary lymph node metastasis.

Materials and Methods: This was a cross-sectional study with a sample size of 75 done in the Department of Pathology, Government Medical College, Kozhikode, Kerala, India from November 2017 to April 2019 with the approval of Institutional Ethics Committee. The demographic data of the patients were collected and histopathological assessment of tumour type, grade, lymphovascular emboli, nodal metastasis and lymphocytic infiltrate was done on Haematoxylin and Eosin (H&E) stained sections. Immunohistochemical evaluation was performed using antibodies against Cluster of Differentiation 4 (CD4) and CD8 and cells were scored separately in the stromal and peritumoural areas. The results were analysed by Analysis of Variance (ANOVA) and Chi-square test. Association between the immune infiltrate and histopathological variables were assessed separately in stromal and peritumoural compartments. Association between the score obtained on H&E and subpopulation score obtained on IHC was also analysed in both the compartments.

Results: Total of 75 subjects were included and the mean age was 52.31 years with a standard deviation of 9.774 years. The degree of stromal TIL infiltrate ranged from 10% to 95%, with a mean of 48.47%. High grade peritumoural infiltrate was seen in 66.67% (50 out of 75 cases). The stromal TILs were significantly higher in higher grade tumours and tumours without nodal metastasis. The peritumoural lymphocytes were also significantly higher in high grade tumours. The scoring of the immune infiltrate on H&E sections and subpopulation score on IHC showed significant association for both stromal and peritumoural compartments.

Conclusion: Stromal TILs and peritumoural lymphocytes were significantly higher in high grade tumours. Stromal TILs were also significantly higher in tumours without axillary lymph node metastasis. No significant association was found between the lymphocytic infiltrate and lymphovascular emboli. The immune infiltrate within breast carcinoma has association with tumour grade and lymph node metastasis.

Keywords: Breast cancer, Immune infiltrate, Immunohistochemistry, Prognostic value

INTRODUCTION

Invasive Breast Carcinoma (IBC) is the most common malignancy in women, accounting for nearly 25% of all malignancies in women [1,2]. The incidence of IBC in India is approximately 25.8 per 100,000 women [3]. Inflammatory cell infiltrate in tumours, referred to as TILs may be involved in immunosurveillance or tumourigenesis. Immunosurveillance is the mechanism by which the host immune effector cells actively search for and eliminate preneoplastic cell clones. Disease progression occurs when the tumour cells eventually escape from the immune control. This dual host protective and protumourigenic roles of the immune system is known as cancer immunoediting [4].

TILs are of two types based on their relationship to the tumour cell nests; intratumoural TILs lie within the cell nests and have cell-to-cell contact with the tumour cells without any intervening stroma, whereas stromal TILs lie in the stroma between the tumour cell nests without any direct contact with the tumour cells. The evaluation of intratumoural TILs in addition to stromal TILs is currently not recommended [5]. TILs are associated with a good prognosis in various malignancies, with improved relapse free and overall survival. The TIL density also shows correlation with tumour depth and Lymphovascular Invasion (LVI) [6]. Peritumoural lymphocytes are the immune infiltrate present at the invasive margin of the tumour. Similar to TILs, they have prognostic and predictive significance, with higher number of peritumoural lymphocytes at the invasive margin of melanoma metastasis correlating with improved response to immune checkpoint inhibition [7].

The immune infiltrate within tumours is heterogeneous. Various studies have shown that CD8+ cytotoxic T lymphocytes are the principal cells involved in tumour immunity, along with Type 1 T helper (Th1) CD4+ T lymphocytes, M1 macrophages, natural killer cells, Th17 CD4+ T cells and dendritic cells; whereas Th2 CD4+ T cells, FOXP3+ (forkhead box P3) regulatory T cells, M2 macrophages, neutrophils and B lymphocytes are likely involved in tumour progression [8,9].

TILs in breast cancer are associated with improved prognosis and response to therapy [5,10]. Relatively fewer studies have been reported from India in this regard. Hence, present study was conducted with the aim to estimate the density of peritumoural and stromal tumour infiltrating lymphocytic infiltrate in breast carcinoma, identify and score their subsets by IHC and assess the relationship between them and the tumour grade, presence of lymphovascular emboli and axillary lymph node metastasis.

MATERIALS AND METHODS

This cross-sectional study was conducted in the Department of Pathology, Government Medical College, Kozhikode, Kerala, India from November 2017 to April 2019. The study was approved by the Institutional Ethics Committee (Ref. No.GMCKKD/RP 2017/IEC/190 dated 15-11-2017). Informed consent was obtained from all patients included in the study.

Sample size calculation: Taking the proportion of cases of breast carcinoma with peritumoural lymphocytes and TILs as 58% [10],

By applying the formula $N=4pq/d^2$

p=prevalence=58 [10],

q=100-p=42

d=precision=20% of p=11.6

Sample size calculated is N=73.

Inclusion criteria: Initial consecutive 75 patients who had undergone mastectomy with proven IBC during the study period were included in the study.

Exclusion criteria: Postchemotherapy and postexcision biopsy mastectomy specimens were excluded from the study.

The clinicopathological parameters evaluated were patient's age, tumour size, tumour stage, histologic subtype and histologic grade. The tumours were subtyped according to the 2012 World Health Organisation (WHO) classification [1] and graded into grades 1, 2 or 3 as per the modified Bloom and Richardson grading [11], by microscopic examination of H&E stained Formalin Fixed Paraffin Embedded (FFPE) sections. The presence of lymphovascular emboli and axillary nodal metastasis were also assessed. The tumour stage was determined according to the American Joint Committee on Cancer (AJCC)/Union for International Cancer Control (UICC) staging criteria [12,13].

The H&E sections were examined for the presence and density of peritumoural lymphocytes and TILs (stromal TILs). The stromal TILs were quantitatively assessed as per the recommendations of the International TILs Working Group, as percentage of stromal TILs [5].

$$\% \text{ Stromal TILs} = \frac{\text{Area occupied by mononuclear inflammatory infiltrate}}{\text{Total intratumoural stromal area}} \times 100$$

The peritumoural lymphocytes were semi-quantitatively scored according to the method adopted by Klintrup K et al., into scores 0, 1, 2 and 3 [14], as given below. The scores 0 and 1 were collapsed into low grade inflammation and scores 2 and 3 into high grade inflammation:

- Score 0=No increase in inflammatory cells.
- Score 1=Mild and patchy increase in inflammatory cells at the invasive margin, but no destruction of invading cancer cell islets by the inflammatory cells.
- Score 2=Inflammatory cells forming a band-like infiltrate at the invasive margin with some destruction of cancer cell islets by inflammatory cells.
- Score 3=Very prominent inflammatory reaction, forming a cup-like zone at the invasive margin, with invariable destruction of cancer cell islets.

Formalin fixed paraffin embedded tissue sections with maximum lymphocytic infiltrate, exclusive of hemorrhagic and necrotic areas, were subjected to manual IHC staining for CD4 (Clone: EP204, Isotype: Rabbit IgG, PathnSitu, Livermore, USA) and CD8 (C8/468, Isotype: Mouse IgG1, PathnSitu, Livermore, USA).

The immunostained slides were examined to identify the individual cell type within the infiltrate. A semi-quantitative measurement of the infiltrate was done under light microscope, independently by two pathologists, based on a modification of the criteria originally proposed by Kreike B et al., as follows [15]:

- Absent- No lymphocytes
- Minimal- <10 lymphocytes per high power field (hpf)
- Moderate- Lymphocytes easily identified, but no large aggregates
- Extensive- Large aggregates of lymphocytes in more than 50% of the tumour

The CD4+ cells and CD8+ cells were individually scored into scores 0, 1, 2 and 3 both in the stromal and the peritumoural compartment, corresponding to absent, minimal, moderate and extensive, respectively as per the criteria proposed by Kreike B et al., [15]. In immunostaining, only strong membranous positivity was considered positive.

STATISTICAL ANALYSIS

The relationship between the lymphocytic infiltrate and the tumour grade, lymphovascular emboli and axillary nodal metastasis were analysed. Qualitative variables were expressed as frequency and percentage, and quantitative variables as mean and standard deviation. The statistical analysis was done using the Chi-square test, t-test or the ANOVA test depending on the nature of the variable. All the data collected was entered in Microsoft Excel and analysis was done with the help of Statistical Package for the Social Sciences software version 18.0 (SPSS Inc., Chicago, USA). A p-value of <0.05 was considered statistically significant.

RESULTS

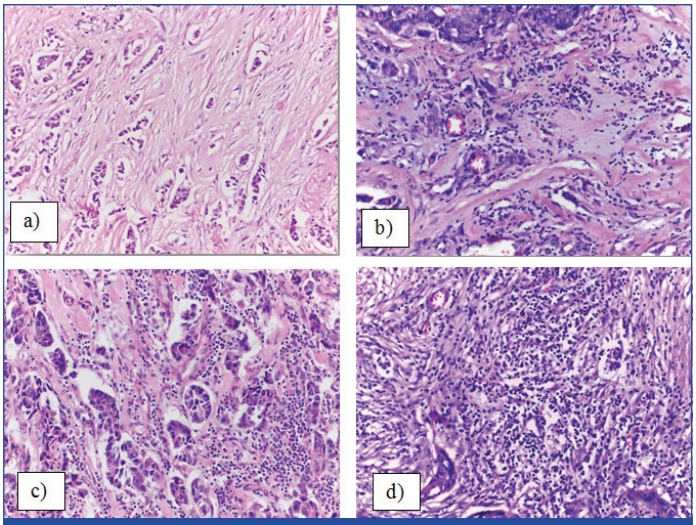
The age of the patients studied ranged from 30 years to 82 years, with a mean age of 52.31 years (standard deviation=9.774 years). Majority of the cases 27 (36%) were in the age group of 50 to 59 years. Of the 75 patients studied, 73 (97.33%) were females and two cases (2.67%) were males. 30 (40%) patients had breast carcinoma on the right side and 45 (60%) had it on the left side.

Out of 75 patients, 71 (94.67%) had IBC, Not Otherwise Specified (NOS) and four cases (5.33%) had IBC with medullary features. Of the 71 cases of IBC, NOS, two cases (2.82%) were grade 1, 59 (83.10%) were grade 2 and 10 (14.08%) were grade 3. Seventeen (22.67%) cases showed lymphovascular emboli, and 33 (44%) cases had axillary node metastasis. Of the 33 patients with axillary node metastasis, 17 (51.52%) had one to three positive nodes, 15 (45.45%) had four to nine positive nodes and one case (3.03%) had >10 positive nodes.

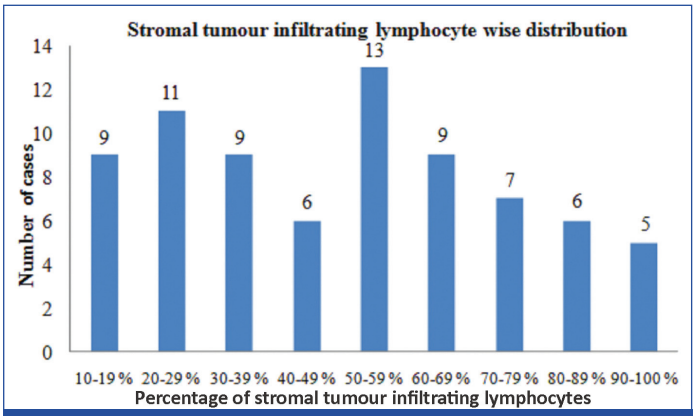
In 14 (18.67%) cases, the maximum tumour dimension was <2 cm (T1), 53 (70.67%) cases measured >2 cm to <5 cm (T2) and four cases (5.33%) cases measured >5 cm (T3). Four (5.33%) cases belonged to T4 stage; all of them showing cutaneous infiltration. 10 (13.33%) patients had stage I disease, 45 (60%) had stage II disease and 20 (26.67%) had stage III disease. None had stage IV disease.

The assessment of stromal TILs was done as shown in [Table/Fig-1]. The degree of stromal TIL infiltrate ranged from 10% to 95%, with a mean of 48.47% (standard deviation=24.203%). The patients were divided into nine groups based on the TIL infiltrate viz., 10-19%, 20-29%, 30-39%, 40-49%, 50-59%, 60-69%, 70-79%, 80-89% and 90-100%. Majority of the present cases 13 (17.33%) were in the TIL group 50-59%. The distribution of cases based on stromal TILs is given in [Table/Fig-2].

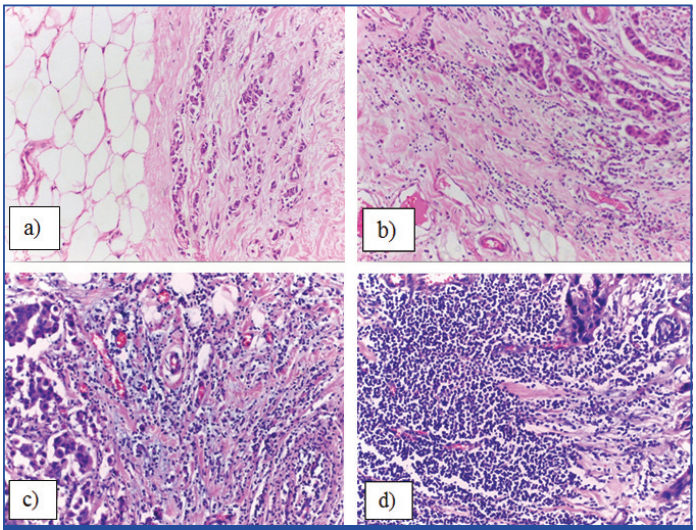
The scoring of peritumoural lymphocytes was done as shown in [Table/Fig-3]. When studied for peritumoural lymphocytes, 25 (33.33%) cases had score 1 infiltrate, 34 (45.34%) had score 2 infiltrate and 16 (21.33%) had score 3 infiltrate. None had score 0 infiltrate. Low grade inflammation (scores 0 and 1) was seen in 25 (33.33%) cases and high grade inflammation (scores 2 and 3) in 50 (66.67%) cases.



[Table/Fig-1]: Stromal Tumour Infiltrating Lymphocytes (TILs): a) Stromal TIL 10%, (H&E 20x); b) Stromal TIL 40%, (H&E 20x); c) Stromal TIL 60%, (H&E 20x); d) Stromal TIL 80%, (H&E 20x).



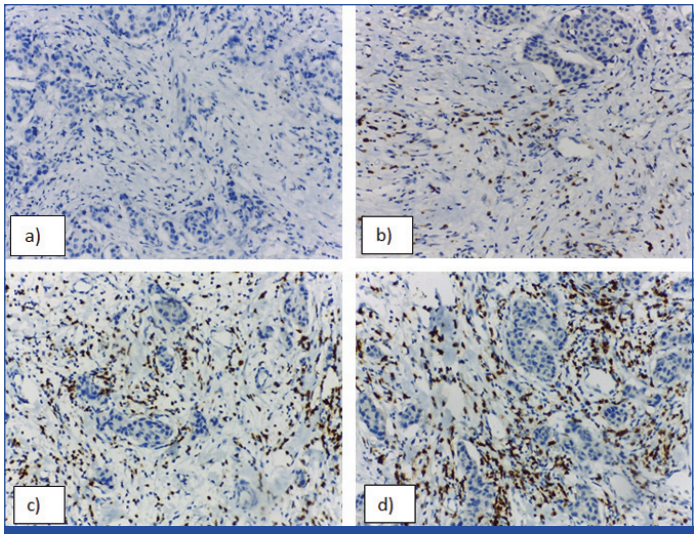
[Table/Fig-2]: Stromal tumour infiltrating lymphocyte wise distribution of cases.



[Table/Fig-3]: Scoring of peritumoural lymphocytes: a) Score 0, (H&E 20x); b) Score 1, (H&E 20x); c) Score 2, (H&E 20x); d) Score 3, (H&E 20x).

The scoring of the lymphoid infiltrate on IHC is given in [Table/Fig-4]. The distribution of cases based on the subpopulations of stromal TILs and peritumoural lymphocytes on IHC is given in [Table/Fig-5].

The association of stromal TILs and peritumoural lymphocytes with tumour grade, Lymphovascular Invasion (LVI) and axillary nodal metastasis is given in [Table/Fig-6]. The lymphoid infiltrate in both compartments were significantly higher (p-value <0.05) in high grade IBC, NOS tumours. All cases of IBC with medullary features had high stromal TILs and high grade peritumoural infiltrate (95% and grade 3 in all 4 cases, respectively). There was no statistically significant association between stromal TILs/peritumoural lymphocytes and presence or absence of lymphovascular emboli (p-values of



[Table/Fig-4]: Scoring of lymphoid infiltrate on Immunohistochemistry (IHC)- CD4: a) Score 0, (IHC 20x); b) Score 1, (IHC 20x); c) Score 2, (IHC 20x); d) Score 3, (IHC 20x).

Stromal TIL/PT score on immunohistochemistry	CD4		CD8	
	Stromal	PT	Stromal	PT
0	8 (10.67%)	3 (4%)	4 (5.33%)	3 (4%)
1	32 (42.67%)	29 (38.67%)	37 (49.33%)	30 (40%)
2	27 (36.00%)	40 (53.33%)	29 (38.67%)	33 (44%)
3	8 (10.66%)	3 (4%)	5 (6.67%)	9 (12%)
Total	75	75	75	75

[Table/Fig-5]: Distribution of cases based on immune cell subsets on Immunohistochemistry (IHC). (Same scoring system was adopted for CD8).
TIL: Tumour infiltrating lymphocytes; PT: Peritumoural lymphocytes

0.454 and 0.324, respectively). The stromal TILs were significantly higher in cases without axillary node metastasis (p-value=0.049), while there was no statistically significant association between peritumoural lymphocyte infiltrate and presence or absence of axillary nodal metastasis (p-value=0.139).

Histologic variables	Subgroups	No. of cases	Stromal TILs*		Peritumoural lymphocytes	
			Mean (%)	Standard deviation (%)	Low grade n (%)	High grade n (%)
Tumour grade	1	2	20	7.071	1 (50%)	1 (50%)
	2	59	44.41	21.195	24 (40.68%)	35 (59.32%)
	3	10	59.50	23.028	0	10 (100%)
	p-value		0.031†		0.041‡	
LVI§	Present	17	46.18	25.528	6 (35.29%)	11 (64.71%)
	Absent	58	51.64	26.497	19 (32.76%)	39 (67.24%)
	p-value		0.454**		0.324‡	
Nodal metastasis	Present	33	42.27	21.618	14 (42.42%)	19 (57.58%)
	Absent	42	53.33	25.247	11 (26.19%)	31 (73.81%)
	p-value		0.049**		0.139‡	

[Table/Fig-6]: Association of stromal TILs and peritumoural lymphocytes with tumour grade, LVI and nodal metastasis
*Tumour Infiltrating Lymphocytes; †ANOVA; ‡Chi square; §Lymphovascular invasion; **t-test
A p-value of <0.05 was considered statistically significant

Association between stromal TILs and peritumoural lymphocytes on H&E and CD4+ and CD8+ score in the corresponding compartment on IHC is given in [Table/Fig-7]. The CD4+ and CD8+ scores were significantly higher in cases with high grade inflammation on H&E in both compartments.

Subset of lymphocytes on IHC*	Score on IHC*	No. of cases based on stromal TIL† subset	Total stromal TILs† on H&E		Total peritumoural lymphocytes on H&E	
			Mean (%)	Standard deviation (%)	Low grade n (%)	High grade n (%)
CD4+ lymphocytes	0	8	23.75	11.877	3 (12.00%)	0 (0%)
	1	32	34.06	16.677	21 (84.00%)	8 (16.00%)
	2	27	63.52	16.221	1 (4.00%)	39 (78.00%)
	3	8	80.00	13.628	0 (0%)	3 (6.00%)
	p-value		<0.001‡		<0.001§	
CD8+ lymphocytes	0	4	20.00	13.540	3 (12.00%)	0 (0%)
	1	37	38.65	19.813	22 (88.00%)	8 (16.00%)
	2	29	59.48	21.312	0 (0%)	33 (66.00%)
	3	5	80.00	12.748	0 (0%)	9 (18.00%)
	p-value		<0.001‡		<0.001§	

[Table/Fig-7]: Association of stromal TILs and peritumoural lymphocytes on H&E with subset score on Immunohistochemistry (IHC).
*Immunohistochemistry; †Tumour infiltrating lymphocytes (TILs); ‡ANOVA; §Chi square
A p-value of <0.05 was considered statistically significant

DISCUSSION

In the present study, patients with IBC were evaluated for the presence and degree of peritumoural infiltration and TILs and their association with tumour grade, lymphovascular emboli and axillary lymph node metastasis.

The majority of patients were in the age group of 50 to 59 years (36%). The age of the patients ranged from 30 years to 82 years, with a mean age of 52.31 years. This was comparable to studies by Chopra B et al., and Augustine P et al., the mean ages in their studies being 50.1 years and 47.79 years, respectively. The peak age groups in their studies were 41 to 50 years and 46 to 55 years, respectively [16,17].

On histologic typing, 94.67% had IBC, NOS and the remaining 5.33% had IBC with medullary features. This was comparable to a study by Netra SM et al, where IBC with medullary features constituted 3.1% of the total cases [18]. Of the 71 cases of IBC, NOS, 2.82% were grade 1, 83.10% were grade 2 and 14.08% were grade 3. Of the total 75 patients, lymphovascular emboli were noted in 22.67% of cases. Both these findings have shown variable results in other studies [10,15,19,20], possibly related to the patient group visiting the specific institutions and the use of IHC in the detection of LVI [19].

Axillary lymph node evaluation showed that 44% patients had nodal metastasis. This was comparable to the study by Chakraborty A et al., where 44.36% patients showed metastasis [20]. Of the 33 patients with axillary node metastasis, 51.52% had one to three positive nodes, 45.45% had four to nine positive nodes and the remaining 3.03% had 10 or more positive nodes. This was in concordance with a study by Mohapatra M and Satyanarayana S, where the positivity rates were 54.7%, 39.6% and 5.7%, respectively [21].

Categorisation based on tumour size showed that 18.67% patients had tumour size up to 2 cm (T1), 70.67% had tumour size >2 cm, but <5 cm (T2) and 5.33% had tumours >5 cm (T3). A 5.33% of patients showed skin infiltration (T4). The predominance of T2 tumours was identical to the study by Kaur M et al., with 70% of tumours in their study being in T2 stage [22].

Stage wise distribution of cases revealed that 13.33% patients presented with stage I disease, 60% with stage II disease and 26.67% with stage III disease. None had stage IV disease. This was comparable to the study by Mohapatra M and Satyanarayana S, where stage I disease was seen in 7.7% cases, stage II disease in 63.4% and stage III disease in 28.9%; none had stage IV disease in their study as well [21].

The degree of stromal TIL infiltrate ranged from 10% to 95%, with a mean of 48.47%. Majority of the present cases were in the TIL group 50-59% (17.33%), followed by 20-29% (14.67%). Low grade inflammation in the peritumoural area was seen in 33.33% cases and high grade inflammation in 66.67% cases.

The stromal TILs were significantly higher in high grade tumours. However, no significant association could be demonstrated between stromal TILs and lymphovascular emboli. Both these findings were similar to those in a study by Ruan M et al., who evaluated patients with triple negative breast cancer who had undergone neoadjuvant chemotherapy [23]. A significantly higher TIL score was demonstrated in high grade triple negative breast cancers by Krishnamurti U et al., as well [24]. A study by Lee HJ et al., also demonstrated that significantly higher TIL scores were associated with high grade tumours and reduced LVI [25].

The stromal TILs were found to be higher in cases without axillary lymph node metastasis. This was similar to a study by Caziuc A et al., who demonstrated reduced likelihood of nodal metastasis in both early stage as well as locally advanced tumours with higher TILs [26].

The present study showed that the peritumoural lymphocytic infiltrate was higher in high grade tumours. This finding was comparable to that in a study by Ahmadvand S et al., in which they demonstrated a significantly higher peritumoural infiltrate of CD3+, CD8+, CD45RO+ and FOXP3+ cells in high grade breast cancers [27]. A similar result was obtained by Mohammed ZMA et al., in which high peritumoural lymphocytic infiltrate was significantly associated with higher tumour grade [28].

The present study failed to show a significant association between peritumoural lymphocytic infiltrate and lymphovascular emboli. This finding was similar to the study done by Al-Saleh K et al., where significant association was demonstrated only between pathological complete response and intratumoural CD8+TILs [10]. The study by Ahmadvand S et al., and Mohammed ZMA et al., also could not demonstrate any association between peritumoural lymphocytic infiltrate and LVI [27,28].

No significant association could be demonstrated between peritumoural lymphocytic infiltrate and axillary node metastasis. This was comparable to the study by Mohammed ZMA et al., where no significant association could be demonstrated between peritumoural lymphocytes and axillary nodal metastasis [28].

A significant association was obtained between the lymphocytes on H&E and the individual CD4+ and CD8+ cell infiltrate on IHC in both stromal and peritumoural compartments. A similar result was obtained by Konig L et al., with significant correlation between the total TILs on H&E and the CD3+, CD4+, CD8+ and CD20+ immune cell subpopulations in IHC [29].

Limitation(s)

The results of this study are dependent upon relatively small sample size. A similar study with a larger sample size might have increased the value of the study. Authors did not use any IHC markers for the detection of LVI, which might have improved the detection rate.

CONCLUSION(S)

Stromal TILs and peritumoural lymphocytes were significantly higher in high grade tumours. Stromal TILs were also significantly higher in tumours without axillary lymph node metastasis. There is no statistically significant association between the lymphocytic infiltrate and LVI. There is significant association between H&E scoring of the immune infiltrate and immunohistochemical scoring of the subpopulations of the immune infiltrate.

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