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Research Article

The Distribution and Prognostic Significance of Tumour Infiltrating Lymphocytes in Invasive Breast Carcinoma of Egyptian Patients

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Abstract

Background: The relationship between tumour infiltrating lymphocytes (TILs) and racial background in invasive breast carcinoma (IBC) is not well studied. In this study, we assessed the association between TILs and various clinicopathological parameters and outcome in a cohort of Egyptian IBC patients.

Methods: This study included a retrospective cohort (n=184) of IBC diagnosed in South Egypt Cancer Institute. All haematoxylin and eosin (H&E) histological sections were retrieved and the percentage of stromal TILs was assessed by 3 pathologists. The interobserver concordance was assessed and the association between TILs and clinicopathological factors and patients' outcome was analysed.

Results: There was an excellent interobserver concordance for TILs assessment (Inter-cluster correlation coefficient ICC=0.9). 70% of the cohort showed TILs density >10%. TILs score was higher in patients aged less than 50 years (p=0.004), higher tumour grade (p=0.001), no special type and special types of breast carcinoma (p=0.001), and

hormonal receptor negativity (p=0.001).Triple negative breast cancer (TNBC) and HER2 enriched molecular subtypes showed the highest TILs density among the molecular classes (p=0.018).There was no significant association between TILs and outcome.

Conclusion: Our study revealed that TILs are associated with features of high-risk IBC. Although the distribution of TILs among various IBC characteristics in Egyptian patients was similar to other ethnicities, there was no association between TILs density and patient outcome. Further epidemiological and comparative studies are warranted.

Keywords: TILs; Breast cancer; Egyptian; Interobserver agreement; Prognostic

1. Introduction

Invasive breast cancer (IBC) is the most common cancer diagnosed in women worldwide and it is the second leading cause of cancer related deaths in females [1]. In Egypt, IBC represents 20% of all primary malignant tumours and ranks the first most common malignancy in females [2]. The choice of systemic therapy for IBC depends on various clinical and pathological parameters as including tumour histological type, grade, hormonal status, HER2 overexpression and proliferation index. Although these factors are vital for patient management, they ignore the role of the surrounding tumour microenvironment especially the immune response, which is critical for tumour progression, invasion and metastases [3]. The tumour immune microenvironment have been shown to modulate therapy response and prognosis, specifically in triple negative breast cancer (TNBC) and human epidermal growth factor receptor 2 (HER2) enriched subtypes, standing for a novel field of translational research in the era of effective immunotherapies [4]. The role of the host immune system in getting rid of malignant cells has been consolidated in the immunoediting theory [5]. Evasion of the host immunity is one of the main hallmarks of carcinogenesis [6]. The International Immune-Oncology Working Group published consensus guidelines for evaluating tumour infiltrating lymphocytes (TILs) using routinely stained haematoxylin and eosin (H&E) sections in IBC. Multiple systemic reviews and meta-analysis have reported the role of TILs in IBC [7, 8]. Moreover, in the recent edition of World Health Organisation (WHO) breast tumours classification, they recommended assessment of TILs in routine practice and introduced the term lymphocyte rich carcinoma as a subtype of IBC of no special type (NST) [9]. Although the role of TILs in IBC prognosis is undeniable, studies assessed the role of TILs among different ethnic and racial backgrounds, to the best of our knowledge, are limited. Thus, in this study we hypothesised that TILs density among Egyptian patients with IBC is unique. We retrospectively evaluated TILs in a cohort of IBC diagnosed and managed in Egypt and investigated its association with different clinicopathological characteristics and patients' outcome.

2. Materials and Methods

2.1 Study cohort

This retrospective study included a cohort of primary IBC (n=184), diagnosed and managed at South Egypt Cancer

Institute, Assiut, Egypt from 2014 to 2016. All available clinicopathological data including patient age at diagnosis, tumour grade, histological subtype, tumour size, lymph node status, lymphovascular invasion (LVI), presence of necrosis, associated ductal carcinoma in situ (DCIS), biomarker defined classes based on both oestrogen receptor (ER) and HER2 status (defined as ER positive/HER2 negative, ER positive/HER2 positive, ER negative/HER2 positive and ER negative/HER2 negative) [9] were collected. In addition, all cases were classified into molecular subtypes according to the immunohistochemical evaluation of ER, progesterone receptor (PR) and HER2 expression into luminal, HER2 enriched and TNBC subtypes. Tumour grade was evaluated according to Nottingham grading system [10], while tumour stage was described using American Joint Committee on Cancer Staging [11]. All cases included in this study were treated primarily by surgery followed by adjuvant therapy either hormonal, chemotherapy or both. Chemotherapy regimen was offered according to the institutional protocol which was mainly CMF for 6 cycles. Patients treated with neoadjuvant therapy were excluded. Outcome data was retrieved in terms of local recurrence free interval (LRFI) (defined as time in months between diagnosis and occurrence of ipsilateral tumour recurrence), distant metastases free interval (DMFI) (defined as time in months between diagnosis and occurrence of DM) and breast cancer specific survival (BCSS) (defined as time in months between diagnosis and patient's last date known to be alive or death from breast cancer). With a median follow up period of 32 months, there were 4 cases (2%) with local recurrence, 16 cases with distant metastases (9%) and 11 cases (6%) deceased from breast cancer.

2.2 Histological evaluation and TILs scoring

All available H&E stained sections from the selected cases were retrieved from histopathology department archive and reviewed for section quality and suitability for TILs evaluation. Moreover, all cases were reclassified according to the recent WHO classification of breast tumours [9, 12]. Stromal TILs have been assessed in H&E stained sections following the international working group recommendations [13]. Briefly, one representative section for each case was selected that showed at least 30%viable invasive tumour tissue. Only TILs within the border of invasive tumour were evaluated. TILs surrounding areas of necrosis, artefacts or extensive fibrosis were not included in the scoring. The average percentage of stromal TILs was reported. In addition, cases with TILs distributed mostly (>95%) at the invasive margin were recorded. The invasive tumour margin was defined as a narrow area at the tumour/host interface with a width of approximately 1mm between the invasive edge of carcinoma tissue and the adjacent non-tumorous fibro-adipose stroma of the breast tissue [14]. Lymphoid aggregates at the invasive margin were recorded if present. Scoring of all cases has been performed by 3 pathologists blinded to patient data to evaluate the inter-observer reliability. The average score for each case was obtained as final scores for purpose of analysis with clinicopathological data and outcome. For the purpose of analysis, TILs were categorised into 3 groups (low <10%, intermediate 11-50%, and high ≥50%) [3].

2.3 Statistical analysis

Statistical analysis wasperformed using SPSS version 20 for windows. The inter-rater reliability was assessed using inter-cluster correlation coefficient test (ICC). Mann Whitney *U* and Kruskal Wallis tests were used to evaluate the

association between TILs as a continuous variable and clinicopathological parameters. Survival analysis was performed by log ranks tests and the Kaplan–Meier curves. Statistical significance was defined as $p< 0.05$. The study was approved by the institutional ethics committee number (17100297) under the title "prognostic significance of tumour infiltrating lymphocytes in invasive breast carcinoma". All patients included were consented to participate in the study and to use their materials in research. All samples were pseudo-anonymised and stored in compliance with the Human Tissue Act. The study was performed in accordance with the Declaration of Helsinki.

3. Results

3.1 Clinicopathologic characteristics

Among cases included in study, there were 157 cases (85%) NST of type, 7 (4%) invasive lobular carcinoma (ILC) and 15 (8%) mixed NST and special type. Clinicopathological characteristics of the patients are shown in Table 1.

Clinicopathological variable	**Frequency (Number/Percentage)**
Age at diagnosis (Years)	
≤ 50	77 (42%)
> 50	107 (58%)
Tumour size (mm)	
<20	7 (4%)
20-50	139 (75%)
>50	38 (21%)
Histological type	
Invasive carcinoma, No special type	157 (85%)
Invasive lobular carcinoma	7 (4%)
Mixed NST and special type	15 (8%)
Pure special type	5 (3%)
Grade	
1	3 (2%)
2	153 (83%)
3	28 (15%)
Oestrogen receptor	
Negative	57 (31 %)
Positive	113 (61 %)
Not available	14 (8 %)
Progesterone receptor	
Negative	67 (36 %)
Positive	101 (55 %)
Not available	16 (9 %)

HER2status	
Negative	82 (45 %)
Positive	33 (18 %)
Not available	69 (37 %)
Molecular classification	
Luminal	120 (65 %)
Her2 enriched	13 (7 %)
TNBC	22 (12 %)
Not available	29 (16 %)
Biomarker defined subtypes	
ER+ HER2+	19 (10 %)
ER+HER2-	53 (29 %)
ER- HER2+	14 (8 %)
ER- HER2-	25 (13 %)
Not available	73 (40 %)
Tumour Stage	
I	10 (5 %)
II	60 (33 %)
II	39 (21 %)
IV	16 (9 %)
Not available	59 (32 %)
Local recurrence	
Yes	4 (2 %)
No	126 (69 %)
Not available	54 (29 %)
Distant metastases	
Yes	16 (9 %)
No	114 (62 %)
Not available	54 (29 %)
State of the patient	
Died	11 (6 %)
Alive	131 (71 %)
Not available	42 (23 %)
Tumour infiltrating lymphocytes	
Mild	56 (30%)
Moderate	117 (64%)
Dense	11 (6%)

Tertiary lymphoid structures	
Present	8 (4 %)
Absent	176 (96 %)
Pattern of TILs distribution	
Invasive margin	66 (36 %)
Whole tumour	118 (64 %)

Table 1: Clinicopathological features of the study cohort.

3.2 TILs scoring concordance and distribution

The interobserver concordnace of TILs scoring between the three observers was excellent (ICC= 0.95; $p<0.001$). The cohort showed unimodal non parametric distribution of TILs. The median TILs density was 15% (range 0-80%). Low TILs density (<10%) was observed in 30% of the cohort, moderate (11-50%) in 64% and high TILs density (>50% of the stroma occupied by inflammatory cells) in 6% of the cases. Figures 1 and 2 show examples of various TILs scores. Lymphoid aggregate at the invasive tumour margin were observed in 8 cases (4%) (Figure 3). TILs were heterogeneously distributed in the whole tumour (invasive margin and tumour centre) in 118 cases (64 %) while 66 cases (36 %) showed >95% of TILs at the invasive margin (Figure 4).

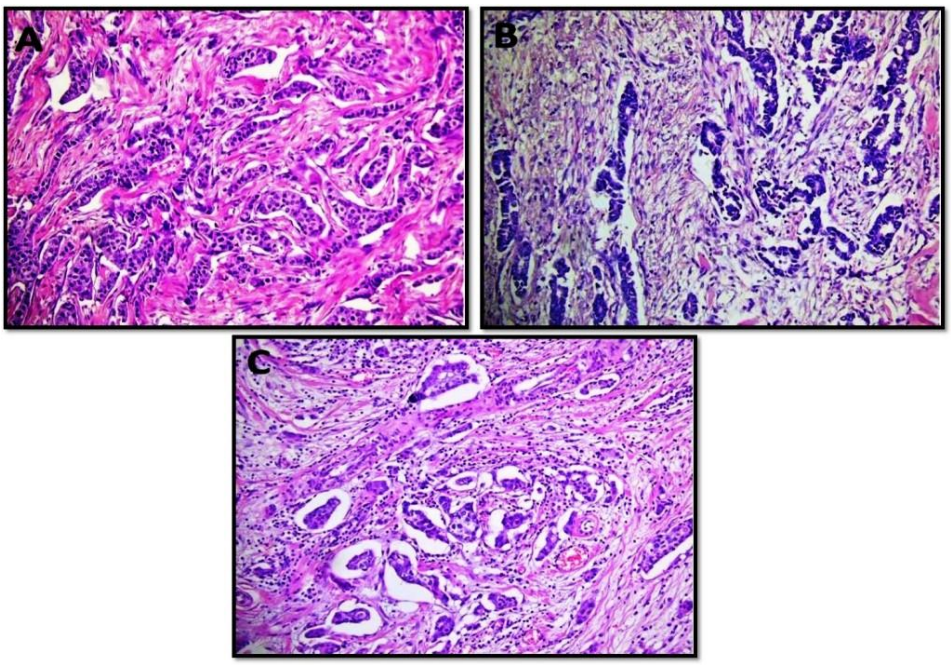

Figure 1: Microphotographic examples of invasive breast carcinomas with low (A and B) and intermediate (C) tumour infiltarting lymphocyte densities. All images at x20 magnifcation.

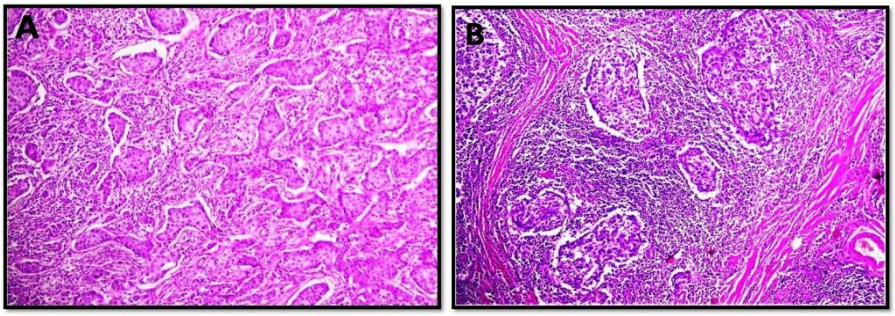

Figure 2: Microphotographic examples of invasive breast carcinomas with high tumour infiltrating lymphocyte densities. All images at x10 maginfcation.

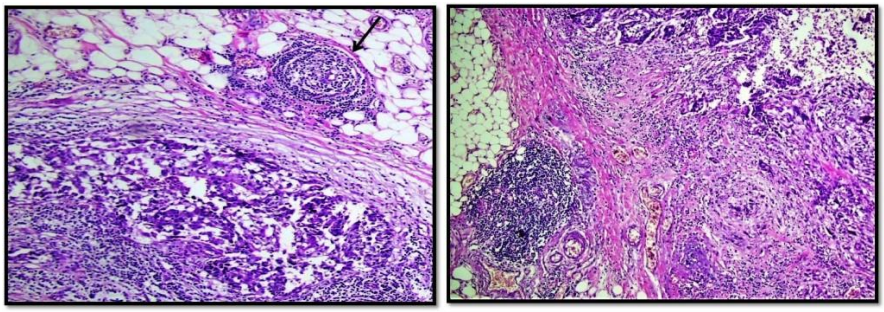

Figure 3: Examples of tertiary lymphoid structure, located at the invasive margin of breast carcinoma case (x10 magnification).

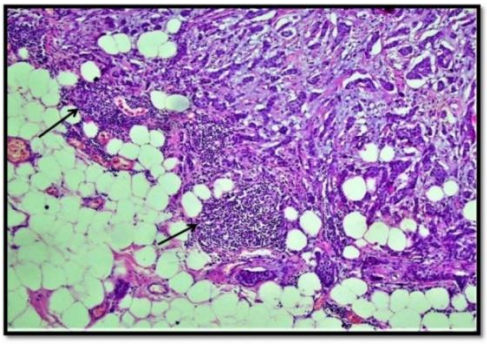

Figure 4: Example of invasive breast carcinoma showing high immune cell density at the invasive margin (arrow) and near complete absence at tumour centre. (x10 magnification).

3.3 Association of TILs and clinicopathological characteristics

The median score of TILs was higher in younger age patients <50 years (p=0.004). TILs were denser in larger (>20mm) than smaller tumours (p=0.01). There was statistically significant difference between the TILs density

among different histological types (p=0.001) where the highest median TILs score was present in NST type while ILC showed the least density. There is a steady increase of TILs density from grade 1 to grade3tumours. Median TILs score was 5, 10and 27.5 % in grade 1, 2 and 3, respectively (p=0.001). TILs were more frequent in cases with hormonal receptors negativity (p=0.001 for both ER and PR). There was no association between TILs and HER2 overexpression (p=0.308). Among molecular BC subtypes, TILs were statistically significant associated with TNBC and HER2 enriched subtypes (p=0.018). Table 2 summarise the association of TILs with different clinicopathological characteristics. No statistically significant association was detected between average stromal TILs and lymphoid aggregates or patterns of TILs distribution. Moreover, neither lymphoid aggregates nor patterns of TILs distribution were associated with any other clinicopathological characters.

3.4 The prognostic significance of TILs

At univariate analysis, tumour stage, age at diagnosis and molecular subtypes showed association with BCSS (Table 3). At multivariate analysis, tumour stage was independent prognostic factor for overall survival**.** There was no significant association between TILs and LRFI (p=0.453), DMFI (p=0.342) nor BCSS (p=0.447); (Figure 5). In TNBC group; two of12 cases (17%) showed local recurrence with TILs level between 11-50% while no events occurred in other two TILs groups. Association of TILs with DM revealed that one patient, 2and nil showed DM in cases with TILs score<10%, 11-50% and >50%, respectively however it did not reach statistical significance (p=0.601); (Figure 6).

Characteristics	**Median TILs score (%)**	***p*-value**
Age at diagnosis		
≤50 years (N = 77)	20.00	**0.004**
>50 years (N = 107)	10.00	
Tumour size		
<20 mm (N = 7)	10.00	**0.010**
20-50 mm (N = 139)	15.00	
>50 mm (N = 38)	10.00	
Histological type		
Invasive carcinoma, NST (N = 157)	15.00	
Invasive lobular carcinoma (N = 7)	5.00	**0.001**
Mixed NST and special type (N = 15)	5.00	
Pure special type (N = 5)	37.50	
Tumour grade		
Grade 1 (N = 3)	5.00	**0.001**
Grade 2 (N = 153)	10.00	
Grade 3 (N = 28)	27.50	
Lymphovascular invasion		

Present (N = 135)	10.00	0.511
Absent (N = 49)	15.00	
Ductal carcinoma in situ		
Present (N = 81)	15.00	0.661
Absent (N = 103)	10.00	
Tumour necrosis		
Present (N = 62)	20.00	0.09
Absent (N = 122)	10.00	
Oestrogen receptor status		
Negative (N = 57)	25.00	**0.001**
Positive (N = 113)	10.00	
Progesterone receptor		
Negative (N = 67)	25.00	**0.001**
Positive (N = 101)	10.00	
HER2 status		
Negative (N = 82)	15.00	
Positive (N = 33)	20.00	0.308
Molecular classification		
Luminal (N = 120)	10.00	
Her2 enriched (N = 13)	25.00	**0.018**
TNBC (N= 22)	25.00	
Biomarker defined subtypes		
ER+, HER2+ (N = 19)	15.00	
ER+, HER2- (N = 53)	10.00	**0.03**
ER-, HER+ (N = 14)	25.00	
ER-, HER2- (N = 25)	25.00	
Tertiary lymphoid structure		
Present (N = 8)	12.50	0.595
Absent (N = 176)	15.00	
Pattern of TILs distribution		
Whole tumour (N = 118)	15.00	0.622
Invasive margin (N = 66)	12.50	

Significant p values are in Bold

Table 2: Association between tumour infiltrating lymphocytes and other clinicopathological features.

Variable	BCSS		LRFI		DMFI	
	Log rank	***p value***	**Log rank**	***p value***	**Log rank**	***p value***
Stage	13.6	0.003	14.1	0.003	175.6	0.001
Age at diagnosis	4.09	0.04	1.03	0.308	0.2	0.649
Molecular classification	14.9	0.001	5.8	0.054	0.27	0.871
Tumour infiltrating lymphocytes	1.61	0.44	1.58	0.45	2.14	0.34

Significant p values are in Bold

Table 3: Univariate analysis of different clinicopathological factors and clinical outcome.

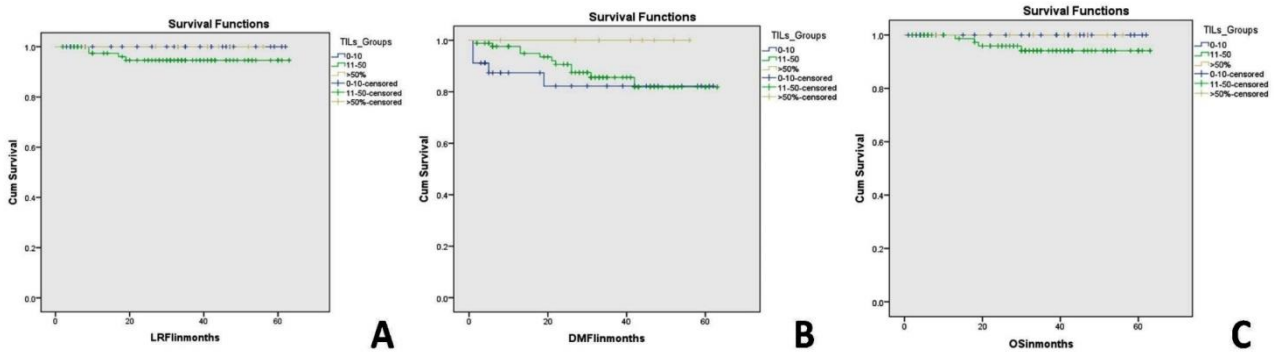

Figure 5: Kaplan Meier survival curves showing the association between tumour infiltrating lymphocytes density and local recurrence free interval (A), distant metastasis free interval (B) and breast cancer specific survival (C) in the whole study cohort.

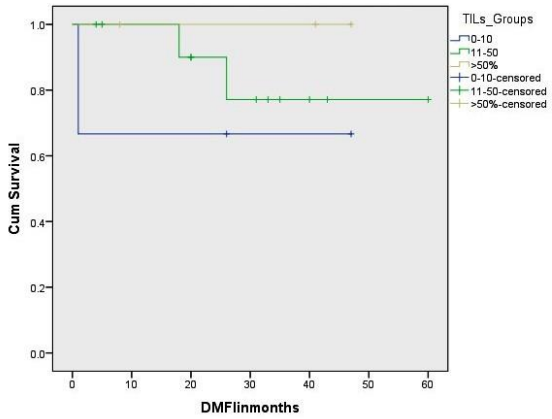

Figure 6: Kaplan Meier survival curves showing the association between tumour infiltrating lymphocytes density and distant metastasis free interval in triple negative breast cancer subtypes. Although it is not statistically significance, there is an obvious difference of the outcome between tumours with low TILs density and other groups.

In HER2 enriched subtype, TILs were not statistically significant associated with DM or BCSS(p=0.705, p=0.578, respectively).There was no prognostic significance of TILs distribution at the invasive margin in terms of LRFI (p=0.857), DMFI (p=0.684) and BCSS(p=0.784). No prognostic significance of lymphoid aggregates formation was observed.

4. Discussion

Tumour infiltrating lymphocytes (TILs) constitute a key component in tumour micro-environment that represent the local immune response against tumour [15]. Based on the emerging importance of TILs in IBC, a consensus guideline to standardise evaluation methodology of TILs assessment in H&E stained sections [13]. One of the main aims of publishing TILs evaluation methodology was to achieve robustness and reproducibility of the assessment method with achievement of good inter-observer agreement between pathologists. In this study, three pathologists have reviewed 184 BC cases and scored TILs as a continuous variable as recommended. Interestingly, scoring of TILs showed excellent interobserver agreement. This supports that the guidelines could consolidate the use of TILs in clinical practice. TILs represent an important prognostic and potentially predictive biomarker in many different solid tumour types such as melanoma [16], non-small cell lung cancer [17] and colorectal carcinoma [18]. A strong association between TILs, prognosis of IBC patients and treatment response has been reported in a number of retrospective series including about 17,000 patients [19, 20]. Difference in TILs value within various ethnicities was reported previously [21]. Despite the large prevalence of BC in Africa, there is a large gap in the data about pathologic features of BC. The prognosis of African patients is less favourable than their counterparts in higher income countries [22]. There is very limited information about TILs in African population which may be different from American and European populations; possibly due to differences in intrinsic tumour features, genetic background and carcinogen exposure [22]. So, better understanding about the prognostic biomarkers in African patients is essential. We retrospectively evaluated TILs in a cohort of IBC from Egypt with median follow up of 32 months. TILs scores were higher in patients <50 years, similar to the report from Tian*et al.* [23] who found that TILs scores were higher in younger patients in studies conducted on Chineese patients. The mechanism of relationship between TILs and age is questionable, however it may be explained by younger patients with BC usually exhibit more aggressive features and more likely to be hormone receptor negative which have more antigenicity and hence more immune reaction [25].

Our results showed statistically significant association of TILs scores with different size groups. Also, median TILs score gradually decrease with higher stage. One study showed that high CD8+ TILs positively correlate with tumour size less than 50 mm [26]. This could be related to the antitumor effect of CD8+ TILs, so they control the growth of the tumour by balancing the anti-tumour and pro-tumour immune responses [26]. Association of TILs with nodal stage revealed that highest TILs scores were in patients without nodal metastases. The correlation between increased TILs and lymph node status was first reported in gastric cancer [27] and melanoma [28]. One previous study on European reported that increased number of TILs was associated with lower number of lymph nodes metastases, suggesting that tumours with higher TILs have less aggressive clinical course [29]. Regarding association of TILs

with different histological types, we found that highest TILs scores were detected in NST carcinoma. Metaplastic carcinoma cases showed absence of hormone receptor expression which may contribute to high TILs amount in this rare special type [30]. Jonej*aet al* reported that PD-L1 expression is significantly higher in metaplastic carcinoma than in NST tumours, which contribute to poor clinical outcome [31]. We demonstrated that TILs are lowest in ILC and mixed invasive ductal carcinoma with special type. Similarly, Desmedt*et al* [32] reported that most ILCs have low TILs level compared with NST tumours in British population. ILCs are characterised by being mostly low grade and hormonal receptor positive tumours which may attribute to low lymphocytic response [32]. The association of TILs with newly defined biomarker types was statistically significant and not surprisingly, TILs were higher in ER negative tumours. The updated classification acknowledged these clinical relevant subtypes of invasive carcinoma as these subtypes are different in pathogenesis, treatment response and prognosis [9]. The overview acknowledges the treatment-relevant subtypes of invasive carcinoma (based on ER and HER2 status) and new data is added to support the differences in pathogenesis, treatment response and prognosis of these clinically relevant groupings.

In agreement with previous studies [23, 33-35], high TILs correlated with higher histological grade and ER negative tumours in our study. High grade carcinomas are commonly of TNBC [36] and HER2 enriched subtypes [37]. The frequency of high TIL levels depend on the molecular subtype of BC [38]. Our study showed that median TILs score was higher in TNBC and HER2 enriched subtypes than luminal subtype. TNBC is usually common among African patients than White and European populations. This was previously described by multiple studies which reported that the median percentage of TILs was highest in ER negative and HER2 negative tumours [38]. Moreover, Papaioannou*et al.*[15] reported that dense lymphocytic infiltrate was observed in HER2 enriched and triple negative patients. This could possibly be due to high somatic mutational load in those specific subtypes, leading to appearance of neo-antigens that stimulate the immunologic response [39]. Our study showed no significant prognostic association between TILs and patient outcome neither for the whole cohort or in TNBC subgroup. This result is in agreement with previous study done by Park *et al* who reported that evaluation of TILs may not be useful in predicting survival in TNBC, on a study included Korean patients [40]. Contrary to the results of our study, results of previous studies that included data of randomised clinical trials evaluating data of 16,000 patients with available clinical follow up data, suggested that TIL expression is associated with prognosis in TNBC patients (19, 41). These studies were conducted mainly on non-African populations; thus such ethnic differences and genetic backgrounds may contribute for this contradictory. Other factors include cohort sample size and difference in management and follow up protocols among various institutions.

TILs were not prognostic in HER2 enriched cases in our study. This is contrary to data from NeoALLTO study trial reporting relationship between recurrence free survival and TILs population in HER2 positive patients [42]. However, the use of antiHER2 (trastuzumab) adjuvant therapy complicate the interpretation of data for HER2 enriched tumours [43] Similar to our results, Loi*et al.* reported that TILs have no prognostic value in hormonal receptor positive breast tumours, although their study included a cohort with different ethnic background [38] . The short median follow-up period in our study may be the reason for the discrepancy in the prognostic value of TILs

between our study and other studies. Another limitation in this study is the lack of defining various types of immune cells using immunohistochemistry for B and T lymphocytes and other inflammatory cells that were used in other studies [44, 45]. The results of the previous studies showed the prognostic value of immunohistochemical analysis of TILs as the different types of TILs surrounding tumour have different roles in tumour suppression or progression. For instance, the negative effects of FOXP3+TILs on survival have been demonstrated in TNBC [44]. Contrary, CD8 + TILs correlated with better survival [45]. Since TILs are heterogeneously distributed, we separately evaluated TILs at the invasive margin and reported presence or absence of tertiary lymphoid structure (TLS), in order to find any clinical implication of tumour heterogeneity. Cases with TILs distributed mostly at the invasive margin and cases with TLS did not show any statistically significant association with patient outcome in our study. In other cancers, for example colorectal cancer, dense inflammatory infiltrate at the invasive margin is a good prognostic factor [18]. Over the past years, multiple studies have attempted to examine the relationship between the inflammatory infiltrate at the invasive margin and survival in IBC. However, the results were conflicting[46].Recently, Romagnoli*et al.*[47**]**looked at quantity and distribution of various immune cell subtypes at invasive margin and tumour centre. They found no statistically significant association in quantity and distribution of TIL ratios and subsets. Moreover, no statistically significant difference was detected when comparing relapse with non-relapse group. Koing *et al* [14] reported that TILs infiltration pattern in primary BC differed in invasive margin and tumour centre significantly, adding evidence that the invasive tumour margin is an immunologically active area .Further studies are needed to understand the exact role and clinical significance of TILs density and different TILs populations in invasive margin. Of note, the retrospective design of this study is a limitation as it decreases the statistical impact of the study while other studies assessed TILs in prospective trials [38, 41, 48]. However, we believe that it may clarify interesting data about association of TILs with pathological and clinical characteristics in Egyptian cohort. TILs have the potential to act as surrogate marker for prognosis.

5. Conclusion

Our study revealed that TILs are higher in younger patients and in special types of breast carcinoma in Egyptian patients similar to other ethnic groups. Moreover, a meaningful correlation was observed with respect to association of TILs with high grade tumours. TILs are higher in TNBC and HER2 enriched subtypes. However, we did not find any prognostic value in our study which may reflect difference behaviours of TILs in IBC of Egyptian population. Various mechanistic and comparative studies involving large cohort of patients from different ethnic and genetic backgrounds are highly warranted to investigate the prognostic and therapeutic value of TILs in IBC.

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