

# Breast Cancer: CD3+ Tumor Infiltrating Lymphocytes Differences among Molecular Subtypes

Nidhal A. Mohammed, Sahera A. Ali, Saad M. Saleh, Ahmed S. Abood

**Abstract**--CD3 is a receptor present on all T lymphocytes. Controversy still surrounds the prognostic role of tumor infiltrating lymphocytes (TILs) within a tumor microenvironment. The present study was done to evaluate CD3 TIL in BC patients and correlation with the molecular subtypes. Sixty-one cases as patients group and seven have been selected as normal group for the study. IHC was used to evaluate the expression of ER, PR, Her2\neu and CD3. Modified Allred scoring was used to evaluate the ER and PR, while Dako Her2 guideline was used for Her2. CD3 assessed by H-SCORE system in two locations (intratumoral and stromal). CD3 shows significant differences in intratumoral lymphocytes and stromal lymphocytes scores among molecular subtypes ( $p = .047$  and  $.045$  respectively). The tumor microenvironment differ according to molecular subtype, moreover, this difference in the microenvironment can modulate the immune response inasmuch CD3+ TIL percentage varies from one molecular subtype to another.

**Keywords:** Breast cancer, CD3, Molecular types, Hormone receptors, Tumor infiltrative lymphocytes.

## 1. INTRODUCTION:

**I**N Iraq, breast cancer (BC) is the commonest type of malignancy in females and there is a general trend towards an increase in the frequency of breast cancer as well as increase incidence in younger age group. Patients under 30 years old age formed about 5% of cases, whereas about 75% of the cases occurred in women older than 40 years. The highest number of cases is between 40-50 years old age groups (1). As BC is a genetically and clinically heterogeneous disease (2), the BC classification systems have been developed in order to organize this heterogeneity and standardize the language.

In addition, BC is immunogenic, the immune system can play a dual role in breast cancer, both promoting tumorigenesis through inflammatory pathways that also suppress adaptive immunity and preventing tumor formation through active immune surveillance (3).

CD3 antigen is a receptor glycoprotein present on all T lymphocytes. Controversy still surrounds the prognostic role of tumor infiltrating lymphocytes (TILs) within a tumor microenvironment. While higher concentration of CD3 TIL has been shown to link with favorable outcome in oropharyngeal cancer (4), a low CD3 count has been reported to predict a shorter disease free survival in colon and cervical cancer (5),(6).

The present study was done to evaluate the density, localization and distribution of CD3 TIL in BC patients. The findings were correlated with the molecular subtypes.

## 2. MATERIALS & METHODS:

Ninety-five of fresh samples and paraffin embedded tissue blocks from female patients with breast mass, during the period between May 2012 till February 2013. Their age ranged from (16 to 70) years. Thirty control samples were taken from normal breast tissue (dead females) in Iraqi center of forensic medicine. Pathological data including: histologic tumor type, tumor grade, tumor stage and lymph node status, were revised and confirmed by a specialist histopathologist. Out of the total ninety-five cases, only sixty-one patients group and out of the thirty normal sample, only seven have been selected as normal group for the study. According to clinic-pathological examination (H&E), the patients distributed into Malignant, Benign and Reactive. In order to approximate the molecular subtypes three markers had been used (ER, PR and Her2) (7) as shown in (Table 1).

Table 1: Approximate Molecular Subtype Using Three Markers

Marker	Luminal A	Luminal B	HER2	Basal-like
ER	+	+	-	-
PR	+	+	-	-
Her2	-	+	+	-

Immunohistochemistry was used to evaluate the expression of ER, PR, Her2\neu and CD3. All of monoclonal Abs, staining kits, Abs diluent, Ag retrieval solution, Mayer's hematoxylin and mounting medium used in this study were produced by DakoCytomation, Denmark. The staining procedures were performed according to manufacturer's instructions. Modified Allred score system (8) was used to evaluate the immune-expression of the ER and PR, while Dako Her2 guideline was used for Her2. In order to evaluate

- Nidhal A. Mohammed, College of Biotechnology, Al-Nahrain University, Iraq E-mail: dr.nidhalmohammed@yahoo.com
- Sahera A. Ali, College of Medicine, Baghdad University, Iraq.
- Saad M. Saleh, College of Education, Al-Iraqia University, Iraq.
- Ahmed S. Abood, College of Education, Al-Iraqia University, Iraq.

the CD3 immunostaining, the semi-quantitative analysis (H-SCORE system), as in (1) was used to assess percentages and staining intensity of the cells stained (9). The H-SCORE was calculated using the following equation:

$$H - score = \sum Pi (i) \quad (i = 0, 1, 2, 3, Pi = 0, 100\%) \quad (1)$$

{(i) means the intensity of staining, i.e. no staining = 0, weak staining = 1, moderate staining = 2 and strong staining = 3. Pi represents percentages of stained cells with intensities varying from 0 to 100}.

Therefore, the H-SCORE ranges from 0 to 300, H-SCORE>0 is considered as positive staining and H-SCORE = 0 is considered as a complete negative staining (10). Two location were scored in each case section, intratumoral and stromal, and two high power field for each location.

Nuclear staining of the cells for both ER and PR was evaluated using modified Allred scoring guideline. According to Dako ER & PR interpretation manual, a positive is defined as TS≥ 3. Membrane staining of the cells was evaluated using the DAKO/HER2 scoring system. Only score +3 has considered a positive and the other scores as negative (11)(11).

According to ER, PR and Her2 results, patients group were classified as luminal-A (ER and/or PR positive, HER2-), luminal-B (ER and/or PR positive, HER2+), HER2-Rich (ER-, PR-, HER2+), and basal (ER-, PR-, HER2-) (11)(12).

In brief, intratumoral T-lymphocytes were defined as T-lymphocytes located within tumor cell nests or in direct contact with the breast cancer malignant epithelial cells, whereas stromal T-lymphocytes were defined as T-lymphocytes in the stroma without direct contact with the cancer cells (13)(13).

The data were statistically analyzed depending on the nature of the character, according to Snedecor and Cochran (1981) and data processing was done by using Statistical Package of Social Science (SPSS) version 22. Data description was presented as means with their standard errors (SE) and standard deviation (SD) were calculated to reflect the size and precision of the estimated values. The independent sample t-test of significance was used for the comparison between two groups. ANOVA test was used to find the differences among three groups or more. The lowest level of significance chosen to be when the probability (p) was less than or equal to 0.05 (p≤0.05).

### 3. RESULTS:

According to the results of ER, PR and HER2 (See Tables 2, 3, 4) the patients group divided into the molecular subtypes. The most frequent molecular subtype is Her2-rich (33%); second group is Luminal B (28%), followed by Luminal A (21%) and Basal-like (18%). See figure (1).

Table 2: Estrogen receptor positivity in patients group.

ER	Frequency	Percent	Valid Percent	Cumulative Percent
Negative	44	72.1	72.1	72.1
Positive	17	27.9	27.9	100.0
Total	61	100.0	100.0	

Table 3: Progesterone receptor positivity in patients group.

PR	Frequency	Percent	Valid Percent	Cumulative Percent
Negative	43	70.5	70.5	70.5
Positive	18	29.5	29.5	100.0
Total	61	100.0	100.0	

Table 4: HER2 positivity in patients group.

Her2	Frequency	Percent	Valid Percent	Cumulative Percent
Negative	24	39.3	39.3	39.3
Positive	37	60.7	60.7	100.0
Total	61	100.0	100.0	

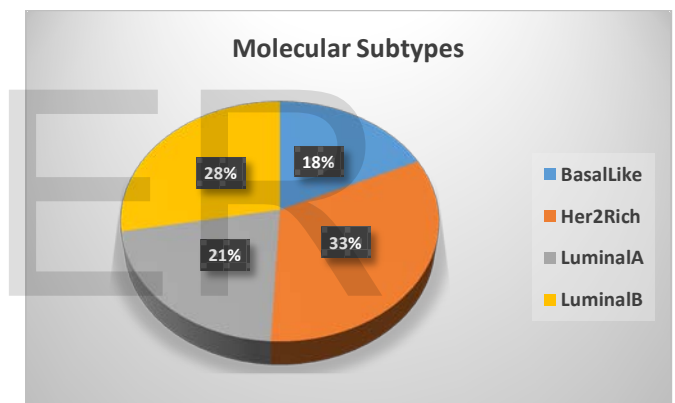


Figure 1: Molecular subtypes frequency in patient group.

With use of independent sample T-test, immunostaining of CD3 show high significant differences between patients and normal groups in both tumor nest and stromal score (p ≤ 0.001 and 0.002 respectively) See Table (5). Moreover, Immunostaining of CD3 shows high significant differences (p ≤ 0.001) between intratumoral lymphocytes and stromal lymphocytes scores in patients group (See Table 6).

Immunostaining of CD3 shows significant differences in intratumoral lymphocytes and stromal lymphocytes scores among molecular subtypes (p = .047 and .045 respectively) See Table (7).

Table 5: Independent sample T-test for CD3 expression in normal and patients group

Marker	Group	Mean	S.D.	S.E.	P value

CD3Tumor	Patient	108.33	63.966	8.957	≤ 0.001
	Normal	17.86	23.780	8.988	
CD3Stroma	Patient	102.45	71.459	10.006	0.002
	Normal	14.29	13.363	5.051	

Table 6: Descriptive data for CD3 expression differences in patients group.

CD3	Range	Min.	Max.	Mean		S.D.	P value
				Stat.	S.E.		
Tumor	300	0	300	108.33	8.957	63.966	≤ 0.001
Stroma	300	0	300	102.45	10.006	71.459	

Table 7: Descriptive data and ANOVA for CD3 expression within molecular subtypes.

CD3	Mean	Std. Dev.	Std. Error	95% Confidence Interval for Mean		Min.	Max.	P value
				Lower Bound	Upper Bound			
Tumor	Luminal-A	141.67	76.376	22.048	93.14	190.19	50	.047
	Luminal-B	95.00	49.721	15.723	59.43	130.57	0	
	Basal-Like	127.27	60.678	18.295	86.51	168.04	50	
	Her2-Rich	81.94	54.101	12.752	55.04	108.85	25	
	Total	108.33	63.966	8.957	90.34	126.32	0	
Stroma	Luminal-A	141.67	76.376	22.048	93.14	190.19	50	.045
	Luminal-B	95.00	49.721	15.723	59.43	130.57	0	
	Basal-Like	118.18	75.076	22.636	67.75	168.62	0	
	Her2-Rich	70.83	65.445	15.426	38.29	103.38	0	
	Total	102.45	71.459	10.006	82.35	122.55	0	

#### 4. DISCUSSION:

Our findings of ER and PR positivity (27.9 % and 29.5%) are lower than the results of local study conducted by Alwan (14). Alwan's reported that ER and PR positivity were demonstrated in (65.1%) and (45.1%) respectively of the studied group. The difference between our findings and the previously mentioned report is due to the studied population size variation, our patients group is smaller (61

than Alwan's (721). Our results of HER2 positivity come in agreement with that of Al-Khafaji (15) in which he stated that there is increase in HER2 expression. On the other hand, HER2 positivity percentage in this study is more than that of Alwan's (14)(14). The differences in the detection results might be due to the same reason previously mentioned.

According to hormones receptors our findings for the molecular subtypes were Her2-rich (33%); second group is Luminal B (28%), followed by Luminal A (21%) and Basal-like (18%). Such as these results are in discordancy with what Malhotra et al., had reviewed. Whereas, they reviewed several reports and stated different frequencies percentages for the molecular subtypes; Luminal A (40%), Luminal B (20%), Basal-like (15-20%) and Her2-rich (10-15%) (16). The explanation for current situation is that our findings yielded from the use of IHC technique and for three markers only, while the reviewed reports by Malhotra et al., (16) had use more sophisticated techniques like PCR and Microarray and for more than three marker. In addition, the ethnic variation between the studied cohorts may play role in the resulted frequencies. Shawarby et al., had reviewed that there are striking differences in compared prevalence patterns in the western and other regionally based studies (17).

the result of high significant differences in the CD3 immuno-expression between the intratumoral and stromal areas for patients group come ( $p \leq 0.001$ ), in some extent, in concordance with the results of Rathore et al., (18)(18) in which the studied group consist of triple-negative BC cases. Explanation for that differences is that might be part of the immune response where the effectors cells activated and accumulated in the site of foreign Ags (19),(20). Beside to the effect of tumor micro-environment which can be modulator to immunological effector cells (21)(22),(23),(24).

Depending upon the molecular subtyping of the patients group the immunostaining for CD3 shows significant differences in both intratumoral and stromal scores among the molecular subtypes ( $p=.047$  and  $.045$  respectively). These findings are consistent with many studies investigate the association between CD3+ tumor infiltrative lymphocytes and certain clinicopathological parameters in regard with the hormones receptors. Rathore et al., (18) had conclude an association between high CD3+ TIL both intratumoral and stromal with survival rate in Basal-Like BC (triple negative BC). Bedri et al., (25) study shows that intratumoral CD3+ TIL were significantly higher in ER/PR negative Her2/neu positive tumors (i.e. HER2-Rich molecular subtype) compared to triple negative breast cancers (i.e. Basal-Like). Calabr'o et al., (26)(26) had stated a poorer overall survival in ER+ patients and better overall survival in ER-patient in association with TIL. Rody et al., (27) reported that there is association between intratumoral and stromal CD3+ TIL with better recurrence free survival in cases who had HER-2+. Our results, with the

support of the previously mentioned studies, suggest that the tumor microenvironment differ according to molecular subtype, moreover, this difference in the microenvironment can modulate the immune response inasmuch CD3+ TIL percentage varies from one molecular subtype to another.

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