



Expression of CD4, CD8 Biomarkers in Invasive Carcinoma of Breast with Clinicopathological Correlation

C. Divyapriya^{a*}, Aarthi Kannan^a and Vijayashree Raghavan^a

^a Department of Pathology, Chettinad Hospital and Research Institute, Chettinad Academy of Research and Education, Rajiv Gandhi Salai, Kelambakkam-603013, Tamilnadu, India.

Authors' contributions

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

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ABSTRACT

Introduction: Tumor infiltrating lymphocytes (TILs) are widely considered a key sign of the immune interaction between host and tumor, and potentially prognostic biomarkers of good or bad outcome in various cancers, including invasive breast cancer (IBC).

Aim and Objectives: To correlate the expression of CD4, CD8 T-lymphocytes in invasive carcinoma breast with established markers of prognosis like tumour size, grade, lymph node status and molecular subtypes mainly ER, PR, Her 2Neu, Ki67 status, mainly the triple negative breast cancers(TNBC).

Methodology: 58 Invasive breast carcinoma proven tissue blocks were subjected to immunohistochemistry and morphometric analysis for positive CD4, CD8 T-lymphocytes were done.

Results: Triple negative breast cancer subtype shows high TILs than other pathologic subtypes. Tumor interface CD8+ cells very well correlated with the pathological higher nodal stage. Majority CD4, CD8 positive cells were populated more towards the stromal and interface of the tumor microenvironment rather than intratumoral.

Conclusion: CD4+ and CD8+ counts may be a valuable independent prognostic tool in predicting the outcome in invasive breast cancer.

Keywords: Breast cancer; invasive breast cancer; CD4 and CD8 markers; clinico pathological correlation; tumour infiltrating lymphocytes.

1. INTRODUCTION

Breast cancer (BC) is a neoplasm characterized by molecular and cellular heterogeneity. It is the leading cause of mortality among women globally [1]. Recent advances in diagnosis and treatment of the breast cancer showed the importance of the role of the new markers in prognosis and target therapies will help in patient survival [2]. A study reviewing several findings have showed the importance of the microenvironment focusing on the function and interaction between immune cells and cancer cells in its role in cancer progression including Breast cancer [3]. It is generally known that regional lymph nodes are a significant immunological defense for tumor expansion [4].

Tumor infiltrating lymphocytes (TILs) have higher specific immunological reactivity against tumor cells. They play dual role i.e., either suppress or promote growth of the tumor [5]. Tumor infiltrating lymphocytes are part of adaptive immunity that migrate into the tumor and stroma, are considered a manifestation of host antitumor response. Moreover, its role in tumor progression or regression is been studied in various solid tumors [6,7]. Tumor cells can express antigens and become targets for a T cell-mediated adaptive immune response. The ability of CD4+, CD8+ lymphocytes to recognize tumor antigens has been well documented, and tumor regression is observed when tumor-reactive T lymphocytes invade cancers more so by CD8 CTLs [8-10]. The anti- and pro-tumor function of TILs in breast cancer is dependent on the TIL subtype. Definitely cytotoxic T cells (CD8+ T cells) have been described to be associated with better clinical outcome in different phenotypes of Breast Cancer [11-14]. The clinical significance of TILs in Breast Cancer is still debatable and the conclusions remain controversial. Generally, assessing the immunogenicity of the neoplasm, and its distribution may determine if an immune response will be prognostically favorable mainly for TNBC and HER2neu overexpressed tumors [15,16]. With successive hormonal and chemotherapeutics, the next modality aims to be immunotherapy in breast cancer. TNBCs are said to have more mutations compared to other subtypes, which could be paramount targets for immunotherapy [8,9,17].

2. MATERIALS AND METHODS

58 formalin-fixed and paraffin embedded tissue blocks along with histopathological slides were retrieved from the archives of the Department of pathology, Chettinad Academy of Research and Education. Core biopsies, lumpectomy specimens, neoadjuvant chemotherapy patients were completely exempted from the study. Estrogen receptor, progesterone receptor, Her2 and Ki67 immunopositive staining for each case was done and evaluated as per American Society of Clinical Oncology/College of American Pathologists Guidelines (ASCO/CAP guidelines). Parameters like pTNM staging, lymph node status, morphological and molecular subtypes, Modified Scarff Bloom Richardson grading. All the recorded data was entered on a detailed proforma designed for this study. Immunohistochemistry:- Formalin-fixed, paraffin embedded tissue sections of 3.5 microns thick. Sections were deparaffinised in xylene followed by hydration in graded ethanol. Antigen retrieval was performed by heating process at 100deg Celsius for 20 mins in Tris-EDTA buffer(pH 7.4). Endogenous peroxidize block in the tissue sections was done for 10 minutes. Sections were covered with appropriate primary antibody for 45 minutes at room temperature. After adequate buffers washes. DAB(3,3'-diaminobenzidine tetrahydrochloride) chromogenis added. Sections were counterstained with hematoxylin, dehydrated with ethanol and xylene, and mounted with DPX(Di-n-butylphthalate in xylene).Tonsillar tissue sections served as positive control for markers CD4+,CD8+.

Microscopic assessment of CD4, CD8 Tcells and scoring: In our study, the CD4 and CD8 Tcells were assessed in immunohistochemical (IHC) slides adapted from Ankita et al study, the scoring of immune stained positive CD4,CD8 TILs was done independently by two pathologists on the same microscopic area. CD4+, and CD8+ TILs were counted in same five randomly selected high power fields (40X magnification) and the counts were averaged for tumor, stroma and tumor interface. The tumor infiltrating lymphocyte count was calculated as: + (1-25 cells), ++ (26-50 cells), +++ (≥51 cells) in the tumor, tumor interface and the stroma separately. Positive TILs upto 25 cells were considered as low TIL count and more than 25 cells (i.e. ++, +++) were considered as high TIL

count in the tumor, tumor interface and stroma separately.

3. RESULTS

In this study, total 58 tissue blocks of patients who underwent modified radical mastectomy (MRM) were only taken and about 63.8% (37/58) had right MRM, 36.2(21/58) had left MRM. Majority in the study group were females (96.6%). Only 2 male subjects and were diagnosed with invasive ductal carcinoma NST. The mean patient's age was 50.03 years (ranging from 35-74 years) Among the study population, majority 20 (34.5%) of them were

belonging to 41 - 50 years. The molecular subtypes showed high triple negative subtype cases (48.3%) than others. Morphological diagnosis of invasive ductal carcinoma NST [77.5%] were predominant cases, followed by invasive carcinoma with medullary like features [15.50%] followed by invasive papillary carcinoma [3.40%], and mucinous carcinoma [3.40%] were observed. Of all cases, (94.8%) were relapse free survivors, 1 patient had recurrence, and 2 patients did not survive. 53 patients of lymph node positive cases observed. The descriptive statistics of all 58 patients were compiled with tabulation.

Table 1. Cross tabulation between Tumour interface CD4 count (High & Low) and other variables

		Tumor interface CD4		Chi sq p value
		High	Low	
pTNMTumour Size	T1 & T2	31 (83.8%)	6 (16.2%)	0.295
	T3 & T4	17 (85%)	3 (15%)	
pTNM Nodal Status	N0 &Nx	20 (76.9%)	6 (23.1%)	0.115
	N1 & above	28 (90.3%)	3 (9.7%)	
pTNM Metastasis	M0	40 (85.1%)	7 (14.9%)	0.315
	Mx	8 (80%)	2 (20%)	
ER	Positive	16 (76.2%)	5 (23.8%)	0.17
	Negative	32 (86.5%)	5 (13.5%)	
PR	Positive	16 (76.2%)	5 (23.8%)	0.17
	Negative	32 (86.5%)	5 (13.5%)	
Her2Neu	Positive	11 (73.3%)	4 (26.7%)	0.159
	Negative	37 (86%)	6 (14%)	
Ki67	1+ & 2+	28 (73.7%)	10 (26.3%)	0.009
	3+	20 (100%)	0 (0%)	
Pathological Subtype	TNBC	24 (85.7%)	4 (14.3%)	0.196
	Luminal A	12 (85.7%)	2 (14.3%)	
	Luminal B	4 (57.1%)	3 (42.9%)	
	Her2 / Neu	8 (88.9%)	1 (11.1%)	

Table 2. Cross tabulation between Intratumoral CD8 count (High & Low) and other variables

		Intratumoral CD8		Chi sq p value
		High	Low	
pTNMTumour Size	T1 & T2	20 (54.1%)	17 (45.9%)	0.77
	T3 & T4	10 (50%)	10 (50%)	
pTNM Nodal Status	N0 &Nx	13 (50%)	13 (50%)	0.716
	N1 & above	17 (54.8%)	14 (45.2%)	
pTNM Metastasis	M0	24 (51.1%)	23 (48.9%)	0.241
	Mx	6 (60%)	4 (40%)	
ER	Positive	6 (28.6%)	15 (71.4%)	0.008
	Negative	24 (64.9%)	13 (35.1%)	
PR	Positive	6 (28.6%)	15 (71.4%)	0.008
	Negative	24 (64.9%)	13 (35.1%)	
Her2Neu	Positive	8 (53.3%)	7 (46.7%)	0.885
	Negative	22 (51.2%)	21 (48.8%)	

		Intratumoral CD8		Chi sq p value
		High	Low	
Ki67	1+ & 2+	16 (42.1%)	22 (57.9%)	0.043
	3+	14 (70%)	6 (30%)	
Pathological Subtype	TNBC	16 (57.1%)	12 (42.9%)	0.011
	Luminal A	5 (35.7%)	9 (64.3%)	
	Luminal B	1 (14.3%)	6 (85.7%)	
	Her2 / Neu	8 (88.9%)	1 (11.1%)	

Table 3. Cross tabulation between Stromal CD8 count (High & Low) and other variables

		Stromal CD8		Chi sq p value
		High	Low	
pTNMTumour Size	T1 & T2	34 (91.9%)	3 (8.1%)	0.393
	T3 & T4	19 (95%)	1 (5%)	
pTNM Nodal Status	N0 &Nx	23 (88.5%)	3 (11.5%)	0.204
	N1 & above	30 (96.8%)	1 (3.2%)	
pTNM Metastasis	M0	43 (91.5%)	4 (8.5%)	0.452
	Mx	10 (100%)	0 (0%)	
ER	Positive	19 (90.5%)	2 (9.5%)	0.356
	Negative	34 (91.9%)	3 (8.1%)	
PR	Positive	19 (90.5%)	2 (9.5%)	0.356
	Negative	34 (91.9%)	3 (8.1%)	
Her2Neu	Positive	14 (93.3%)	1 (6.7%)	0.404
	Negative	39 (90.7%)	4 (9.3%)	
Ki67	1+ & 2+	33 (86.8%)	5 (13.2%)	0.11
	3+	20 (100%)	0 (0%)	
Pathological Subtype	TNBC	25 (89.3%)	3 (10.7%)	0.14
	Luminal A	13 (92.9%)	1 (7.1%)	
	Luminal B	6 (85.7%)	1 (14.3%)	
	Her2 / Neu	9 (100%)	0 (0%)	

Table 4. Cross tabulation between Tumour Interface CD8 count (High & Low) and other variables

		Tumor interface CD8		Chi sq p value
		High	Low	
pTNMTumour Size	T1 & T2	34 (91.9%)	3 (8.1%)	0.266
	T3 & T4	20 (100%)	0 (0%)	
pTNM Nodal Status	N0 &Nx	23 (88.5%)	3 (11.5%)	0.049
	N1 & above	31 (100%)	0 (0%)	
pTNM Metastasis	M0	44 (93.6%)	3 (6.4%)	0.554
	Mx	10 (100%)	0 (0%)	
ER	Positive	19 (90.5%)	2 (9.5%)	0.33
	Negative	35 (94.6%)	2 (5.4%)	
PR	Positive	19 (90.5%)	2 (9.5%)	0.33
	Negative	35 (94.6%)	2 (5.4%)	
Her2Neu	Positive	13 (86.7%)	2 (13.3%)	0.223
	Negative	41 (95.3%)	2 (4.7%)	
Ki67	1+ & 2+	34 (89.5%)	4 (10.5%)	0.174
	3+	20 (100%)	0 (0%)	
Pathological Subtype	TNBC	26 (92.9%)	2 (7.1%)	0.175
	Luminal A	14 (100%)	0 (0%)	
	Luminal B	5 (71.4%)	2 (28.6%)	
	Her2 / Neu	9 (100%)	0 (0%)	

MICROSCOPIC IMAGES

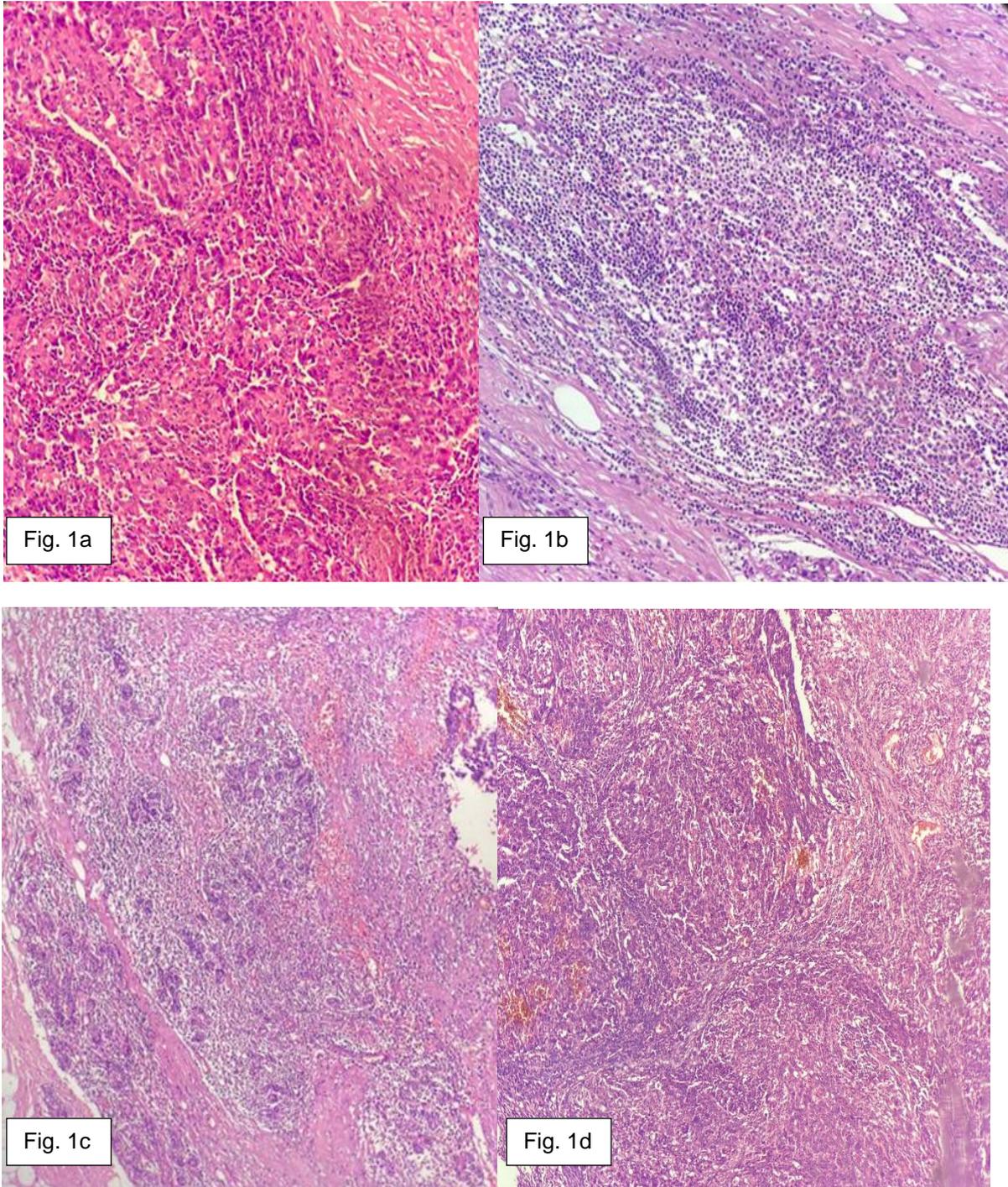
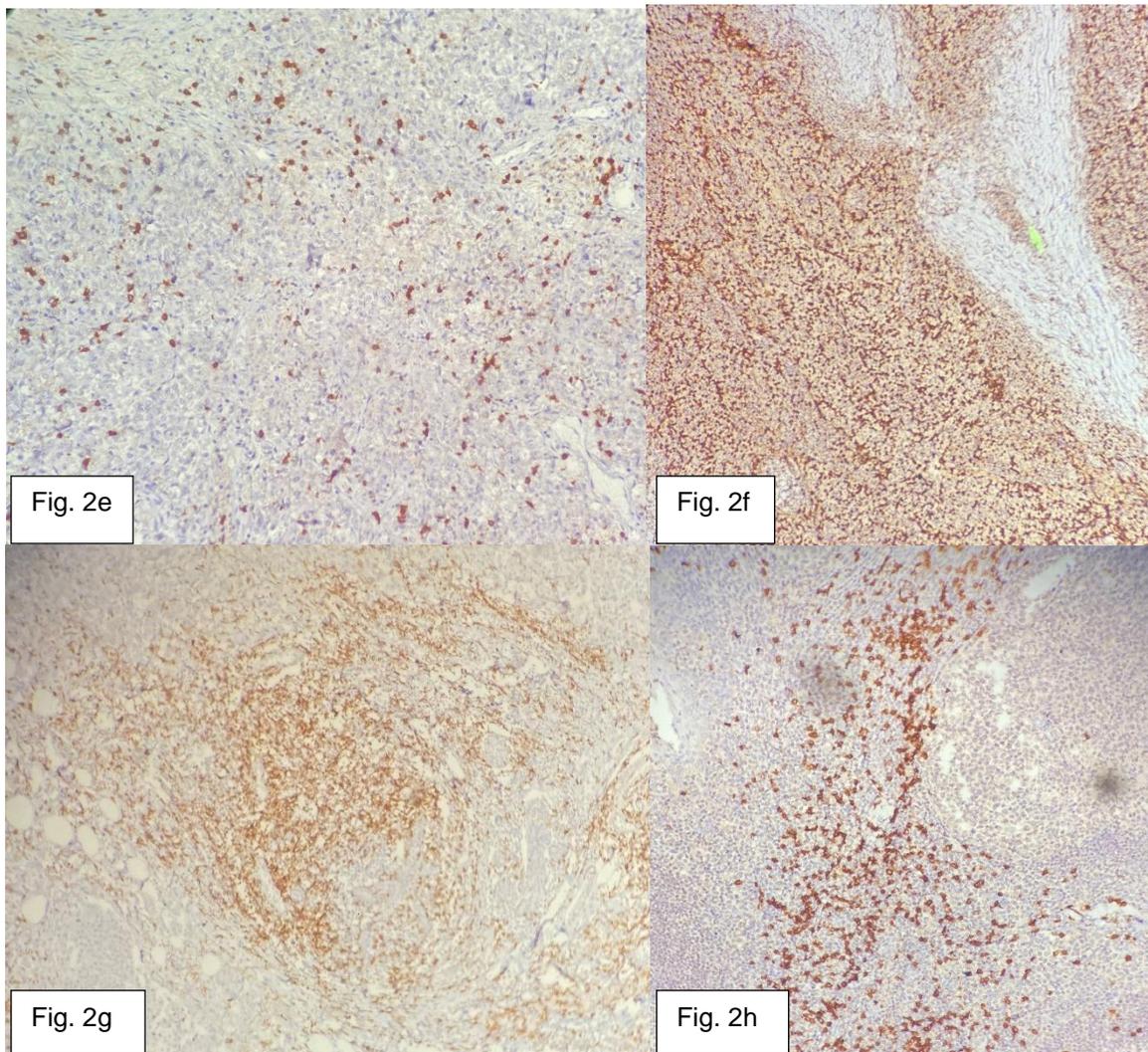


Fig. 1(a). Intratumorallymphocytes(x40 magnification, H&E)
Fig. 1(b). Tumor interface lymphocytic infiltrate(x40 magnification, H&E)
Fig. 1(c). Stromal lymphocytic infiltrate seen extending to the adjacent normal breast terminal duct lobular unit(x20),
Fig. 1(d). Extensive lymphoplasmacytic infiltrates in the stroma and intratumoral(x40)



**Fig. 2(e,f). Intratumoral CD8, CD4 immunopositive T-lymphocytes(x40,x20)
Fig. 2(g). Tumor interface CD8 positive Tcells(x20)
Fig. 2(h). Stromal CD8 positive TCells (x40)**

4. DISCUSSION

In the present study, the correlation between Intratumoral, stromal and tumor interface CD4+, CD8+ marker expression with pathological variables and molecular subtypes were as follows:-

Stromal CD4 count when correlated with negative hormonal receptor status(ER,PR) was significant(p value =0.05). When compared to the tumor stage, low intratumoral CD4 and high stromal and interface CD4 count was found in T1,T2 stages of tumor and in all positive nodal status as well. Collectively CD4 intratumoral expression was low in relation to all the parameters like tumor size, lymph nodes and all

molecular subtypes. Majority, of TNBC showed low intratumoral CD4 count and high stromal and interface CD4 count. Low tumor stage(T1,T2) showed high intratumoral CD8 positive expression and also significant correlation was observed with triple negative subtype(ER-,PR-,Her2-) of p value(<0.05) which was statistically significant. Whereas stromal CD8+ didn't show any significant difference compared to any of the parameters, but high stromal and interface CD8 count is seen compared to intratumoral CD8 count. Tumor interface CD8+ cells very well correlated with the pathological higher nodal stage. Again, high CD8 count is seen more in triple negative breast cancers. Also noted that both stromal and interface CD4, CD8 infiltrate were high in Her2 and TNBC cases than the

intratumoral sites. CD8 T cells were high compared to CD4 T cells around the normal breast TDLU. Moreover, suggests immune response in these two subtypes is different from hormonal receptor subtype.

Rafal Matkowski et al (2009) studied 88 breast cancer patients with ductal histology. Along with the CD4 and CD8 expression, the type, density, localization and distribution of tumour infiltrating lymphocytes (TILs) was studied. The patients with high expression of CD4 or CD8 had lymph node involvement and worse cancer specific overall survival in this study [18].

Sahar M A Mahmoud et al (2011) analysed 1334 tissue microarray cores. The study results revealed that the total number of CD8(+) cells was positively correlated with tumour grade, patient's age during the diagnosis, molecular markers expression. They observed that the Stromal CD8+ T cell lymphocytes were independently associated with better prognosis and patient survival [19]. Also in our study, stromal CD8+ counts were high with good prognosis.

Ankita Singh Rathore et al (2014) In this study, immunohistochemistry of CD3, CD4 and CD8 T-cell markers were used on 150 breast cancer tissue sections. On analysing each parameter, it showed that the high (++) intratumoral CD4+ count showed the highest survival compared to CD3+ and CD8+ count the least when compared to respective low (+) counts. In contrast, the high CD8+ ($P < 0.001$) stromal count showed the highest survival followed by CD4+ and CD3+ the least. They concluded that each of them are better independent predictors of favourable survival outcome in infiltrating ductal breast carcinoma [20].

Ezzeldin M Ibrahim et al (2014) meta-analysis study showed, the prognostic value of tumour-infiltrating lymphocytes in triple-negative breast cancer (TNBC) by extracting data of 2,987 patients with early stage breast cancer from 8 studies and concluded that the increased TILs were significantly associated with improved survival outcome in early TNBC and further recommended that it can be considered as a strong prognostic factor for TNBC [17]. Also stated, Invasive ductal histology is the predominant type to be involved [21].

Hirofumi Matsumoto et al. study found that high levels of CD8+ iTILs and CD4+ sTILs were

significantly associated with better clinical outcomes in TNBC. The role of CD4+ TILs in antitumour responses is often to stimulate the CD8+ TILs and to attack tumor cells. The CD4+ T-cells benefit the CD8+ CTLs in maintaining the tumour immunity which happens in three phases, viz. by primary induction, effector maintenance, and memory of CD 8+ CTL responses [22].

Kim et al reported that declined number of CD8+ TILs in breast tumours were significantly related with lymph node metastasis (as seen in our study too), high stage and higher proliferative index [23].

Triple negative breast cancers (TNBC) are most likely to have tumors with >50 % lymphocytic infiltrate, has the greatest survival benefit from each 10 % increase in TIL. ER+ve, PR+ve, HER2- tumors tend to have the least immune infiltrate [24]. T cells were the predominant immunophenotype being noted in 81% of tumors, compared to B cells in predicting the biological behavior. They came to a conclusion that, probably favors neoplastic progression rather than acting as an antitumor immune response [25].

Kurozumi et al. recently investigated the relationship between TILs and prognosis in 294 cases and reported that high stromal TILs expression was a good prognostic marker in ER-negative cancer subtype [26].

Running text- Clinicopathological comparison of T-cell Biomarkers CD4, CD8 influence on tumour infiltrating lymphocytes (TILs) in invasive breast cancer depicts the immunological interaction of host immunity and tumor, which shows progression or regression of tumor cells in invasive breast carcinoma. The prognostic significance is mainly needed in triple negative breast cancers (TNBCs), where therapy is questionable. Emergence of immunotherapy in various cancers, including breast cancer imply the significance of T-cell population of tumor infiltrating lymphocytes which can promote value to the treatment protocol and predict the life expectancy of patients with Triple negative invasive breast cancer. As with this study, triple negative breast cancer subtype showed high TILs than other pathologic subtypes. Tumor interface CD8+ cells very well correlated with the pathological higher nodal stage. Majority CD4, CD8 positive cells were populated more towards the stromal and interface of the tumor microenvironment rather being intratumoral.

5. CONCLUSION

In conclusion of this study, it is to highlight that immune markers like CD4, CD8 T cells expression might benefit patients particularly in triple negative breast cancer and Her2 overexpressed subtypes which is more immunogenic than the hormonal subtypes. Understanding the impact of specific subsets of immune cells that infiltrate tumors and its microenvironment is important for making rational decisions in the development of targeted therapies in invasive breast carcinomas. As shown in our study, lymph node involvement was seen in cases with high CD8 cells in the tumor interface. So, in addition to ER, PR, Her2 and Ki67, CD8 can also be used in patients to assess the prognosis.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Ethical Clearance for this study got approved from the Institutional Human Ethical Committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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