Original Article

Tumor Infiltrating Lymphocytes Predicting Long-Term Outcomes in HER2-Negative Breast Cancer Patients with Visceral Metastases

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Abstract

Background: Tumor-infiltrating lymphocytes (TILs) were found to be associated with a better clinical outcome in specific subtypes of breast cancer.

Aim: To study the association between TILs and the prognosis of Egyptian patients with HER2-negative breast cancer metastatic to the viscera.

Methods: This prospective study included 100 patients with HER2-negative metastatic breast cancer. Intratumoral TILs, stromal TILs, and CD4 and CD8 were examined in the pathological specimens and their relationship with survival and response to treatment was studied.

Results: At a median follow-up period of 43 months, the median overall survival was 44.7 months (95%CI: 39.2-50.2) and the 5-year overall survival rate was 28%. A high level of CD8+ve TILs was associated with significantly longer overall survival (p<0.001) and progression-free survival (p=0.043). There was no significant correlation between intratumoral TILs, stromal TILs, or CD4+ve and overall survival.

Conclusions: A higher level of CD8+ve TILs is associated with better overall as well as progression-free survival in HER2-negative breast cancer with visceral metastases.

Introduction

Breast cancer is a significant public health issue expected to cause more than one million new cases and nearly half a million fatalities annually. It is the most prevalent cancer among women in Egypt, accounting for one-third of all cancer cases in this group. [1] Mortality because of breast cancer is thought to be over 11%, according to the Egyptian registry, making it the second leading cause of cancer-related death after liver cancer. [2]

Although therapeutic approaches for breast cancer have improved, there is still a need for new biomarkers to direct treatment in the metastatic scenario. In primary breast cancer, tumor-infiltrating lymphocytes (TILs) were found to be related to prognosis for many years. [3]

In a process known as the elimination phase of immune recognition and eradication of malignant cells, CD8+ T cells and CD4+ T cells were discovered by Dunn et al. in 2004 to be the primary components of immune-surveillance related lymphocytes and to be capable of recognizing and eliminating malignant cells. [4]

Although it is generally acknowledged that having a high TIL infiltration is associated with a better prognosis, there is much debate over the TIL subpopulations and the observed response. [5] TILs

are determined to be T cells in around 75% of cases, and CD8+ T cells are associated with better clinical outcomes. [6] Specifically, type 1 T helper (Th-1) cells from CD4+ T cells are a favorable prognostic indicator and are frequently linked to improved overall clinical outcomes. [6, 7]

According to Denkert et al., a high number of TILs has drawn significant interest as a biomarker for predicting pathological complete response (pCR) following neoadjuvant chemotherapy. The proportion of intratumoral TILs (iTILs) as a continuous variable was shown to correlate independently with pCR by the same research group. [8] While TILs have a prognostic utility in the adjuvant breast cancer treatment setting, there is a dearth of information in the metastatic scenario. [9] Although iTILs and stromal TILs (sTILs) often correlate, certain studies have discovered a greater connection with one or the other. [8, 11]

This study aimed to examine the relationship between the infiltration by TILs and the treatment outcome in Egyptian patients with metastatic HER2-negative breast cancer.

Methods

This was a prospective observational study that included 100 patients with breast cancer and was conducted at Assiut University Hospitals between January 2015 and January 2021.

Patients

The inclusion criteria were as follows: age between 18 and 70 years, Eastern Cooperative Oncology Group (ECOG) performance scale of 0 to 2, pathologically proven malignant breast cancer positive for HER2, visceral metastases, and scheduling for chemotherapy. Patients with bone as the only metastatic sites were excluded as well as those expected to be non-compliant.

Evaluation of tumor-infiltrating lymphocytes (TILs)

The Pathology Department of Assiut University Hospital provided the paraffin blocks. Histological analysis was done on the hematoxylin and eosin-stained slides and the HER2, estrogen receptors (ER), and progesterone receptors (PR) immunostained slides. HER2 stain scores of 0 or +1 were regarded as negative. If ER / PR expression was more than 10%, it was regarded as positive. [10]

Sections of 3–4 m thick formalin-fixed paraffin-embedded tissue blocks were cut and mounted on positively charged slides. Utilizing antigen retrieval in Tris EDTA, a DAKO automatic immunostainer was employed (pH9). A second kit for envision detection was used. Mayer's hematoxylin was used as a counterstain for the tissue slices.

Stromal TILs were recognized as lymphocytes dispersed throughout the tumoral stroma and do not directly interact with carcinoma cells while Intratumoral TILs (iTILs) are those in tumor nests that have cell-to-cell contact with carcinoma cells. Infiltrating lymphocytes were evaluated and calculated under a microscope at five high-power fields. The median lymphocyte percentages within tumor cell clusters (iTILs) and within the stroma (sTILs) were calculated. Then it was determined how many CD4-positive and CD8-positive lymphocytes were present in iTILs and sTILs.

Treatment and follow up

Patients who had never had chemotherapy were treated with an anthracycline/taxane regimen. Those who already received an anthracycline/taxane regimen before were treated with other protocols. Subtypes of luminal breast cancer were treated with vinorelbine and capecitabine, whereas those with the triple-negative disease were treated with gemcitabine and platinum. Following the completion of systemic chemotherapy, patients with ER/PR-positive disease were offered hormonal therapy. Patients with the triple-negative disease were kept under follow-up

End Point Definition

The primary endpoint was progression-free survival (PFS), which was correlated with the proportion of iTILs and other evaluated factors (sTILs, and CD4 and CD8 positivity). Progression-free survival was defined as the duration from the commencement of treatment until the first sign of a progressing, relapse, or death from any cause.

Secondary end-points included the response to 1st line chemotherapy for the metastatic disease and overall survival (OS) which was defined as the duration from commencing treatment to death from any cause. The relationship between iTILs and other variables and response to treatment and OS was studied as well.

Statistical Methods

Using IBM SPSS Statistics for Windows, version 22.0. (Armonk, NY: IBM Corp), all data were gathered, tabulated, and statistically analyzed. Continuous variables were expressed as the mean

and SD or median and range, and categorical variables as number and percentage. When appropriate, the Pearson Chi-square test or Fisher's exact test was used to compare the distribution of categorical variables. The Kaplan-Meier method was used to estimate time-to-event distributions, and distributions were compared using the two-sided exact log-rank test. All tests were two-sided. Statistical significance was defined as *p*-value <0.05.

Results

Ninety-three out of the 100 enrolled patients (93%) met the inclusion criteria and seven patients were excluded because of the inadequate tumor tissue available to complete the full pathological investigations. Patient and tumor characteristics and the results of immunohistochemistry (IHC) studies are illustrated in Table 1.

Table 1: Patient and tumor characteristics and immunohistochemistry results (*n*=93)

	n	%
Age (years)		
< 50	39	41.9
≥ 50	54	58.1
Menopausal status		
Premenopausal	44	47.3
Postmenopausal	49	52.7
Family history		
Negative	49	52.7
Positive	44	47.3
Side		
Left	57	61.3
Right	33	35.5
Bilateral	3	3.2
CA 15.3		
High	34	36.6
Unknown	59	63.4
Grade		
2	74	79.6
3	19	20.4
Lymphovascular invasion		
Absent	26	28
Present	67	72
Site of 1ˢᵗ metastases		
Lung	48	51.6
Liver	17	18.3
brain	3	3.2
Multiple sites	25	26.9
Estrogen receptor		
Negative	29	31.2
Positive	64	68.8

	n	%
Progesterone receptor		
Negative	51	54.8
Positive	42	45.2
Molecular subtype		
Luminal A/B like	64	68.8
Triple-negative	29	31.2
CD8 IHC staining		
Negative	46	49.5
Positive	47	50.5
CD4 IHC staining		
Negative	40	43
Positive	53	57
sTILs IHC staining		
Negative	42	45.2
Positive	51	54.8
iTILs IHC staining		
< 5%	46	49.5
≥ 5%	47	50.5

iTILs: Intratumoral tumor-infiltrating lymphocytes, **sTILs:** Stromal tumor-infiltrating lymphocytes, **IHC:** Immunohistochemistry

Their median (range) age was 53 years (30-68) and the mean (SD) was 51.7 (9.3). Twenty-one (23%) patients had visceral metastases as their initial illness presentation (denovo metastatic), while 72 cases (77%) were initially diagnosed with a non-metastatic disease and developed visceral metastases later in the course of their disease. Approximately 67 (72%) patients showed positive lymphovascular invasion (LVI), and 74 (79.6%) patients had grade 2 tumors. Patients with ER-negative tumors included 29 (31.2%) and those with PR-negative tumors comprised 51. (54.8%). The molecular subtyping was luminal A/B-like in 67 (72%) patients and triple-negative (TN) in 26 (28 %).

Figure 1 demonstrates examples of IHC with different percentages of iTILs and sTILs in breast cancer.

Patients were followed up for a median of 43 months (36-60 months). Regarding the survival data, we noticed that; the median PFS for the first- and second-line chemotherapy was 13 months (95% C I 8.97 – 17.02), and 7 months (95% CI 6.24 – 7.75) respectively. There was a significant correlation between PFS after 1ˢᵗ line chemotherapy and iTILs staining as well as with sTILs, CD4, and CD8 (Table 2). The median OS for the studied patients was 44.7 months (95%CI: 39.2-50.2), and the 5-year OS rate was 27.8%. Only CD8 has a significant correlation with OS following 1ˢᵗ line therapy (Table 2).
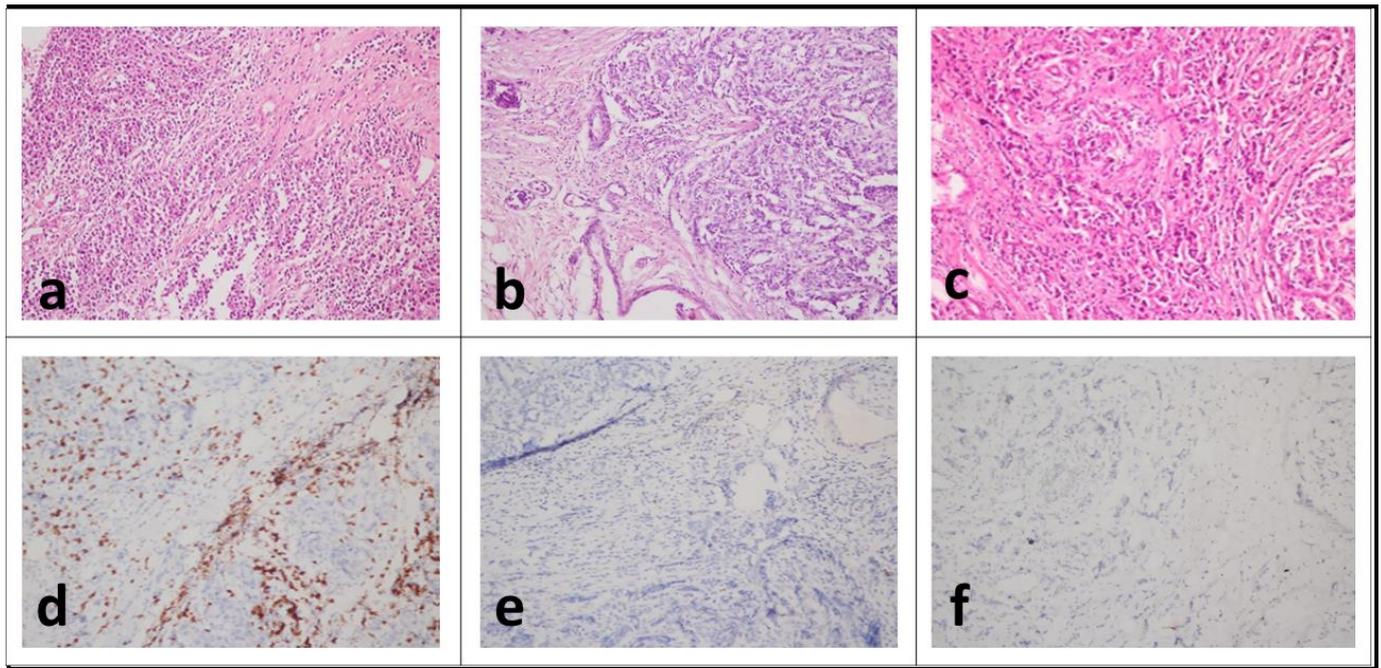
Figure 1: Examples of staining for intratumoral tumor-infiltrating lymphocytes (iTILs) and stromal TILs (sTILs): a) Invasive ductal carcinoma (IDC) with 60% iTILs and 40% sTILs (H&E 200X), **b)** IDC with <10% iTILs and 50% sTILs (H&E 200X), **c)** IDC with 30% iTILs and 50% sTILs (H&E 200X), **d)** All infiltrating lymphocytes are positive for CD8 (CD8 immunostaining, 200X), **e)** <10% of lymphocytes positive for CD4 (CD4 immunostaining, 200X), **f)** All infiltrating lymphocytes are negative for CD4 (CD4 immunostaining, 200X)

Table 2: Overall and progression-free survival following 1ˢᵗ line treatment according to iTILs, sTILs, CD4, and CD8

		Progression-free survival			Overall survival		
		Median	95% CI	*p*-value	Median	95% CI	*p*-value
iTILs	<5%	14	10.9 - 17.1	0.010	46.4	36.5 - 56.3	0.91
	≥5%	11	6.6 - 15.4		43.7	40.6 - 46.7	
STILs	Negative	14	4.3 - 23.7	0.025	40.6	26 - 55.1	0.703
	Positive	13	9.3 - 16.7		45	40.9 - 49	
CD4	Negative	16	13.2 - 18.8	<0.001	46.6	39.4 - 53.8	0.773
	Positive	7	5.8 - 8.2		42.2	37.1 - 47.4	
CD8	Negative	7	5.2 - 8.8	0.043	34.8	26.3 - 43.4	<0.001
	Positive	15	12.4 - 17.6		53.1	48.5 - 57.7	

CI: Confidence interval, **iTILs:** Intratumoral tumor-infiltrating lymphocytes, **sTILs:** Stromal tumor-infiltrating lymphocytes

The relationship between the results of IHC and the studied variables is shown in Table 3. iTILs had a significant correlation with family history and being metastatic at presentation; while sTILs correlated significantly with lymphovascular invasion only. CD8 positivity had a significant correlation with tumor size and nodal status at presentation. On the other hand, CD4 positivity correlated significantly with age, family history, grade, and PR status.

There was a significant correlation between the status of iTILs, sTILs, and CD4 and the site of metastases (*p*-value: 0.034, <0.001, and 0.010; respectively). There was no significant correlation between CD8 status and the site of metastases.

As for the correlation with the response to 1ˢᵗ line chemotherapy for metastatic disease, TILs, STILs and CD8 had a significant correlation with the response (Table 4).

Table 3: The relationship between iTILs, sTILs, CD4, and CD8 and clinicopathological variables

	iTILs			sTILs			CD4			CD8		
	-ve	+ve	*p*	-ve	+ve	*p*	-ve	+ve	*p*	-ve	+ve	*p*
	n(%)	*n*(%)		*n*(%)	*n*(%)		*n*(%)	*n*(%)		*n*(%)	*n*(%)	
Age (years)												
< 50	19(49)	20(51)	0.903	19(49)	20(51)	0.56	12(31)	27(69)	0.044	22(56)	17(44)	0.257
≥ 50	27(50)	27(50)		23(43)	31(57)		28(52)	26(48)		24(44)	30(56)	
Menopausal												
No	22(50)	22(50)	0.922	21(48)	23(52)	0.639	15(34)	29(66)	0.102	26(59)	18(41)	0.08
Yes	24(49)	25(51)		21(43)	28(57)		25(51)	24(49)		20(41)	29(59)	
Family history												
Negative	19(39)	30(61)	0.031	22(45)	27(55)	0.957	10(20)	39(80)	<0.001	26(53)	23(47)	0.466
Positive	27(61)	17(39)		20(46)	24(55)		30(68)	14(32)		20(46)	24(55)	
Side												
Left	27(47)	30(53)	0.204	24(42)	33(58)	0.145	22(39)	35(61)	0.105	25(44)	32(56)	0.127
Right	16(49)	17(52)		15(46)	18(55)		18(55)	15(46)		18(55)	15(46)	
Bilateral	3(100)	0		3(100)	0		0	3(100)		3(100)	0	
Grade												
2	37(50)	37(50)	0.838	33(45)	41(55)	0.829	36(49)	38(51)	0.031	36(49)	38(51)	0.758
3	9(47)	10(53)		9(47)	10(53)		4(21)	15(79)		10(53)	9(48)	
LVI												
Negative	15(58)	11(42)	0.325	19(73)	7(27)	0.001	12(46)	14(54)	0.704	17(65)	9(35)	0.057
Positive	31(46)	36(54)		23(34)	44(66)		28(42)	39(58)		29(43)	38(57)	
T*												
T2	30 (57)	23(43)	0.366	24(45)	29(55)	0.491	27(51)	26(49)	0.353	26(49)	27(51)	0.005
T3	7(47)	8(53)		9(60)	6(40)		5(33)	10(67)		13(87)	2(13)	
T4	8(38)	13(62)		8(38)	13(62)		7(33)	14(67)		6(29)	15(71)	
Unknown	1(25)	3(75)		1(25)	3(75)		1(25)	3(75)		1(25)	3(75)	
N*												
0	4(31)	9(69)	0.432	7(54)	6(46)	0.162	7(54)	6(46)	0.259	13(100)	0	0.001
1	10(50)	10(50)		5(25)	15(75)		10(50)	10(50)		11(55)	9(45)	
2	23(54)	20(47)		24(56)	19(44)		14(33)	29(67)		17(40)	26(61)	
3	8(62)	5(39)		5(39)	8(62)		8(62)	5(39)		4(31)	9(69)	
Unknown	1(25)	3(75)		1(25)	3(75)		1(25)	3(75)		1(25)	3(75)	
M*												
M0	40(56)	32(44)	0.03	36(50)	36(50)	0.084	31(43)	41(57)	0.987	34(47)	38(53)	0.426
M1	6(29)	15(71)		6(29)	15(71)		9(43)	12(57)		12(57)	9(43)	
TNM stage*												
II	19(53)	17(47)	0.184	16(44)	20(56)	0.275	17(47)	19(53)	0.369	21(58)	15(42)	0.266
III	22(55)	18(45)		21(53)	19(48)		14(35)	26(65)		16(40)	24(60)	
IV	5(29)	12(71)		5(29)	12(71)		9(53)	8(47)		9(53)	8(47)	
CA15.3												
High	19(56)	15(44)	0.347	11(32)	23(68)	0.061	15(44)	19(56)	0.871	16(47)	18(53)	0.726
Unknown	27(46)	32(54)		31(53)	28(48)		25(42)	34(58)		30(51)	29(49)	
ER												
Negative	12(41)	17(59)	0.3	15(52)	14(48)	0.395	12(41)	17(59)	0.832	18(62)	11(38)	0.104
Positive	34(53)	30(47)		27(42)	37(58)		28(44)	36(56)		28(44)	36(56)	
PR												
Negative	23(45)	28(55)	0.297	23(45)	28(55)	0.906	27(53)	24(47)	0.042	27(53)	24(47)	0.391
Positive	23(56)	18(44)		19(46)	22(54)		13(32)	28(68)		18(44)	23(56)	

***At initial diagnosis, iTILs:** Intratumoral tumor-infiltrating lymphocytes, **sTILs:** Stromal tumor-infiltrating lymphocytes, **LVI:** Lymphovascular invasion, **ER:** The estrogen receptor, **PR:** Progesterone receptor

Table 4: Relationship between iTILs, sTILs, CD4, and CD8 and the best response to 1st line treatment of metastatic disease

	Response to 1st line treatment				*p*-value
	CR (*n*=9)	PR (*n*=49)	SD (*n*=18)	PD (*n*=17)	
iTILs					
Negative	6 (13)	27 (59)	12 (26)	1 (2.2)	<0.001
Positive	3 (6)	22 (47)	6 (13)	16 (34)	
STILs					
Negative	3 (7)	25 (60)	9 (21)	5 (12)	0.382
Positive	6 (12)	24 (47)	9 (18)	12 (24)	
CD4					
Negative	6 (15)	26 (65)	5 (13)	3 (8)	0.017
Positive	3 (6)	23 (43)	13 (25)	14 (26)	
CD8					
Negative	0	25 (54)	12 (26)	9(20)	0.011
Positive	9 (19)	24 (51)	6 (13)	8(17)	

CR: Complete response, PR: Partial response, SD: Stable disease, PD: Progressive disease, **iTILs**: Intratumoral tumor-infiltrating lymphocytes, **sTILs**: Stromal tumor-infiltrating lymphocytes

Discussion

To the best of our knowledge, this study is among the first conducted in Egypt to evaluate the prognostic significance of TILs in patients with metastatic HER2-negative breast cancer.

In this group, a high level of iTILs and sTILs was linked with a better PFS. This finding is consistent with the results of Luen et al. who suggested a link between TILs and survival in cases of advanced HER2-positive breast cancer. [12] Additionally, in the BIG 02-98 adjuvant study, Loi et al. found a relationship between TILs and better OS in patients with triple-negative breast cancer. [13]

The CD8 and CD4 positivity of lymphocytes was examined in this study and we found a significant correlation between CD4 and CD8 positivity and PFS. CD8+ve lymphocytes were positively correlated with OS which is concordant with Ma et al. data. [14] Furthermore, our results matched those of Song et al. who suggested that elevated levels of CD8+ve T lymphocytes represent a novel independent predictor of PFS. [15] Mahmoud et al. found that tumor-infiltrating CD8+ve T lymphocytes have antitumor activity as judged by their favorable effect on patients' survival and could potentially be exploited in the treatment of breast cancer. [16]

The nodal status and tumor size correlated significantly with CD8+ve cells in our study. This is consistent with the findings of Mahmoud et al. [16] We also found that CD4+ve lymphocytes correlated significantly with age, tumor grade, and PR status. This is supported by the findings of Matkowski et al., who reported a strong association between CD4 expression and lymph node status and lymphatic infiltration intensity. [17]

Our findings show a very strong correlation between sTILs and lymphovascular invasion. Additionally, we found a strong association between iTILs infiltration and the presentation of metastatic disease from the start. This is supported by the findings of Miyoshi et al. who described a substantial correlation between TILs and nodal metastasis, ER status, PR status, tumor grade, and Ki67 labeling index. [18]

In the current study, there was a significant correlation between iTILs IHC staining and the first site of metastasis, specifically lung metastasis. This is consistent with the finding of Takada et al. that higher iTILs density was associated with lung metastasis, which was the most frequent site of distant metastasis in their study that included ER-positive/HER2-negative patients. [19]

This study has limitations that include the small number of patients recruited which is related mainly to the challenges in retrieving the paraffin blocks. Also, it the difficulty in taking a biopsy from distant metastases like small pulmonary nodules to assess the level of TILs in the metastatic site and to compare it with TILs in the primary tumor. This can be assessed in a larger study comparing the differences in the percentage of TILs in the primary and metastatic sites.

Conclusion

In the current study, iTILs, sTILs, CD8 positivity, and CD4 positivity correlated significantly with PFS. CD8+ve lymphocytes positive were linked to increased OS. Given that they are straightforward and affordable markers, we propose that iTILs, sTILs, CD4, and CD8 lymphocytes be used as prognostic tools in the metastatic phase of breast cancer and later may be useful as predictive markers for various types of treatment to provide a better service to our patients despite the country's limited resources.

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