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RESEARCH ARTICLE

The Programmed Death-1 Receptor, Programmed Death-1 Ligand (PD-1/PD-L1) and apoptosis in breast cancer patients: A potential Mechanism of immune escape

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ABSTRACT

Background: Interaction between programmed cell death ligand-1 (PD-L1) and its receptor PD-1 is a major inhibitory pathway in maintaining an immunosuppressive tumor microenvironment. The expression of PD-L1 in various solid tumors is proposed to function in preventing T-cell mediated tumor killing, and activating tumor -suppressive cell populations through different mechanisms. The B-cell Lymphoma-2 (Bcl-2) is a key anti-apoptotic protein has been described as mediators of cancer progression. The expression of PD-L1 on activated T-cells supports their survival. **Objectives:** Evaluate the expression of PD-1, PD-L1 in relation to apoptosis among patients with breast cancer. All parameters were correlated with each other and with the clinicopathological features of the disease. **Patients and Methods:** This case-control study was conducted on 55 breast cancer patients with different stages of the disease. In addition, 20 age-matched normal healthy individuals were included in the study as a control group. Patient groups divided into early stage disease and advanced stage disease. The percentage of PD-1 and PDL-1 were measured in blood samples of all subjects using flowcytometry. Quantitative detection of Bcl-2 protein was assessed using enzyme linked immunosorbent assay. **Results:** PD-L1 expression was significantly associated with tumor grade, lymph node involvement, tumor size, vascular invasion and negative hormonal receptors. PD-1 expression was significantly associated only with lymph-node involvement. A significant positive correlation was existed between serum Bcl-2 and PD-L1+ expressing granulocytes. **Conclusions:** The direct correlation between PD-L1+ expression and serum Bcl-2 concentration may explore a role of apoptotic machinery in the pathogenesis of breast cancer.

Keywords: breast cancer, apoptosis, immune escape

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INTRODUCTION

Breast cancer is the most common malignant tumor in women worldwide (Siegel et al., 2017). Advances in diagnosis and therapy have significantly improved the survival of breast cancer patients, however, recurrence and metastasis remain the leading cause of breast cancer death (Berry et al., 2005). Cancer cells can maintain an immunosuppressive microenvironment that favors tumor progression by expressing immune inhibitory signals (Postow et al., 2015).

The interaction between programmed cell death ligand-1 (PD-L1 or CD274) and its receptor PD-1 is major inhibitory pathway in maintaining immunosuppressive tumor microenvironment. Interestingly, inhibition of immune checkpoint regulator PD-L1 or PD-1 is a new anticancer therapy (Iwai et al., 2002, Brahmer et al., 2012,). PD1 is a cell surface membrane protein, member of B7 family of immune checkpoints, which is activated by its ligands PD-L1 and PD-L2. PD-1 is expressed on T cells, B cells, natural killer T cells, monocytes and dendritic cells (DC)

after activation. PD-1 is not present on naive T cells, but is induced on CD4+ and CD8+ T cells after TCR activation and remains high in case of persistent stimulation with antigen (Bally et al., 2016).

In addition, PD-1 induced on T cells by common gamma-chain cytokines such as interleukins (IL-2, IL-7, IL-15 and IL-21), which are important for survival and expansion of T cells (Boussiotis et al., 2014). PD-L1 plays a significant role in tumor evasion by promoting the activation of the PD-1 / PD-L1 pathway (Dermaniand Kohlan, 2019). The expression of PD-L1 has been observed in various solid tumors including breast cancer, colon cancer, renal cancer and others. Once engaged, the PD1/PD-L1 pathway is proposed to function in preventing T-cell mediated tumor killing, protecting tumor cells and activating tumour-suppressive cell populations through different mechanisms (Goodman et al., 2018).

Apoptosis is a form of programmed cell death that is characterized by distinct structural and molecular changes resulting in cell death without the release of cellular contents (Pfeffer and Singh, 2018). Apoptosis is divided into two pathways, intrinsic and extrinsic. Both of these processes are responsible for cell killing and are important for the normal development of an organism as well as for the removal of damaged cells (Chung, 2018).

The B-cell Lymphoma-2(Bcl-2) is a member of the Bcl-2 family of regulator proteins that regulate apoptosis, either by inducing or inhibiting apoptotic cell death. Bcl-2 is known to be a key anti-apoptotic protein, located at position 18q21.33 which is an integral outer mitochondrial membrane protein that blocks the apoptotic death of some cells such as lymphocytes (Warren et al 2019). Bcl-2 proteins have been described as fundamental mediators of cancer progression. The expression of PD-L1 on activated T-cells supports their survival such that PD-L1 deficient T-cells express lower Bcl-xl, which is an antiapoptosis gene, than wild type cells and are more sensitive to apoptosis in vivo (Pulko et al., 2011). Tumor cells exploit this pathway by the expression of PD-L1 to survive immune surveillance. Antitumor T-cells can upregulate PD-L1 on tumor cells through the production of IFN-γ that is a dual-edged cytokine capable of inducing apoptosis and also facilitating tumor dormancy (Liu et al., 2017).

Tumor cells co-opt the PD1/PD-L1 axis results in suppression of anti-tumor adaptive responses through mechanisms involving induction of cytotoxic T-lymphocyte (CTL) suppression, exhaustion, apoptosis and decreased cytokine production (Black et al., 2016). In addition to interfering with CTL function, engagement of PD-1 with PD-L1 increase tumor cell resistance to pro-apoptotic signals including those delivered by cytotoxic immune effectors (Fas ligation) (Lin et al., 2018). However, until now only few studies have reported a possible link between PD-1/PD-L1 and apoptosis among breast cancer patients. Therefore, understanding the role of PD/PD-L1 and apoptosis in breast cancer may provide new strategies in the development of new therapeutic approaches to manage the disease.

This study aimed to evaluate the expression of the programmed death-1 receptor, programmed death-1 ligand in relation to apoptosis among patients with different stages of breast cancer. In addition, all parameters were correlated with each other and with the clinicopathological features of the disease.

PATIENTS AND METHODS

This case-controlled study was conducted on 55 breast cancer patients with different stages of the disease who presented to the Surgery Department outpatient clinic and Cancer Management and Research Department, Medical Research Institute, Alexandria University, Alexandria, Egypt, from April 2017 to November 2018.

An informed written consent was taken from all subjects included in this study according to the rules approved by the ethical committee of the Medical Research Institute IORG#: IORG0008812 and according to the Helsinki declaration (World medical declaration of Helsinki, 2014) Patients with autoimmune diseases or any diseases affecting the immune system were excluded from the study. Also, 20 age-matched normal healthy individuals who are comparable to the control group.

Patients' Characteristics

Patient groups comprising breast cancer patients were divided into two groups: early-stage (stages I-II) and advanced stage (stages III-IV) of the disease (Giuliano et al., 2018). Clinical data of the studied groups were collected prospectively. These data included history tracking, clinical examination to detect the site of the tumor and the presence of enlarged lymph nodes. Radiological investigations including mammogram, abdominal ultrasound and chest x-ray.

The diagnosis of invasive breast cancer was made by fine-needle aspiration cytology (FNAC) or core-needle biopsy of the breast tumor. The patients with the early-stage disease were operated by modified radical mastectomy or breast conservative surgery. Pathological evaluation of the tumor included histologic subtype of the tumor, grading, size of the tumor, axillary lymph node status, presence or absence of vascular invasion. The metastatic disease was proved by radiological studies or pathological assessment or both. Blood samples were collected from breast cancer patients at the presentation before starting chemotherapy.

Assessment of estrogen, progesterone receptors

Assessment of estrogen, progesterone receptors (ER, PR) and human epidermal growth factor receptor-2(Her-2) status was carried by immunohistochemistry. Formalin-fixed, paraffin-embedded tissue blocks with tumour were used for immunohistochemical staining. Antigen recovery, imunocytochemistery was performed after epitope retrieval with a polymer-based detection system (Envision plus, Dako, Carpinteria, CA) using mouse monoclonal antibodies for ER and PR. Nuclear staining in more than 10% of tumor cells was considered positive for ER and PR (Williams et al., 2009).

Assessment of Her-2

The HER2 protein expression in paraffin sections was detected by immunostaining using Herceptin kit (HercepTest, Dako, Carpinteria, CA) according to manufacturer's instructions. Immunostaining was performed by incubation with rabbit anti-human monoclonal anti-HER-2 (Maixin Biotech., Fuzhou, China). HER-2 results were determined based on the maximum area of staining intensity, according to the package insert and ASCO/CAP guidelines (Williams et al., 2009). Cases with equivocal Her-2 were confirmed by fluorescent in situ hybridization FISH was performed using the US FDA approved PathVysion. HER2 DNA probe kit (Abbott Molecular Inc., USA), a dual coloured probe comprising locus-specific identifier (LSI) HER2 was used to detect HER2 gene amplification status (Press et al .,2016).

Sampling Technique

Peripheral venous blood samples (5ml) were obtained from all subjects under the study. Three ml in EDTA coated vacutainers for measurement of PD-1 and PDL-1 expression. Two ml of venous blood in plain vacutainers for serum separation that was allowed to clot for 20 minutes at 37 °C followed by one hour at 4°C. Serum was then separated by centrifugation at 1500 rpm for 10 minutes and preserved at -80°C until use for Bcl-2 measurement.

Assessment of PD-1and PDL-1 serum level

The percentage of PD-1 (CD279) and PDL-1 (CD274) expressing on lymphocytes, monocytes and granulocytes were measured in EDTA blood samples of all subjects under the study using flowcytometry (BD FACS Calibur) according to manufacturer's instructions.

Peripheral blood mononuclear cells (PBMC) isolation and flowcytometric analysis:

The gating strategy was used in the analysis of intracellular signaling pathway in PBMCs. Blood was collected in polystyrene tubes and PBMCs were isolated by density gradient centrifugation before cryopreservation for 15 minutes. The stains used for the two markers were as follow; CD279 PE (Phycoerythrin), and CD274 FITC respectively (Fluorescein isothiocyanate) (BD Biosciences). Samples were washed to remove excess dye and stained with fluorochrome-conjugated antibodies. The detection was done by using CD279 and CD274 monoclonal antibodies respectively. All tubes were analyzed by flow cytometer. Cell subtypes were identified based on their scattering properties as either monocytes or lymphocytes or granulocytes (Dey, 2018).

Assessment of Bcl-2 serum level using (ELISA) technique

Anti-apoptotic protein (Bcl-2) levels were measured using commercially available ELISA kit for hematological and biochemical investigations. The assay was used for quantitative detection of B-cell leukemia/lymphoma-2 (Bcl-2) protein using biotin double antibody sandwich technology, detection range (0.3ng/ml - 90ng/ml) (Human B-cell leukemia/lymphoma 2 ELISA kit, Bioassay Technology Laboratory) (Kosacka et al., 2016).

Bcl-2 molecules present in serum samples were absorbed to the microwells which were coated with anti-Bcl-2 monoclonal antibody. Then a biotin-conjugated monoclonal anti-Bcl-2 antibody was added to bind to Bcl-2 captured by the first antibody. Following incubation time, unbound biotin-conjugated anti-Bcl-2 was removed by washing, and then streptavidin-horseradish peroxidase (HRP) was added to bind to the biotin-conjugated anti-Bcl-2. A colored product which proportional to the amount of human Bcl-2 present in serum that terminated by acid and optical absorbance was measured at 450 nm according to the manufacturer's recommendation.

Statistical Analysis

Statistical analysis was carried out using Graph pad 7software (Prism, La Jolla, USA). The sample size was calculated using t-test (Kelsey et al., 1996). A Student's T-test was used to compare between two groups, while for more groups, ANOVA test was used and Chi-Square test for proportion data. The test performed was unpaired and two-tailed. Results are expressed as mean ± SEM or SD. P values were considered significant when $p \leq 0.05$.

RESULTS

This study included 75 age-matched females; 20 normal healthy females as a control group and 55 breast cancer patients. The patients were classified into two groups: early-stage (I-II) included 24 patients and advanced stage (III-IV) included 31 patients. The mean age of all patients was 46.24±9.85 years; ranged 32-70 years, while in healthy individuals, it was 41.4±10.54 years ranged 30-65 years.

Age distribution of the studied groups is shown in (Table 1). There was no difference between the studied groups.

Clinicopathological characteristics of the studied patients are summarized in (Table 2). There was a statistically significant difference between the early and advanced stages regarding tumour grade and tumour size. While, there was no statistically significant difference between the two groups regarding lymph node involvement, histologic subtype of the tumor and vascular invasion. Concerning the hormonal status in all patients, 41/55 patients (74.54%) were negative for Her-2 relative to 14/55 patients (25.45%) were positive for Her-2. Regarding positivity and negativity of ER and PR 39/55 patients (70.9%) were positive for both ER and PR and 16/55 patients (29.09%) for both ER and PR were negative.

Immunological investigations

The positivity and negativity of PD-1 and PD-L1 expressing lymphocytes, monocytes and granulocytes were determined for all subjects under study using flowcytometry of all subjects under study using relevant monoclonal antibodies (Table 3).

Regarding PD-L1 positivity showed a non-significant difference between the patient groups (52.72%) and control group (40%) (p=.086). The expression of PD-L1 has been studied in different leukocytes. In lymphocytes, the mean % of PD-L1+in all positive patients (29 patients) was 24.38±12.25 compared to that of control individuals which were 5±2.67 (range 1.3-9).

In early and advanced stages the mean PD-L1+ %was 20.4±7.51(range 11-37) and 25.95±13.69 (range 12-60) respectively, there was significant increase in the mean % of PD-L1+between different groups (P= <0.0001) (Figure 1B).

PD-1,89.09% of patients (49/55) were positive, while 100% of the control group were positive for PD-1. There was no statistically significant difference between the two groups (p=0.117). The mean % of PD-1+expressing lymphocytes in all positive patients (49patients) was 17.02 ±7.14compared to that of control individuals which were 6.26±6.57 (range 2.7-26).

Table 1. Age distribution and relation between the studied groups (n=75)

Age (year)	Total patients(n = 55)		Early (n= 24)		Advanced (n=31)		Control (n = 20)		F	P
	No	%	No	%	No	%	No	%		
≤ 40	21	38.18	9	37.5	12	38.7	12	60	1.68	0.302
>40	34	61.81	15	62.5	19	61.29	8	40		
Min-Max	32-70		32-67		33-70		30-65			
Mean± SD.	46.24±9.85		46.33±11.35		46.16±8.73		41.4±10.54			
Median	44		43.5		44		38			

F: F for ANOVA test , P: value for among the groups , *: Statistically significant at p < 0.05

Table 2. Clinicopathological parameters of the studied patients (n=55)

Parameter	Total patients (n=55)		Early-stage (n=24)		Advanced-stage (n=31)		X^2	P
	No	**%**	**No**	**%**	**No**	**%**		
Grade								
II	41	74.54	3	12.5	5	16.12	3.766	0.023*
III	14	25.45	21	87.5	26	83.87		
Lymph node Involvement								
N0								
N1	13	23.62	10	41.66	3	9.67		
N2	17	30.90	9	37.5	8	25.80	3.47	0.324
N3	18	34.54	5	20.83	13	41.93		
	7	10.90	0	00.00	7	22.58		
Type of tumor								
IDC	51	92.72	22	91.66	29	93.54		
Mixed type	3	5.45	1	4.16	2	6.45	1.404	0.495
ILC	1	1.81	1	4.16	0	0.00		
Vascular invasion								
No	6	10.90	3	12.5	3	9.67		
Yes							0.1109	0.7391
	49	89.09	21	87.5	28	90.32		
Size of tumor								
T1	7	12.72	6	25	1	3.22	6.063	0.048*
T2	38	69.09	15	62.5	23	74.19		
T3	10	18.18	3	12.5	7	22.58		
Her-2 status								
Negative	41	74.54	20	83.33	21	67.74	1.733	0.188
Positive	14	25.45	4	16.66	10	32.25		
ER								
Negative	16	29.09	6	25	10	32.25	1.483	0.223
Positive	39	70.9	18	75	21	67.74		
PR								
Negative	16	29.09	6	25	10	32.25	1.483	0.223
Positive	39	70.90	18	75	21	67.74		

X^2: Chi-square test IDC: invasive ductal carcinoma, ILC : invasive lobular carcinoma, N0 negative axillary lymph nodes, N1 (1-3 positive axillary lymph nodes), N2 (4 – 9 positive axillary lymph nodes), N3 (≥ 10 positive axillary lymph nodes), T1 (≤ 2cm), T2 (> 2 – 5cm), T3 (> 5cm), ER: estrogen receptors, PR: progesterone receptors, Her2: human epidermal growth factor receptor-2, P: p value for comparing between the two groups, *: Statistically significant at p < 0.05

Table 3. Distribution of positivity and negativity of PD-1 and PD-L1 among the studied groups (n=75)

	Total patients(n=55)		Early-stage (n= 24)		Advanced-stage(n=31)		Control (n=20)		Test of significant	P
	No	%	No	%	**No**	%	**No**	%		
PD-1										
PD-1-	6	10.9	4	16.66	2	6.45	0.0	0.0	$\chi 2$=4.28	0.117
PD-1+	49	89.09	20	83.33	29	93.5	20	100		
PDL-1										
PDL-1-	26	47.27	15	62.5	11	35.4	12	60	$\chi 2$=4.9	0.086
PDL-1+	29	52.72	9	37.5	20	64.5	8	40		

$\chi 2$: Chi-square test, P: p value for comparing among the studied groups, *: Statistically significant at p < 0.05

In early and advanced stages the mean PD-1+ %was 17.01±5.84(range8.71-30)and 17.03±8.02 (range10-41) respectively, with non-significant difference between different groups (P= 0.919) (Figure 1C). Bcl-2 level was assessed using the enzyme-linked immunosorbent assay (ELISA) technique (Table 4) The mean Bcl2 serum concentration in all patients was 60.22±39.45U/ml; while its level in control individuals was 27.56±1.172U/ml . There was a significant increase in the mean Bcl-2 serum concentration in the advanced-stage group compared to the early stage-group (p <0.0001) and also between the advanced- stage and control group. We carried out a χ2 analysis to determine the association between several clinicopathological parameters and PD-1/PD-L1percentage.There were no significant associations were found between the positivity of PD-1 and different clinicopathological characteristics (Age, tumor stage, tumor grade, tumor size, vascular invasion, ER, PR and Her-2). PD-1 was significantly associated only to lymph-node involvement (Table 5).

The presence of PD-L1 positivity was significantly associated with tumor grade II (58.62%), lymph node involvement N2 (41.37%), tumor size T2 (58.62%), positive vascular invasion (100%), negative ER (51.72%) and negative PR (51.72%). Although it was associated with advanced -age and negative Her-2, there was no significant difference. (Table 6).

Correlation studies

The results revealed a significant negative correlation between serum Bcl-2 and early-stage and lymph node involvement. While the correlation between serum Bcl-2 and other clinicopathological parameters as age, tumor grade, type of tumor, vascular invasion or hormonal status did not show any statistical significance. (Table 7). Statistical analysis of the studied factors showed a significant positive correlation between serum Bcl-2 concentrations and PD-L1+ expression. (Table 8). Otherwise, the correlation between Bcl-2 serum levels and PD-1+ expression didn't show any statistical significance (Table 9).

Table 3. Distribution of positivity and negativity of PD-1 and PD-L1 among the studied groups (n=75)

	Total patients**(n=55)**		Early-stage **(n= 24)**		Advanced-stage(n=31)		Control **(n=20)**		Test of significant	P
	No	%	No	%	**No**	%	**No**	%		
PD-1										
PD-1-	6	10.9	4	16.66	2	6.45	0.0	0.0	4.28	0.117
PD-1+	49	89.09	20	83.33	29	93.5	20	100		
PDL-1										
PDL-1-	26	47.27	15	62.5	11	35.4	12	60	$\chi 2$=4.9	0.086
PDL-1+	29	52.72	9	37.5	20	64.5	8	40		

$\chi 2$: Chi-square test, P: p value for comparing among the studied groups, *: Statistically significant at p < 0.05

Table 4. Serum levels of Bcl-2 in breast cancer patients and control group(n=75)

Bcl-2 level (U/ml)	Control (n =20)	Total (n =55)	Early (n=24)	Advanced (n=31)	F	P
Min – Max	25.75- 29.8	28.14-200	28.14-43.87	44.26-200		
Mean ±SD	27.56±1.172	60.22±39.45	33.94±5.051	80.75±42.45	29.68	<0.0001*
			P1=0.7259, P2= <0.0001*, P3= <0.0001*			

F: ANOVA test, p: p value for comparing between the different groups, *: Statistically significant at p < 0.05, P1: p Value for comparing between Early and Control, P2: p Value for comparing between Advanced and Control, P3: p Value for comparing between Early and Advanced

Table 5. Comparison between percentages of PD-1 (PD-1+/ PD-1–) expression according to the clinicopathological parameters in breast cancer patients. (n=55)

Clinicopathological parameters	Breast cancer patients (n=55)				Test of sig. ($\chi 2$)	P
	PD-1+(n =49)		PD-1– (n = 6)			
	No	%	No	%		
Age (year)						
≤ 40	18	36.73	3	50	0.3985	0.5279
>40	31	63.26	3	50		
Tumor stage						
Early	20	40.81	4	66.66	1.452	0.2282
Advanced	29	59.18	2	33.33		
Tumor grade						
II	35	71.42	6	100	2.3	0.1294
III	14	28.57	0	0.00		
Lymph node involvement						
N0						
N1	12	24.48	1	16.66		
N2	12	24.48	5	83.33	9.188	0.0269*
N3	18	36.73	0	0		
	7	14.28	0	0		
Tumor size						
T1	7	14.28	0	0		
T2	33	67.34	6	100	2.763	0.2512
T3	9	18.36	0	0		
Vascular invasion						
Yes	44	89.79	5	83.33	0.2297	0.6317
No	5	10.20	1	16.66		
ER						
Negative	15	30.61	0	0	2.459	0.1169
Positive	34	69.38	6	100		
PR						
Negative	15	30.61	0	0	2.459	0.1169
Positive	34	69.38	6	100		
Her-2						
Negative	35	71.42	6	100	2.3	0.129
Positive	14	28.57	0	0		

$\chi 2$: Chi-square test, P: p value for comparing between the studied groups

DISCUSSION

Programmed cell death-1 (PD-1) and programmed cell death ligand-1 (PD-L1) axis play a key role in physiological immune homoeostasis and represent a mechanism of immune escape (Dong et al., 2017). Several studies have focused on PD-1/PD-L1 expression in various malignancies; however, few studies have investigated the expression of PD-1 and PD-L1 in breast cancer patients (Jiang et al.,2019). B-cell lymphoma-2 (Bcl-2) is the founding member of the Bcl-2 family of regulator proteins that regulate apoptosis, either by inducing or inhibiting apoptotic cell death (Campbell and Tait, 2018).

The role of Bcl-2 differs depending on its interaction with other members of the Bcl-2 family. Pro-apoptotic and anti-apoptotic signals are tightly regulated in normal breast epithelial cells. Dysregulation of this balance is required for breast tumorigenesis and increases of acquired resistance to treatments. (Warren et al., 2019). Therefore, this study aimed to evaluate the role of programmed death-1 receptor and programmed death ligand-1 (PD-1/PD-L1) expressing lymphocytes, monocytes and granulocytes, as a potential mechanism of immune escape in breast cancer patients. Also, serum levels of Bcl-2 were analyzed among patients with different stages of breast cancer. In addition, all parameters were correlated with each other and with clinicopathological parameters of the disease.

The study was conducted on a total of seventy-five females; fifty-five of them represented the breast cancer patients at early (24 patients) and advanced (31 patients) stages and 20 age-matched female represented the control group.

Table 6. Comparison between percentages of PD-L1 (PD-L1+/ PD-L1–) expression according to the clinicopathological parameters in breast cancer patients. (n=55).

Clinicopathological parameters	Breast cancer patients (n=55)				Test of sig. ($\chi 2$)	P
	PD-L1+ (n =29)		PD-L1– (n = 26)			
	No	%	No	%		
Age (year)						
≤ 40	11	37.93	10	38.46	0.0016	0.9678
>40	18	62.06	16	61.53		
Tumor stage						
Early	9	31.03	15	57.96	3.961	0.0466*
Advanced	20	68.96	11	42.30		
Tumor grade						
II	17	58.62	23	88.46	6.155	0.013*
III	12	41.37	3	11.53		
Lymph node involvement						
N0	5	17.24	8	30.76		
N1	5	17.24	12	46.15	12.45	0.006*
N2	12	41.37	6	23.07		
N3	7	24.14	0	0		
Tumor size						
T1	3	10.34	4	15.38		
T2	17	58.62	22	84.61	9.649	0.008*
T3	9	31.03	0	00.00		
Vascular invasion						
Yes	29	100	20	76.93	7.512	0.0061*
No	0	00	6	23.07		
Hormonal status						
ER						
Negative	15	51.72	0	0	18.49	<0.0001*
Positive	14	48.27	26	100		
PR						
Negative	15	51.72	0	0	18.49	<0.0001*
Positive	14	48.27	26	100		
Her-2						
Negative	24	82.75	17	65.38	2.18	0.139
Positive	5	17.24	9	34.61		

$\chi 2$: Chi-square test, P: p value for comparing between the studied groups

The results showed that the mean age of all patients was 46.24±9.85 years and that of the control group was 41.4±10.54years. There was no significant difference between the studied groups regarding age. Botti G et al. (2017) have included 238 TNBC samples and the age of patients ranged 24–93 years, with an average age of 57 years. This difference is due to larger studied samples and a wider range of age.

Regarding the clinicopathological parameters of patients in this study, we noticed that the majority of patients (36.36%) were stage III followed by stage II (30.9%), stage IV (20%) and stage I (12.72%). According to the tumor grade, the results revealed that the grade II was the commonest among our patients, represented by 87.5% in early-stage and 64.51% in an advanced- stage with a statistically significant difference between the two groups.

Wang et al. (2016) found that 36% (36 breast cancer patients out of 100 patients) were grade II. The discrepancy between findings concerning tumor grade may be due to the variability in case numbers and also to ethnic differences. Concerning lymph node involvement, the present study showed that the lymph node involvement (N2) was the commonest among all breast cancer patients.

The present study showed that the majority of studied cases had large-sized tumors (>2-5 cm), since 23/31 patients (74.19%) of advanced stage and 15/24 patients (62.5%) of early-stage, were allocated to this tumor size. Regarding hormonal status, our results showed no significant difference between early and advanced-stage breast cancer patients regarding Her-2, ER and PR status.

Table 7. Correlation between serum Bcl-2 levels and clinicopathological parameters in breast cancer patients. (n=55).

Clinicopathological parameters	Bcl-2 (U/ml)	
	r_s	P
Age (year)	0.1035	0.4522
Tumor stage		
Early	-0.5499	<0.0001*
Advanced	0.3096	0.0215*
Tumor grade		
II	-0.167	0.167
III	0.2230	0.2230
Lymph node involvement		
N0	>0.9999	00
N1	-0.3284	0.0144*
N2	0.2686	0.0474*
N3	0.07716	0.5755
Vascular invasion		
Yes	0.07532	0.5847
No	-0.07532	0.5847
ER		
Negative	0.243	0.0728
Positive	-0.243	0.0728
PR		
Negative	0.243	0.0728
Positive	-0.243	0.0728
Her-2		
Negative	-0.0106	0.439
Positive	0.0106	0.439

*: Statistically significant at p <0.05, rs: Spearman coefficient.

Table 8. Correlation between PD-L1+ expression and serum Bcl-2 level in breast cancer patients (n=55).

PD-L1+		Bcl-2 serum concentration	
		rs	**P**
Lymphocytes	Percentage	0.082	0.571
	MFI	-0.021	0.882
Monocytes	Percentage	0.291	0.889
	MFI	-0.179	0.389
Granulocytes	Percentage	0.503	0.033*
	MFI	0.303	0.221

*: Statistically significant at p < 0.05, r_s: Spearman coefficient

MFI: Mean fluorescent intensity

Table 9. Correlation between Bcl-2 serum concentration and PD-1expression in breast cancer patients (n = 55).

PD-1+		Bcl-2 serum concentration	
		r_s	P
Lymphocytes	Percentage	- 0.141	0.331
	MFI	-0.175	0.227
Monocytes	Percentage	0.431	0.007*
	MFI	-0.048	0.775
Granulocytes	Percentage	-0.157	0.406
	MFI	-0.043	0.818

*: Statistically significant at p < 0.05.
r_s: Spearman coefficient.
MFI: Mean fluorescent intensity

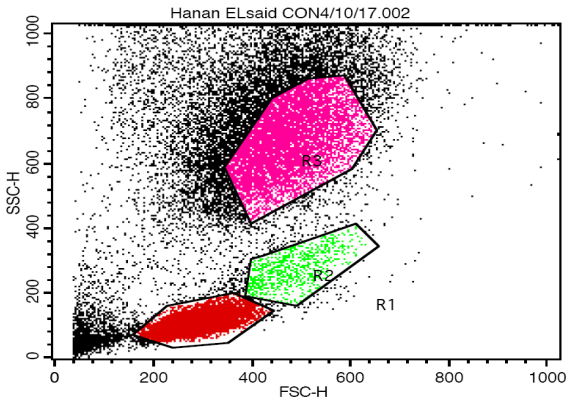

Figure 1A. Gating of lymphocytes, monocytes and granulocytes.

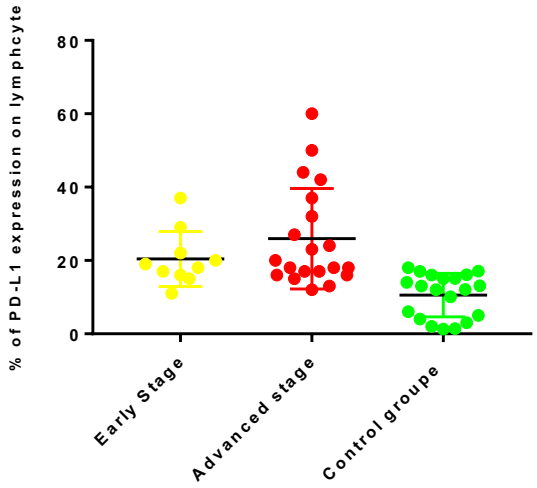

Figure 1B. Percentage of PD-L1+ (CD274) expressing lymphocyte in breast cancer patients and control group. n of early-stage = 9, advanced stage = 20 and control = 8. The means of PD-L1 are significantly different when P< 0.001.

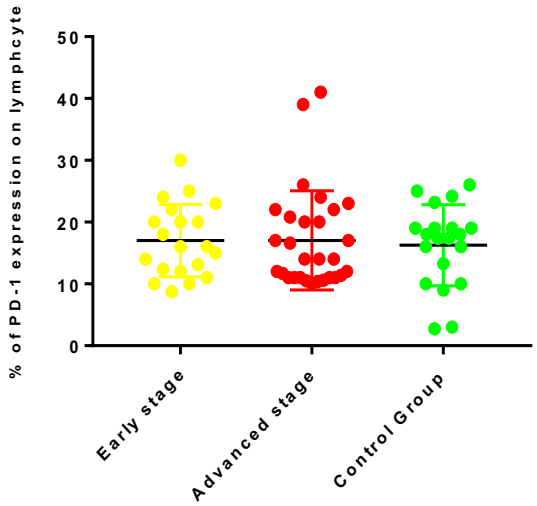

Figure 1C. Percentage of PD-1 (CD279) expressing lymphocyte in breast cancer patients and control group. n of early-stage = 20, advanced stage = 29 and control=20. There is no significant difference among the means.

Regarding the positivity and negativity of PD-1/PD-L1, PD -L1positivity showed a non-significant difference between the patient groups and control group, although the mean % of PD-L1+ in lymphocytes was significantly different among the studied groups.

Moreover, PD-1 showed no statistically significant difference between the patients and control groups. The mean % of PD-1+expressing lymphocytes showed also a non-significant difference between different groups.

Consistent with our results, Schalper et al. (2014) reported PD-L1 mRNA expression in 58% of their breast cancer specimens. Similar results were reported by Shen and Zhao (2018) showed that 54% of breast cancer patients were positive. In agreement with our results. Li et al. (2018) showed that the positive expression of PD-L1 was significantly higher in cancerous tissues than that of in tumour-adjacent normal tissues. Zang et al. (2017) also showed that the PD-1 expression level on CD4+ and CD8+ T cells was significantly increased in cervical cancer, compared with that in healthy control. In contrast, Muenst et al. (2014) reported that the expression of PD-L1 was present in 23.4% of breast cancer patients and its expression associated with poor prognosis in breast cancer.

The divergent results obtained could be at least partially related to study population ethnicity and number of patients or specimens. In addition, the variations could be related to technical methods and data scoring. The precise mechanisms driving PD-L1 upregulation in breast cancer remain elusive. Several studies suggested that multiple factors present in the tumor microenvironment may promote increased PD-L1 expression by tumors (Kinter et al., 2008). This is likely a mechanism whereby tumor cells evade the anti-tumor immune response of tumor-specific T-cells. Therefore, it has become very clear that malignant cells must be able to successfully evade immune surveillance to progress and metastasis. Tumor cells use a variety of different pathways to achieve this goal (Lisiecka and Kostro , 2016).

PD-L1 upregulation can reflect either lack or presence of antitumor immunity in the specific tumor microenvironment. Therefore, theoretically, higher PD-L1 expression can be correlated with either worse or improved prognosis across different tumor types (Xiang et al., 2018).

However, in breast cancer patients the relationship between PD-L1 expression and prognosis remains unclear. Some studies have shown that positive PD-L1 was associated with significantly poor survival (Lisiecka and Kostro, 2016), but other studies could not confirm these findings (Park et al., 2016). These discrepancies may be due to different thresholds were used to determine expression positivity and because the studies included populations of different races. Comparisons of different studies reporting PD-L1 expression in various cancers are hindered by the use of different methodologies, different thresholds, different antibodies and specimens from different areas. Thus, future studies should make an effort to use standardized quantitative assays to measure PD-L1 expression (Ilie and Hofman, 2017).

In this study, we investigated the relationship between PD-L1 expression and clinicopathological parameters. According to our results, patients with positive lymph node, advanced tumor stage, histological grade II, tumor size T2, ER, PR and Her-2 negativity had higher PD-L1 expression levels. These results are consistent with other studies, Zhang et al. (2017) indicated that PD-L1 expression was associated with positive lymph node metastasis, higher histological grades, estrogen receptor (ER) negativity and triple-negative breast cancer (TNBC). Li et al (2018) showed that PD-L1 positive expression was not related with the patients' age, menopausal history, family history, tumor size, and tumor location ($p > 0.05$), but it was related with the clinical stage, lymph node metastasis and histopathological grade.

Regarding the relation between positivity and negativity expression of PD-1 and clinico-pathological parameters, the results showed that PD-1 expression was associated with lymph node involvement only ($p<0.0269$). Otherwise, correlation between PD-1+/PD-1$^-$expression and other clinico-patholgical parameters did not show any statistical significance ($p > 0.05$).

Ghebeh et al. (2008) found that PD-1 was expressed in up to 70% of tumor-infiltrating lymphocytes compared to 30% in normal breast tissue and its expression was associated with histological grade, ER and PR status. Muenst et al., (2013) observed that the presence of PD-1 TIL was significantly associated with tumor size, tumor staging system (TNM), tumor grade and lymph node status. Also, the same authors found that the presence of PD-1+ TIL was negatively associated with ER and PR but with no significant association with Her-2 expression.

Regarding the relationship between positivity of PD-1/PD-L1 expression in breast cancer patients, the results showed a highly significant association between PD-1 and PD-L1 expression in early and advanced breast cancer patients.In agreement with these results, Gatalic et al. (2014) PD-L1 and PD-1 were expressed concurrently in various tumors. It was also reported that the rate of concurrent PD-L1 and PD-1 expression in breast cancers regardless of tumor subtypes was 29%; however, the rate in TNBCs was significantly higher than that in non-TNBCs (45% vs 13%-17% respectively). In breast cancer, Bcl-2 has been reported to be favorable prognostic factor, especially in the luminal A subtype (Escórcio-Dourado et al. 2017).

The present study showed a significant increase in the mean Bcl-2 serum concentration in patients compared to healthy individuals. These results are in agreement with Hwang et al. (2012) who demonstrated that the expression of Bcl-2 was frequent in breast cancer patients compared to healthy control. It has been reported that Bcl-2 is up-regulated by estrogen in breast cancer, through a direct consequence of transcriptional induction. Also, Chen et al. (2015) observed that the engagement of PD-1 with its ligand leads to inhibition of T-cell activation via mechanisms that include blocking of proliferation, induction of apoptosis and regulatory T-cell differentiation and therefore immune inhibition. Therefore, it's not surprising that increased expression of pro-survival Bcl-2 protein is found in many cancer types.

In this study, there was a significant increase in the mean of serum Bcl-2 concentration in the advanced stage and lymph node involvement N2. While the correlation between serum Bcl-2 and other clinicopathological parameters did not show any statistical significant (p= > 0.05). In agreement with our result, Cecka et al. (2008) reported that there was an association between Bcl-2 protein expression and stage of breast cancer.

On the contrary, Eom et al. (2016) observed that Bcl-2 level was increased in early stages of breast cancer and its upregulation was related to favorable prognosis. In addition, Hwang et al. (2018) observed that the expression levels of Bcl-2 and mRNA were strongly associated with the status of ER, PR and Her-2. This discrepancy between our results and previous results may be due to the number of subjects was relatively small which considered as a limitation of our study. Moreover, different methods used in the analysis was another factor, in our study the serum samples were employed to measure the serum concentration of Bcl-2 using ELISA while other investigators performed their studies on tissue samples using immunohistochemistry and the statistical analysis might be limited, especially in the subgroup analyses.

Finally, our results showed that Bcl-2 serum concentration correlated positively with positive PD-L1+ expressing granulocytes (r=0.503, p=0.033). While the correlation between serum Bcl-2 and PD-1+ expressing lymphocytes, monocytes and granulocytes did not show any statistical significance. However, till now only a few studies have reported a possible link between PD-1/P-L1 and apoptosis among breast cancer patients.

CONCLUSIONS & RECOMMENDATIONS

The direct correlation between PD-L1+ expression and serum Bcl-2 concentration may explore the role of apoptotic machinery in the pathogenesis of breast cancer. Understanding the regulation of PD-1/PD-L1 in cancer will be one of the utmost importance for defining their roles as predictive markers and for optimizing strategies for cancer immunotherapy. Future studies should make an effort to use standardized quantitative assays to measure PD-L1/PD-1 expression to solve the discrepancies between different results.

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Conflicts of interest

The authors have no conflict of interest to declare

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