MAK HILL Publications Research Journal of Medical Sciences

Research Article
doi: 10.36478/makrjms.2024.7.469.473

OPEN ACCESS

Key Words
TIL- tumor infiltrating lymphocytes, stromal tumor infiltrating lymphocytes (sTIL), intratumoral tumor infiltrating lymphocytes (iTIL), H and E-hematoxylin and eosin

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Received: 27 April 2024
Accepted: 19 June 2024
Published: 25 June 2024

Citation: Y. Aishwarya, B.M. Vara Prasad and Atira Mirza, 2024. A Study on Histopathological Evaluation of Tumor Infiltrating Lymphocytes (TIL's) in Correlation With Molecular Subtypes, Tumor Grade and Stage of Breast Cancer. Res. J. Med. Sci., 18: 469-473, doi: 10.36478/makrjms.2024.7.469.473

A Study on Histopathological Evaluation of Tumor Infiltrating Lymphocytes (TIL's) in Correlation With Molecular Subtypes, Tumor Grade and Stage of Breast Cancer

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Abstract
Tumour-infiltrating lymphocytes (TILs) exhibit anti-tumoral immunity and have the potential as a prognostic marker, especially in triple-negative and human epidermal growth factor receptor 2-over expressing breast cancer. The study aims to evaluate the correlation between TILs and molecular subtypes, tumour grading and stage of breast cancer. Materials and The study was a cross-sectional study carried out at Ramaiah Medical College over three months from May to July 2023, comprising 40 cases of breast carcinoma. Breast cancer tissue was examined using Haematoxylon and Eosin (H and E) and Immunohistochemistry (IHC) staining, in addition to the assessment of lymph node involvement, tumour grading and staging. In the study of 40 cases, the most common histological type was invasive ductal carcinoma (95%). The Luminal A subtype was the most common variant observed. It was associated with low levels of Stromal tumour infiltrating Lymphocytes (sTIL) and Intratumoral infiltrating lymphocytes (iTIL) scores in comparison with triple-negative and Her-2 positive tumours, which had higher levels of sTILs and iTILs. In the present study sTIL and iTIL scores increased with tumour grade, size, mitosis and staging. The study concludes that tumor infiltrating lymphocytes are an easily accessible, affordable marker for estimating the tumor grade and stage of breast cancer and must be incorporated in every screening test panel for breast cancer.

INTRODUCTION

Molecular subtypes have a significant prognostic value in evaluation of breast cancer[1]. Although the exact relationship between breast cancer and the host's immune response is unknown, tumor infiltrating lymphocytes (TIL) are emerging as a potential immunological marker. Galon was the first one to discover that TIL has a predictive prognostic value in patients with colon cancer[2-4]. TILs initiate an immune response against tumor growth and metastasis. Detection of TIL in the cancer tissue has shown to be a reproducible immunological marker that can be implemented in the standard investigations protocol for grading and staging of breast cancer. In patients with pathological complete response (PCR), TIL has been detected at high levels[5]. However, estimating TIL in patients with PCR had variable results. The presence of TIL was highly consistent in patients with triple-negative breast cancer than in HER 2 positive breast cancer and was the least consistent in luminal cases6. Higher levels of TIL, especially in breast stromal tissue, increase the anti-tumor effects of therapy[7].

MATERIALS AND METHODS

A retrospective study was conducted in the Department of Pathology, M.S Ramaiah Medical College and Hospital, Bangalore. The study was conducted over three months, i.e. from May 2023- July 2023, during which haematoxylin and eosin(H and E) stained slides of 40 invasive breast carcinoma cases were examined.

Inclusion Criteria: The tissue specimens of all patients with invasive breast cancer who had undergone modified radical mastectomy in the institution were included in the study.

Exclusion Criteria: patients who had in-situ carcinoma were excluded.

A 5µm thin Hematoxylin and Eosin (H and E) stained slide was selected for all cases and clinico-pathological details such as histologic type, size of tumor, tumor grading, tumor stage IHC etc were recorded. Two pathologists performed histopathological scoring of TILs on H and E stained sections. Two scoring systems were applied to evaluate Stromal tumour infiltrating lymphocytes (sTIL) and Intratumoral Lymphocytes (iTu-Ly). sTIL was scored according to the guidelines of International TIL's working group (2014) the percentage of sTIL was determined using 200X magnification within the borders of the invasive tumour.

A semi-quantitative scoring system (H-score) was used to score iTu-Ly, which included the grade of lymphocytic infiltration (grade 0-3) and the percentage of tumour harbouring in each grade.

iTu-Ly was graded from 0-3, 0 = virtually absent lymphocytes, 1= few intra tumoral lymphocytes seen under 40X, 2 = lymphocytes are frequent in number and can be easily recognized under 10X, 3= large number of lymphocytes obscuring the tumor.

The final score was determined by multiplying the tumour grade with its corresponding percentage of cells present and then summing up the product, which resulted in a score ranging from 0-300. For example, for a tumour with 50% Grade 0, 20% Grade 1, 20% Grade 2 10% Grade 3, the final score would be 50 *0 +20*0+20*2+10*3 = 140.

The molecular subtypes of breast cancer were identified by immunohistochemistry staining for estrogen receptors (ER), progesterone receptors (PR) and Her2 neu on tumour blocks. The intensity of immunofluorescence was compared with that of the controls. Depending upon the presence or absence of the immunohistochemical marker, each case was allotted to a molecular subtype.

Statistical Analysis: Data was represented by percentages and mean +/-SD as applicable. All data was compiled and analyzed using ANOVA and student t-tests. Pearson's Correlation coefficient was used to calculate the degree of correlation between different variables. $P<0.05$ shall be considered significant.

RESULTS AND DISCUSSIONS

Forty patients with breast cancer were included in the present study. The most common type of carcinoma, accounting for more than 97.5% of the cases, was the infiltrative ductal carcinoma variant. The

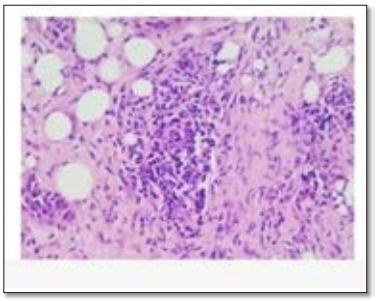

Fig 1: Image of sTIL on hematoxylin and Eosin stained slide (40x) of breast carcinoma-sTIL score 10-15%

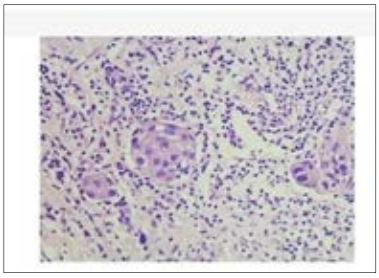

Fig 2: Image of iTIL on Hemotoxylin and Eosin stained slide (40x) of breast carcinoma-i TIL score 2

Table 1: stromal TIL levels and intratumoral TIL levels with Molecular subtypes of breast cancer correlation.

		Luminal A	Luminal B	Her-2neu enriched	Triple-negative breast cancer	p-value
sTIL score	<10% (n = 7)	7	0	0	0	0.001, statistically significant
	10-50% (n =9)	9	0	0	0	
	>50% (n =24)	2	3	7	12	
	Total (n = 40)	18	3	7	12	
iTIL levels	<100 (n = 21)	18	3	0	0	0.001, statistically significant
	100-200 (n = 16)	0	0	7	9	
	>200 (n = 3)	0	0	0	3	
	total	18	3	7	12	

Table 2 shows the correlation between stromal TIL levels and intratumoral TIL levels with TNM staging.

		Stage 1	Stage 2	Stage 3	Stage 4	p-value
sTIL score	<10% (n = 7)	4	3	0	0	0.001, statistically significant
	10-50% (n =9)	0	9	0	0	
	>50% (n =24)	0	16	5	3	
	total	4	28	5	3	
iTIL levels	<100 (n = 21)	4	17	0	0	0.001, statistically significant
	100-200 (n = 16)	0	8	5	3	
	>200 (n = 3)	0	3	0	0	
	total	4	28	5	3	

Table 3: stromal TIL levels and intratumoral TIL levels with Grades of breast cancer correlation.

		Grade 1	Grade 2	Grade 3	p-value
sTIL score	<10% (n = 7)	3	4	0	0.001; statistically significant
	10-50% (n =9)	0	9	0	
	>50% (n =24)	0	17	7	
	total	3	30	7	
iTIL levels	<100 (n = 21)	3	18	0	0.002; statistically significant
	100-200 (n = 16)	0	9	7	
	>200 (n = 3)	0	3	0	
	total	3	30	7	

second most common type observed in the present study was met aplastic carcinoma (2.5%). Breast cancers were categorized into molecular subtypes based on their presence or absence of immunohistochemical staining for hormone receptors-estrogen receptors (ER), progesterone receptors (PR) and Her-2 neu receptors. 45% of the cases had Luminal A molecular subtype while 30% had triple-negative molecular subtype of carcinoma. The majority of the cases had grade 2 carcinoma (75%). According to the TNM stage, most cases (70%) belonged to stage 2.

Luminal, A molecular subtype of breast cancer, was the most common variant observed in the present study (45%), followed by Triple-negative breast cancer (30%). The sTIL percentage was more than 50 % in 24 cases (60%). 17.5% of the patients had sTIL percentage <50%. While all patients with Luminal B type, Her-2 neu variant and Triple-negative variants of breast cancer had sTIL levels >50%, the majority of the patients with Luminal A variant of breast cancer had sTIL scores <10%. The correlation between the percentage of sTIL and molecular subtypes was statistically significant (p-value = 0.001).

Most of the patients had iTIL levels <100 (52.5%). All patients with Luminal A and B variants had iTIL levels <100. There was a statistically significant correlation between the molecular subtype of breast cancer with sTIL and iTIL levels. Triple-negative e breast cancer (TNBC) had the highest mean values of both stromal TIL levels and intratumoral TIL levels. In contrast, Luminal A variants of breast cancers had the lowest levels.

Most of the patients had Stage 2 breast cancer at the time of presentation (70%). Most patients with stages 2, 3 and 4 had sTIL levels > 50%, while Stage 1 had sTIL <10%. Most patients with Stage 1 and Stage 2 cancers had iTIL levels <100 (10% and 42.5%). The correlation between sTIL and iTIL levels with TNM staging was statistically significant.

Grading was done using the modified Bloom-Richardson grading of Breast carcinoma. Most of the patients had Grade 2 breast cancer (75%). Most of the patients with grade 2 and grade 3 breast cancers had sTIL scores >50% (42.5% and 7.5%). Most of the patients with Grade 1 and Grade 2 breast cancer (7.5% and 45%, respectively) had iTIL levels <100. The correlation between sTIL levels and iTIL levels with grades of breast cancer was found to be statistically significant. With an increase in tumour grade, the levels of sTIL and iTu-Ly were also found to be increasing.

The correlation coefficient was used to determine the strength of the correlation between sTIL and iTu-Ly and various clinicopathological factors of breast cancer, which was found to be significant (correlation coefficient = 0.05).

Breast cancer is the most frequently encountered malignancy in women worldwide. The immune response in breast cancer patients is an emerging research field. The presence of cytotoxic lymphocytes (Tumor Infiltrative Lymphocytes-TILs) in breast cancer suggests an ongoing immune response against tumor cells. Determination of levels of TILs has a promising future as a prognostic immunological marker as well as

a biomarker for therapy response, especially in patients with TNBC (Triple Negative Breast Cancer)[1]. The type, density and location of TILs exhibit different values to estimate the prognosis and progression of breast cancer [2]. Few studies have observed that higher levels of TIL are associated with greater anti-tumour effects of chemotherapy[8].

In the present study, the majority of the patients had the Luminal A variant of breast cancer, followed by triple-negative breast cancer. Most patients with the Luminal A variant had sTIL scores <10% and iTIL levels <100. While those with triple-negative breast cancer had sTIL scores >50% and iTIL levels >200. In a meta-analysis study done by Carsten *et al.,* who evaluated the predictive response to chemotherapy in patients with TNBC, Her-2 neu variants and Luminal-Her2 negative variants of breast cancer, observed that higher levels of TIL are associated with survival benefits in patients with TNBC and Her-2 variants[9]. Kurozumi[4] evaluated the expression of TIL in ER-negative and ER-positive breast cancer patients. They observed that high TIL expression was associated with poor prognosis in ER-positive patients and good prognosis in ER-negative patients.

TILs are mononuclear lymphocytic cells that act by affecting the immunosurveillance of tumours, facilitating the activation of other T cells facilitating the differentiation of inflammatory ductal carcinoma via an ATP-mediated mechanism. Few studies have shown that levels of TIL in patients with breast cancer not only serve as a potential prognostic marker but also aid as a marker in mitigating the toxic effects of chemotherapy. It serves as an affordable screening marker in countries like India, where there is a lack of sufficient resources in rural areas[8]. Murthy[10] analyzed a total of 20928 patients with breast cancer varying between stages I-III. They developed predictive models for breast cancer-specific survival (BCSS), which included TIL as a biomarker.

Li[11], in their study, observed that a threshold of 20% TILs had the highest prognostic value in patients with TNBC and HER2-positive breast cancer patients in response to neoadjuvant chemotherapy. Stanton[12] observed that breast cancer with high immune infiltrates (high levels of TILs) have the highest tendency to respond to immune checkpoint inhibitor therapy. In contrast, those breast cancers with low immune infiltrate might benefit if they are pre-treated with interventions which can increase TIL load, such as cytotoxic chemotherapy and vaccine therapy. The incorporation of assessment of TIL levels in the staging and grading of breast cancers has been suggested by the International TIL's working group to standardize treatment protocols[6].

CONCLUSION

This study concludes that TIL is a reliable, affordable easily available marker of immune infiltration load, highlighting anti-tumor immunity in breast cancer. This study also emphasizes the need for the inclusion of TILs in protocols for screening and staging of breast cancer.

Acknowledgments: The authors would like to express their gratitude to the staff of the Department of Pathology for extending their valuable support in conducting this study.

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