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RESEARCH ARTICLE

Breast Cancer: Molecular Subtype Tumor Microenvironment and CD8+ Tumor Infiltrative Lymphocytes

Nidhal A. Mohammed [1]*, Sahera A. Ali[2], Ahmed S. Abood [3]and Nabeel W. Rasheed [2]

1. College of Biotechnology, Al-Nahrain University, Iraq.
2. College of Medicine, Baghdad University, Iraq.
3. College of Education, Al-Iraqia University, Iraq

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Corresponding Author

Nidhal A. Mohammed

Abstract

The immune response to cancer development may be defined by the tumor infiltrating lymphocytes. Nevertheless, their significant association remains controversial in breast cancer. This study conducted to assess CD8 infiltrating rate differences among 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 CD8. ER, PR and Her2\neu were used for the molecular subtyping. CD8 assessed by H-SCORE system in two locations (intratumoral and stromal). CD8 shows no significant differences in intratumoral lymphocytes and stromal lymphocytes scores among molecular subtypes (p=.322 and =.151 respectively). There is no differences in the tumor infiltrating rate of CD8+ T-lymphocytes among the molecular subtypes.

Introduction

Breast cancer is a heterogeneous disease composed of different subtypes. Clinically, the classification of breast cancer depends on the expression of three biomarkers: estrogen receptors (ERs), progesterone receptors (PRs) and human epidermal growth factor receptor 2 (HER2) [(1)]. The role of immune system in cancer development and progression is not fully understood or elucidated. Even the efforts in the comprehensive researches it still be one of the most challenging issue in immunology. The emergence of the cancer immunoediting hypothesis not only indicates a role for the immune system in the active elimination of immunogenic tumor cells, but also emphasizes the importance of immunity in promoting the outgrowth of less immunogenic tumor cell variants ([2)]. Cytotoxic CD8+ T lymphocytes are decisive players of tumor-specific cellular adaptive immunity that respond to and kill tumor cells presenting major histocompatibility complex class I loaded with tumor-associated antigen peptide on their surface [(3), (4)]. The potential effects of the inflammatory cell infiltrate in breast cancer are numerous and complex. The statement that the immune response inhibit or enhance cancer development cannot be dramatized in a particular fashion without specifically clarify which immune cell phenotype participates in each process [(5)]. The present study was carried out to assess the density, localization and distribution of CD8 TIL in BC patients. The findings were correlated with the molecular subtypes.

Material and 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) ([6] as shown in (Table 1).

Table 1: Approximate Molecular Subtype Using Three Markers (Brenton *et al.*, 2005).

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

The expression of ER, PR, Her2 and CD8 were evaluated by using IHC technique. DakoCytomation produced all of monoclonal Abs, staining kits, Abs diluent, Ag retrieval solution, Mayer's hematoxylin and mounting medium used in this study. All procedures were carried out according to instructions of manufacturer. immunoexpression of the ER and PR assessed by modified Allred score system [7], while Her2 by Dako Her2 guideline . 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-) [8], [9]. In order to evaluate the CD8 immunostaining, the semi-quantitative analysis (H-SCORE system) was used to assess percentages and staining intensity of the cells stained [10], [11]**.** The H-SCORE was calculated using the following equation:

H-SCORE =∑ Pi (i) (i = 0,1,2,3, Pi = 0,100%).

Two location were scored in each case section, intratumoral and stromal, and two high power field for each location. Intratumoral T-lymphocytes are those T-lymphocytes located within tumor cell nests or in direct contact with the breast cancer malignant epithelial cells. Whereas stromal T-lymphocytes are those T-lymphocytes in the stroma without direct contact with the cancer cells [12].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 ($p \leq 0.05$).

Result and Discussion

According to our published data of ER, PR and HER2 results (in press), the patients group classified into the molecular subtypes. With use of independent sample T-test, immunostaining of CD8 show no significant differences between patients and normal groups in both tumor nest and stromal score (p = 0.295 and 0.667 respectively). See Table (2).

Table 2: Independent sample T-test for CD8 expression in normal and patients group.

Marker	Group	Mean	Std. Deviation	Std. Error Mean	P value
CD8Tumor	Patient	23.47	39.990	5.713	0.295
	Normal	7.14	18.898	7.143	
CD8stroma	Patient	7.65	24.582	3.512	0.667
	Normal	3.57	9.449	3.571	

Immunostaining of CD8 shows high significant differences ($p \leq 0.001$) between intratumoral lymphocytes and stromal lymphocytes scores (see table 3). Immunostaining of CD8 shows no significant differences in intratumoral and stromal lymphocytes scores among clinical groups (p=.083 and =.427) (see table 4).

Table 3: Descriptive data for CD8 expression in patients group.

CD8	Range	Min.	Max.	Mean		Std. Dev.	P value
				Statistic	Std. Error		
Tumor	200	0	200	23.47	5.713	39.990	≤ 0.001
stroma	100	0	100	7.65	3.512	24.582	

Table 4: Descriptive data and ANOVA for CD8 expression within clinical groups.

CD8		Mean	Std. Dev.	Std. Error	95% Confidence Interval for Mean		Min.	Max.	P value
					Lower Bound	Upper Bound			
Tumor	Malignant	38.10	51.611	11.262	14.60	61.59	0	200	.083
	Benign	11.76	19.995	4.850	1.48	22.05	0	50	
	Reactive	13.64	30.339	9.148	-6.75	34.02	0	100	
	Total	23.47	39.990	5.713	11.98	34.96	0	200	
Stroma	Malignant	11.90	30.227	6.596	-1.85	25.66	0	100	.427
	Benign	1.47	6.063	1.471	-1.65	4.59	0	25	
	Reactive	9.09	30.151	9.091	-11.16	29.35	0	100	
	Total	7.65	24.582	3.512	.59	14.71	0	100	

Immunostaining of CD8 shows no significant differences in intratumoral lymphocytes among molecular subtypes (p=.322), as well as, in stromal lymphocytes scores (p=.151) (see table 5).

Table 5: Descriptive data and ANOVA for CD8 expression within molecular subtypes.

CD8		Mean	Std. Dev.	Std. Error	95% Confidence Interval for Mean		Min.	Max.	
					Lower Bound	Upper Bound			
Tumor	LuminalA	22.73	32.509	9.802	.89	44.57	0	100	.322
	LuminalB	9.09	20.226	6.098	-4.50	22.68	0	50	
	BasalLike	40.91	65.453	19.735	-3.06	84.88	0	200	
	Her2Rich	21.88	30.104	7.526	5.83	37.92	0	100	
	Total	23.47	39.990	5.713	11.98	34.96	0	200	
stroma	LuminalA	11.36	30.339	9.148	-9.02	31.75	0	100	.151
	LuminalB	0.00	0.000	0.000	0.00	0.00	0	0	
	BasalLike	20.45	40.028	12.069	-6.44	47.35	0	100	
	Her2Rich	1.56	6.250	1.563	-1.77	4.89	0	25	
	Total	7.65	24.582	3.512	.59	14.71	0	100	

Our study findings for CD8 immuno-expression shows no significant differences between the patients and normal groups in both tumor nest and stromal score ($p = 0.295$ and 0.667 respectively), such results may be due to the relatively small normal group in compare with the patients group.

We found out that there is a high significant differences ($p \leq 0.001$) between intratumoral and stromal CD8+ lymphocytes in patients group. These findings come in concordance with the results of Chen et al., [12] and Liu et al., [13]**.** Construing for this result is that the effectors cells activated and accumulated in the site of foreign Ags [3], [4]. In addition to the effect of tumor micro-environment which can be modulator to immunological effector cells [14], [15], [16], [17].

Our immunohistochemical study of CD8+ lymphocytes shows that the highest means were obtained with the malignant group both intratumoral and stromal (38.10 S.D ±51.611 and 11.90 S.D. ±30.227 respectively). Dobrzanski et al., [18] and Schillaci et al., [19]studies provided support to our findings by their results which conclude that the CD8+ T cell-mediated type 1 immune responses can enhance the function of distinct endogenous CD8+ T cells by selectively modulate the differentiated and non-differentiated T cell. This modulation take place via CD8+ T-lymphocytes localization and activation, which lead to accumulation of these cells in the tumor microenvironment and eventually facilitate their antitumor function in breast cancer.

When we used ANOVA test to find out the differences in immunoexpression of CD8 among the molecular subtypes, there was no significant differences in CD8 scores in both intratumoral and stromal (p=.322 and =.151 respectively). Several research articles were reviewed to compare our results with their findings, some of them come in concordance with our results and others not. Liu et al., [13], Chen et al., [12] and Mahmoud et al., [5] findings were in disagreement with ours, each of them had found out a significant differences in CD8+ TILs among molecular subtypes of BC. On the other hand, Liu et al., [20] findings were in agreement with our findings in each of intratumoral and stromal. These conflicting results are another scene in the vista of Breast cancer and CD8+ lymphocytes. Whereas, the prognostic effect of CD8+ CTLs is still a matter of debate. While one study demonstrated that both the total number and the distant stromal CD8+ CTLs significantly associated with better prognosis of breast cancer and its subtypes (ER negative, HER-2 negative and basal-like cancers) [5], another study showed that neither intratumoral nor stromal CD8+ CTLs had protective effect on survival of breast cancer patients [20]. The construing to what previously mentioned is that might be the difference in cohort size between our study and the reviewed researches, whereas, our study groups is relative smaller than others. In addition, immunohistochemical technique, which used in our study in order to approximate the molecular subtypes, is less sophisticated than gene expression detecting techniques that had been used in the compared studies. Finally, the conflicting results in the role of CD8+ lymphocytes in BC molecular subtypes might be due to the ethnic variation between the studied cohorts.

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