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Evaluation of the Role of Tumor-Infiltrating Lymphocytes and CD8+ Cytotoxic Lymphocytes in the Survival of Patients with Breast Cancer

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ABSTRACT

Background: This study aimed to evaluate the significance of tumor lymphocyte infiltration (TIL) and the number of CD8+ T cells in breast cancer and their relationship with the other clinicopathological factors and overall survival (OS) was investigated.

Materials and Methods: The studied samples were breast cancer patients (2005-2017) referring to the medical oncology departments for treatment. Pathologic samples of breast cancer patients were evaluated in terms of TIL and positive immunohistochemical staining for CD8 cytotoxic cells.

Results: 299 patients were entered into the study, 3 male and 296 female. Their mean follow-up period was 61 months. Statistical findings indicated that lymph involvement is more accompanied by low TIL within the tumor (0.011). Correlations were observed between the estrogen, progesterone receptors, P53 state, and TIL; which were significant by P-value<0.049, P-value=0.024, P-value =0.002, respectively. With any Ki67 value, the number of patients with less than 30% TIL was more considerable than the two other groups with lymphocyte cut-off of 30-50% and more than 50%. Comparison of the OS of patients with positive and negative CD8 cytotoxic lymphocytes in 45 patients with lymphocyte infiltration of equal or more than 40% showed that the OS results were in favor of patients with CD8+ cytotoxic lymphocyte (0.022). Out of 299 patients, 17 died.

Conclusion: Our findings showed that in cases of CD8+ cytotoxic lymphocytes in tumors, the OS of the patients will be enhanced which can act as an independent.

Keywords: Breast cancer; CD8+ cytotoxic lymphocytes; Overall Survival; Tumor-infiltrating lymphocytes

INTRODUCTION

One of the major objectives of breast cancer researche is to find new prognostic and predictive factors to determine the survival rate of the patients. The presence of tumor lymphocyte infiltration(TIL) cells is one of the factors which has been studied in

the last decade[1].The prognostic relevance of lymphocyte infiltration in the tumor has been more frequently observed with positive CD8 + lymphocytes[2]. Prognostic association of most of the positive CD8 lymphocytes (TIL) has been observed which is the major part of the host immune response against the foreign antigens, also known as the cancer cell killing cells. Cytotoxic $CD8^+$ lymphocyte cells produce gamma interferon[3].Tumor-infiltration of CD8+ lymphocytes can be regarded as an independent factor to evaluate the response to therapy. The immune system's impacts on the response to the treatment have been evaluated in cancer patients to promote prognostic influences. This approach has been also employed in immune checkpoint inhibitor treatments[4]. These treatments have shown promising outcomes in breast cancer cases with triple-negative subtypes and positive Her2-Neu[5].Tumor lymphocyte infiltration can show synergetic impacts on chemotherapy. This means that these lymphocytes can increase the sensitivity of cancer cells to chemotherapy. In some studies, tumor lymphocyte infiltration was reported as the prognostic factor predicting the response to the treatment, in particular when chemotherapy included anthracyclines or taxanes [6,7]. The aim of the present study is to evaluate the role of Tumor lymphocyte infiltration and the consequent effect of CD8+ lymphocytes in overall survival (OS) and disease-free survival (DFS) of breast cancer. Investigation of their relationship with clinicopathological characteristics of the patients and other prognostic factors, such as luminal sub-types, is the other goal of this study.

MTERIALS AND METHODS

The studied samples were breast cancer patients (2005-2017) from the western provinces of Iran referring to the medical oncology departments of medical science university-affiliated hospitals possessing a file or being in follow-up; who cooperated in delivering their pathological samples to the pathology centers. These samples were re-evaluated in our center. In the case of residual tumor, the required cuts were conducted and hematoxylin-eosin staining was carried out. Two pathologists examined the percentage of lymphocyte infiltration. If infiltration exceeded 30%, immune-histochemical staining was also performed for CD8. Based on hematoxylin-eosin staining the grade of the sample was classified into three groups: below 30%, 30-50% and above 50%. Clinicopathological data of the patients were extracted from their files and recorded in a checklist; these parameters included their age, tumor size, cancer grade, lymph node metastasis, distant metastasis, hormone receptor condition, Her2/neu score, Ki67, vascular involvement, perineural, P53, and luminal status. In this research, the association between the lymphocyte infiltration and CD8+ cytotoxic lymphocytes of the tumor with the clinicopathological data of the patients was assessed and its impact on the patient survival was studied.

RESULTS

299 breast cancer patients were entered into the study.3 of them were male and the remaining 296 were women.Their average follow-up period was 61 months ranging from a minimum of 7 to a maximum of 146 months (mean and median of 61 and 51, respectively). The average age of the patients was 47.21.The age distribution of the patients revealed that 6 of them were in the age range of 20-29; 61 were 30-39 years old; while 120, 81, and 31 patients were in the age ranges of 40-49, 50-59 and above 60, respectively.The Staging our patients is shown in Table 1. This table also presents the hormone receptor and Her2neu status of the patients. In terms of luminal classification, only 236 patients could be luminally evaluated: 78 of them were luminal A, 101 patients were in luminal B group; 30 patients were classified in positive triple-negative while 27 of them were in Her 2neu-rich group.Table 1 demonstrates the relationship between luminal classification and TIL. The patients were also evaluated in terms of lymphocyte infiltration which revealed that 173 patients had TIL range of 0-10%, 48 of them showed TIL in the range of 11-20%; TIL of 29 patient varied from 21 to 30%; 10 patients had TIL range of 31-40%; 15 patient had TIL of 41-50% and TIL of 24 patients was higher than 50%. Three TIL cut off groups were used to investigate the relationship with the other clinicopathological factors (<30%, 30-50%, and >50%). Statistical analysis showed that the

higher the involved lymph nodes, the lower the TIL will be. This difference was significant with the p-value of 0.011 this implies that the higher the disease stage in terms of lymph node involvement, the lower the TIL (Table 2).The relationship between the TIL and estrogen and progesterone receptors was also addressed. There was a significant relationship between the progesterone receptor state and TIL (P-value<0.024), (Table 2). In cases with positive progesterone receptors, TIL was less than 30% in most of the patients. In the majority of patients with negative progesterone receptor, TIL was less than 30% suggesting the TIL as an independent factor in relation to the progesterone receptor. This analysis was also conducted for the estrogen receptor, which was significant with p-value of 0.049 (Table 2). In terms of TIL relationship with tumor size, tumor grade and vascular and perineural, no specific association was observed (P.V>0.05) (Table 2).The association of P53 with TIL was also addressed. 262 patients were evaluated. Among 135 patients with negative P53, 122 patients had TIL<30%; 3 patients' TIL ranged in 30-50% and 10 patients showed TIL values above 50%. Among 127 patients with positive P53, TIL of 98 patients was below 30%, 17 of them exhibited TIL values between 30 and 50% and 12 patients had TIL>50%. This difference was significant with a P-value of 0.002. The relation between Ki 67 and TIL was also assessed. Among 163 patients with Ki 67 in the range of 10-30%, 141 of them had TIL<30%. In 7 patients, TIL ranged from 30 to 50%; while TIL of 15 patients was below 30%. Among 38 patients with 31<Ki 67<60%, the TIL of 31 patients fell below 30%; 5 patients showed TIL values in the range of 30 to 50% and TIL of 2 patients exceeded 50%. Out of 12 patients whose Ki 67 was higher than 70%, the TIL of 4 patients was less than 30%; 6 patients had TIL values in the range of 30 to 50% and the TIL of 2 patients exceeded 50%. Regardless of Ki 67 level, the number of patients with TIL<30% was significantly higher than the other two groups. CD8 staining was conducted in 23 patients with TIL values 30%. 21 patients showed positive while 2 of them exhibited negative results. Out of 10 patients with infiltration of 31 to 40%, only 6 patients showed CD8+ results. Among 13 patients with TIL range of 41-50%, 7 patients exhibited $CD8^+$ results while 6 patients showed negative outcomes. CD8 status of 16 patients with TIL>50% was positive while 8 patients showed negative results. Study of patients with TIL levels equal or higher than 40% (45 patients) revealed that 28 patients had $CD8^+$ while 17 of them were $CD8^-$. This difference was statistically significant (p-value=0.022). As shown in Table 2, no association was detected between the tumor size and grade and TIL. Seventeen patients of the 299 studied patients died. The OS of the patients was 131.5 months (Figure.1) while their DFS was 124.6 months (Figure.1). No significant difference was observed in the OS of the patients and their DFS for three TIL levels (Figure 2). Based on immunohistological staining for CD8 cytotoxic lymphocytes, the mean survival of the CD8+ and CD8-patients was 119.33 and 105.67 months showing a significant difference (p-value=0.03)(Figure 3).

Table 1: Staging our patients; classification of luminal in our patient and TIL; Estrogen, Progesterone, Her2neu status Lymph node status our Patients

Hormone Receptor	Negative	Weakly Positive	Strongly Positive	P
Progesterone Receptor (n/%)	81(27.1%)	11(4%)	205(69.2%)	-
TIL%, PR□ (Number of patients)	62 ;<30% 10;30-50% 9; >50%	7;<30% 1;30-50% 3;>50%	180;<30% 13;30-50% 12;>50%	0.024
Estrogen Receptor (n/%)	81(27.1%)	13(4.63%)	203(68.22%)	
TIL %,ER□ (Number of patients)	62; <30% 10;30-50% 9; >50%	9;<30% 1;30-50% 3;>50%	178;<30% 13;30-50% 12;>50%	0.049
Her2neu (TIL %) (Number of patients)	148(score 0,1)	82(score 2)	69(score 3)	0.223
Luminal/ TIL %	TIL 30%: NEGATIVE (p. v:0.59)	TIL 30%: POSITIVE (p. v 0.59)	TIL 50% :NEGATIVE (p. v:0.05)	TIL 50%: POSITIVE (p. v:0.05)
Luminal A (Number of patients:78)	64 out 78	14 out 78	74 out 78	4 out 78
Luminal B (Number of patients:102)	77 out 103	25 out 102	88 out 102	14 out 102
TNBC□(Number of patients:30)	17 out 30	13 out 30	21 out 30	9 out 30
HER2 RICH(Number of patients:27)	20 out 27	7 out 27	23 out 27	4 out 27
Staging (Total patients) IA: 24 pts (8%)	Lymph Node/ TIL % N0 :103 patients N1:92	**TIL %; 30%**	**TIL; %,30-50%**	**TIL; %,>50%**
IIA: 96 pts (32.1%)	patients N2 :65 patients N3:25	TIL ;<30% ,93 pts	TIL ;30%-50%,4 pts	TIL; >50%,11 pts
IIB: 59 pts (19.7%)	patients Nx: 9 pts	TIL ;<30%,83 pts	TIL;30%-50%, 5 pts	TIL; >50%, 4 pts
IIIA: 73 pts (24.4%)		TIL; <30%,46 pts	TIL ;30%-50%,13 pts	TIL; >50%,6 pts
IIIB: 2 pts (0.7%)		TIL; <30%, 20 pts	TIL; 30%-50% , 3 pts	TIL; >50%, 2 pts
IIIC: 22 pts (7.4%)		TIL; <30%, 9 pts	TIL; 30%-50%, 0 pts	TIL; >50%, 21 pts
IV: 19 pts (6.4%)				

α: Progesterone β: Estrogen λ: Triple Negative Breast Cancer

Table 2: Relation TIL and other clinicopathological in our patient

		TIL; 30% Negative	TIL;30% Positive	TIL; 30-50% Negative	TIL 30-50% Positive	TIL; >50% Negative	TIL; >50% Positive	P.value TIL; >50%
Size	T1	47(20.6%)	14(19.7%)	59(21.5%)	2(8.0%)	53(20.3%)	8(21.1%)	0.74
Til;30%(P. value 0.97)	T2	146(64.0%)	45(63.4%)	172(62.8%)	19(76.0%)	167(64.0%)	24(63.2%)	
	T3	33(14.5%)	11(15.5%	40(14.6%)	4(16.0%)	39(14.9%)	5(13.2%)	
Til ;30-50% P.vlue:0.39	Tx	2(0.9%)	1(1.4%)	3(1.1%)	0(0.0%)	2(0.8%)	1(2.6%)	
Grade	1	37(17.7%)	5(8.1%)	39(15.7%)	3(13.0%)	40(16.7%)	2(6.3%)	0.29
Til ;30%(P. value 0.07)	2	138(66.0%)	41(66.1%)	167(67.3%)	12(52.2%)	156(65.3%	23(71.9%)	
	3	34(16.3%)	16(25.8%)	42(16.9%)	8(34.8%)	43(18.0%	7(21.9%)	
Til ;30-50% P.vlue:0.1								
Progesterone	Neg	54(23.8%)	27(38.6%)	71(26.0%)	10(41.7%)	65(25.1%)	16(42.1%)	0.024
Til ;30%(P. value 0.01)	Pos	173(76.2%)	43(64.4%)	202(74.0%)	14(58.3%)	194(79.4%)	22(57.9%)	
Til ;30-50% P.vlue:0.09								
Estrogen	Neg	57(25.1%)	24(34.3%)	71(26.0%)	10(41.7%)	64(24.7%)	17(44.7%)	0.049
Til ;30%(P. value 0.13)	Pos	170(74.9%)	46(65.7%)	202(74.0%)	14(58.3%)	195(75.3%)	21(55.3%)	
Til ;30-50% P.vlue:0.09								
P 53	Neg	116(57.4%	19(31.7%	132(54.5%)	3(15.0%)	123(53.5%	12(37.5%	0.009
Til ;30%(P. value 0.001)	Pos	86(42.6%	41(68.3%	110(45.5%)	17(85.0%)	107(46.5%	20(62.5%	
Til ;30-50% P.vlue:0.001								
Lymph Node	No	84(36.8%)	24(33.8%)	104(38.0%)	4(16.0%)	94(36.0%)	14(36.8%)	0.16
Til ;30%(P. value 0.2)	N1	75(32.9%)	17(23.9%)	87(31.8%)	5(20.0%)	86(33.0%)	6(15.8%)	
	N2	44(19.3%)	21(29.6%)	52(19.0%)	13(52.0%)	52(19.9%)	13(34.2%)	
Til ;30-50%	N3	17(7.5%)	8(11.3%)	22(8.0%)	3(12.0%)	21(8.0%)	4(10.5%)	
P.vlue:0.002	Nx	8(3.5%)	1(1.4%)	9(3.3%)	0(0.0%)	8(3.1%)	1(2.6%)	

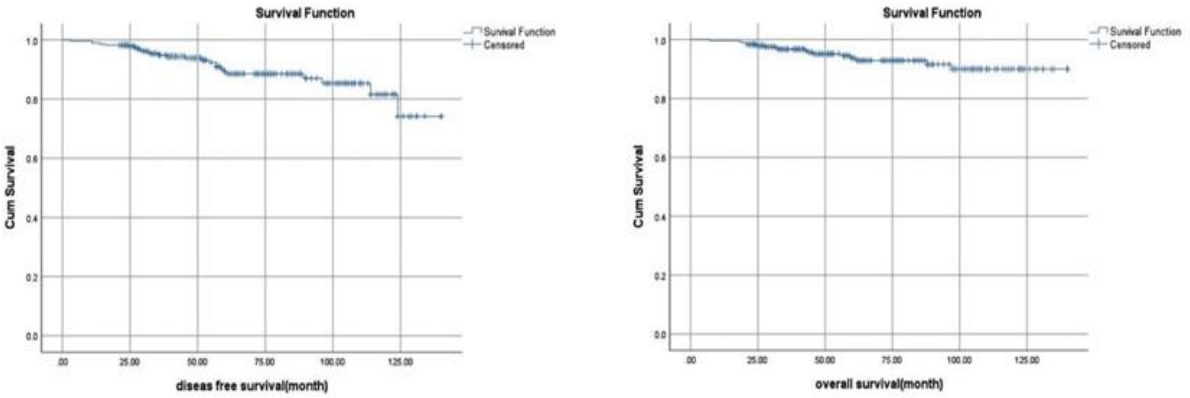

Figure 1. Overall survival and disease free survival in total our patients

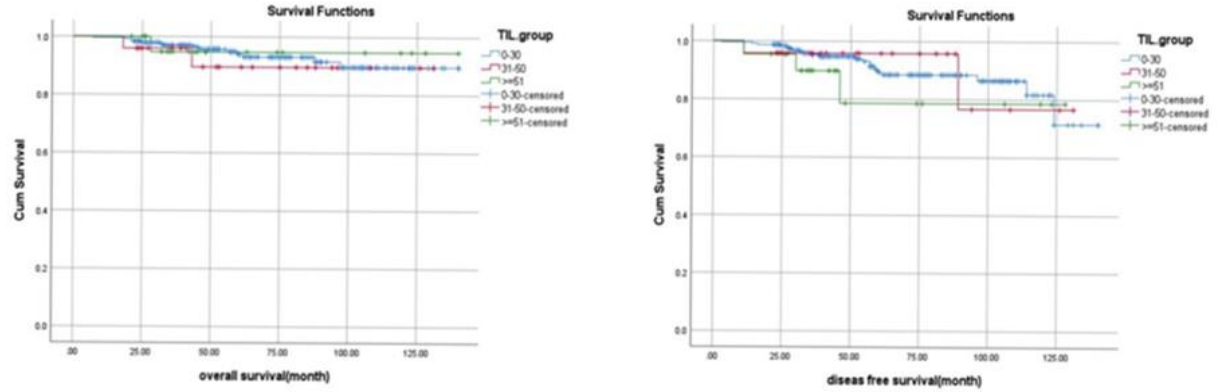

Figure 2. Overall survival our patients based of TIL 30%,30-50%,TIL>50%(Right), Disease free survival based of TIL 30%, 30-50%, TIL>50 %(Left)

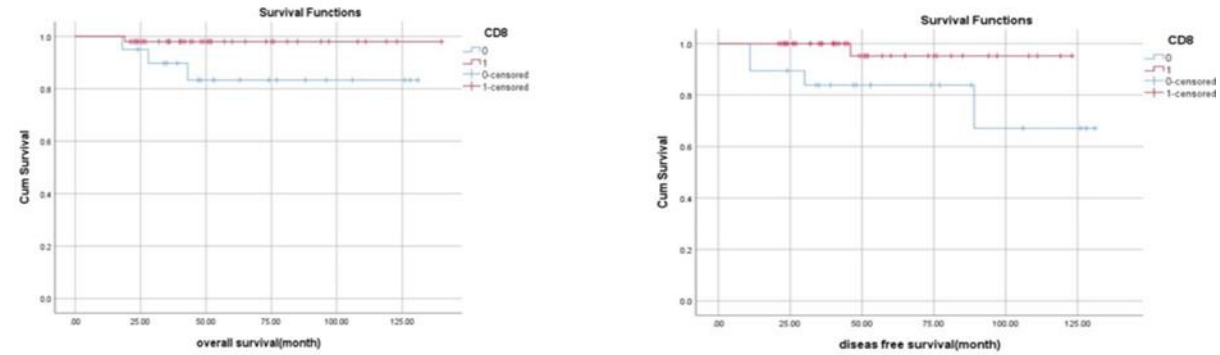

Figure 3. Overall survival our patients based of positive CD8 (Left), Disease free Survival based of negative CD8 (Right)

DISCUSSION

TIL has been recognized as a prognostic factor of disease outcomes in several types of cancer including breast cancer. Depending on the luminal subtype classification, TIL may have different impacts on the patients' survival[8]. In this study, the relationship between the TIL level and CD8 cytotoxic lymphocytes with clinicopathological features of the patients was also assessed. Not only the lymphocyte infiltration percentage but also the TIL phenotype can influence the disease pattern.CD4-type T-helper cells can facilitate the antigen presentation through cytokine secretion but CD8 cytotoxic lymphocytes are however necessary for the destruction of tumor cells. Type-II CD4+ T-helper cells can inhibit the function of cytotoxic cells; but since they also contribute to lymphocyte-B proliferation[9], they may activate anti-inflammatory responses resulting in further tumor growth. In some studies on patients with invasive breast cancer, the highest clinical benefits and survival was observed in the cases with TIL values over 50%[10]. Some other studies have assessed the relationship between TIL and various luminal subtypes of breast cancer.For example in some of the studies, reduced recurrence and death risks (by 34%) were reported among the triple-negative subtype patients with TIL of 30%[11]. The relationship between the TIL and estrogen and progesterone receptors was also addressed. There was a significant relationship between the progesterone receptor state and TIL (P-value<0.024), (Table 2). In cases with positive progesterone receptors, TIL was less than 30% in most of the patients. In the majority of patients with negative progesterone receptor, TIL was less than 30% suggesting the TIL as an independent factor in relation to the progesterone receptor. This analysis was also conducted for the estrogen receptor, which was significant with p-value of 0.049 (Table 2). Our study indicated the following results: in the cases with positive estrogen and progesterone, high-percentage TIL is significantly less likely. This indicates declined immune response in the cases of positive estrogen and progesterone. In terms of luminal classification, the patients could be categorized in Luminal A and Luminal B groups. The association of lymph node with lymphocyte infiltration was also assessed in this research. In the case of 30<TIL<50%, this relationship was statistically significant as the TIL value was low for any type of lymph node involvement. This suggests the decrease of the immune response in the cases of the involved lymph nodes. These results are in line with some studies while opposing some others. Some studies have stated that TIL expression is not an important prognostic marker in positive lymph node patients[12]. Among the patients with no lymph node involvement, the prognosis of high-TIL patients is better than the low-TIL ones. Some studies, however, reported no relationship. In the present study, the patients of three different TIL groups (below 30%, 30-50% and above 50%) showed no significant difference in terms of OS and DFS rates. These results are not consistent with some previous studies stating that high TIL is a proper prognostic factor. Such inconsistency could be attributed to the low number of group members. In our research, in the cases with positive CD8 in the tumor, the OS of the patients was significantly higher. Similar to some studies, these results indicate that the immune response of CD8 lymphocytes can increase the patients' survival. As mentioned before, some chemotherapy agents (in particular anthracycline, taxanes, and cyclophosphamides) may induce specific immune system responses giving rise to the death of cancer cells[6,7]. It has been reported that the anthracycline-based chemotherapies can increase the invasion of CD8+ cytotoxic lymphocytes which produce gamma interferon. Taxanes can also enhance the immunomodulatory impacts on the immune system cells. In fact, taxane-containing chemotherapy regimen can play a significant role in the elimination of the tumor cells due to the activation of the immune system[6,7]. The applied regimen in this study contained taxanes, anthracycline, and cyclophosphamides and 5flouracl ; thus increase in the patients' survival is anticipated. In some studies with CD8 cell percentage below 14%, a two-fold increase can be observed in mortality among triple-negative cancer patients. It has been reported that the majority of the patients (75% with reduced CD8, TIL<14%) may die in less than 2.1 years (on average), while the patients with high TIL values (above 14%) survived 15.4 years after diagnosis (on

average)[13].This reflects a relationship between the low TIL level (CD8-type) and triple-negative subtype outcomes. In terms of luminal classification, in our study, the majority of triple-negative subtype patients have TIL values above 50%, which is in line with most of the previous researches in this field. Due to the small population, it was not possible to evaluate the percentage of CD8 lymphocytes based on luminal classification; thus, we could not investigate the patients' prognosis according to their $CD8^+$ cells and luminal classes. Chemotherapy or vaccination interventions could be employed in cancers with low to moderate TIL levels. Chemotherapy drugs such as cyclophosphamide can decrease the T-helper $CD4^+$ lymphocytes which may be helpful in longer survival of the patients[6]; this drug was included in the chemotherapy regimen of our study.High-CD8 tumor cells may respond better to the immune checkpoint inhibitor treatments. In some studies, triple-negative breast cancer possessed higher levels of fatigued acting cells relative to luminal A indicating the immune suppression among the triple-negative patients. PD1/PDL1 is more likely to be positive in triple-negative patients[14]. The immune-related treatments have offered significant clinical benefits for survival.

CONCLUSION

Our study shows that the presence of $CD8^+$ cytotoxic lymphocyte tumor infiltration is strongly correlated with improved OS of the patients. CD8 lymphocyte factors are the major part of immune reaction.

CONFLICTS OF INTEREST

The authors have declared no potential conflicts of interest.

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