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Research article

Evaluation PD-L1, CD8 and CD20 as early predictor and tracking markers for breast cancer (BC) in Egypt

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ABSTRACT

Background: Breast cancer (BC) is considered as a common type of cancer threatening women throughout the world. Therefore, development of early predication biomarkers for BC got more concern especially for Egyptian females. This study was aimed to evaluate PD-L1, CD8, and CD20 as early prediction breast cancer biomarkers.
Methods: Flow cytometry (FC), immunohistochemistry (IHC), Western Blot, and q-PCR were used to compare PD-L1, CD20, and CD8 levels in tissues and blood samples of Breast Cancer and controls.
Results: Blood samples showed a significant increase in PD-L1, CD20, and CD8 compared to controls ($p<0.005$). A Significant correlation was shown between PD-L1, CD8, and CD20 in tissue and breast cancer subtypes. Whereas, invasive lobular carcinoma (ILC) was characterized by superior PD-L1 and CD20 levels compared to invasive ductal carcinoma (IDC). FC studies on Blood showed 83% and 45.7% PD-L1 expressions for IDC and ILC, respectively. CD20 in ILC and IDC were 78.2% and 62.5%, respectively. Nevertheless, CD8 was 74.2% for IDC and 67.7% for ILC. Whereas, FC studies for PD-L1, CD20, and CD8 in ILC in tissues gave 34.4%, 30.2% and 35.1%, respectively. In addition, IDC tissue samples showed 16%, 12.5, and 13.5% for PD-L1, CD20, and CD8. The moderate stage of adenocarcinoma caused expression of PD-L1 within inflammatory cells, while expression was within neoplastic glandular cells in late stage.
Conclusion: PD-L1, CD8, and CD20 are considered as early predictor and tracking markers for breast cancer.

1. Introduction

Breast cancer (BC) is a common type of cancer threatening women throughout the world and it is considered the second cause of death after lung cancer (Cianfrocca and Goldstein, 2004; Ahmedin Jemal et al., 2004). According to WHO, 23081 (35.1%) of female cancer patients in Egypt are diagnosed with BC in 2018 (Sung et al., 2021). Most of the women who got BC had their menopause, but about (20%) are under 50 years old (Yarnold, 2009). BC is a miscellaneous disease that can be categorized into subtypes; the breast tissues that are comprised of lobules, or glands for milk production, and the ducts that connect the lobules to the nipple. The remaining breast is composed of connective, fatty, and lymphatic tissues (Isakoff, 2010). Most BC is infiltrating or invasive, these cancers have penetrated through the glandular or ductal wall where they started and spread into surrounding tissues (Morrow et al., 2016). While multifaceted approaches include surgery, chemotherapy, and radiation therapy, they have slightly enhanced survival and have developed chemo-resistance and the recurrence of the disease (Pierga et al., 2012). In this way, it is imperative to develop more effective therapy and predictive biomarkers for early diagnosis of BC, because this disease classically produces no indications when the tumor is small and more effectively cured (Siegel et al., 2014).

PD-L1(CD274) is an immunoinhibitory molecule belonging to the B7-CD28 super-family. It suppresses the activation of T cells leading to the progression of tumours. PD-L1 is predominantly expressed in various solid malignancies that are on the surface of tumour cells and antigen-presenting cells for example brain, oesophagus, lung, thymus, breast, thyroid, squamous cell carcinoma of the head and neck, gastrointestinal tract, liver, colorectum, pancreas, kidney, bladder, adrenal cortex, urothelium, skin, and ovary (Wang et al., 2016; Thierauf et al., 2015; Gadiot et al., 2011; Chowdhury et al., 2016). PD-L1 binds PD-1 to constrict the cellular immune response by promoting T-cell apoptosis or exhaustion. This interaction causes the inhibition of T cell activation, the induction of T-cell apoptosis, and the promotion of tumour immune escape (Chen and

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Mellman, 2013). Blocking antibodies that target PD-1 and PD-L1 eliminate the inhibition and increase the immunity of T-cell to recognize cancer cell and destroy it. This is a promising therapeutic approach that has accomplished an impressive response rate in patients who have PD-L1-overexpressing (Salgado et al., 2014; Sanmamed and Chen, 2014; Stovgaard et al., 2019). The expression of PD-L1 in different tissue specimens has been studied such as kidney, malignant melanoma, non-small cell lung cancer, colon cancer, and oesophageal cancer. However, only a few reports of PD-L1 and the role of CD20 in breast cancer tissue have been published (Dong et al., 2002; Konishi et al., 2004; Cierna et al., 2015; Zhang et al., 2017).

Many studies reflected variable PD-L1 expression patterns within TC and Tumour Infiltrating Lymphocytes (TILs) with prognostic values with BC. Some investigations confirmed PD-L1 expression in BC as a marker of poor prognosis (Chae et al., 2016; Stovgaard et al., 2019). The association of PD-L1 expression in BC with a more favorable prognosis (Cottrell and Taube, 2018; Uhercik et al., 2017) was detected.

The objective of this study was the evaluation of PD-L1, CD8, and CD20 as early predictor biomarkers for breast cancer in Egypt. To achieve this goal, flow cytometry, immunohistochemistry (IHC), quantitative PCR (qPCR), and western blotting techniques were employed to compare PD-L1, CD20, and CD8 in blood, tissues, and control samples.

2. Methods

2.1. Sample collection

50 Blood and tissues samples of Egyptian patients (females), just diagnosed with breast cancer, were collected during surgery (with their consent) from Oncology Center Mansoura University (OCMU). Patients taking any kind of treatment and suffering from other diseases were excluded. Patients diagnosed with invasive ductal carcinoma (IDC) and with invasive lobular carcinoma (ILC) were included. In addition, 15 healthy control blood samples. There are no other diseases included according to our protocol (Table 1). The median age at diagnosis was (54.6 ± 14.7) years that range from 21 to 84 years. The majority of diagnosed patients were invasive ductal carcinoma IDC (74%), and the rest were invasive lobular carcinoma ILC (26%). This study was approved by the Ethics Committee of at Mansoura University Sci-ch-M-2020-38.

2.2. Separation of lymphocytes

Blood was collected into EDTA-containing tubes and then diluted with phosphate buffer solution (PBS) (1:1). This diluted blood was added slowly to another tube containing Ficoll Hypaque, and was centrifuged at 2000 rpm for 20 min. The upper layer (plasma) was discarded using a Pasteur pipette, and then the lymphocytes were transferred to a centrifuge tube that was washed with PBS and centrifuged again for 10 min. The supernatant was excluded, 1 ml ice-cold ethanol was added to the pellet, and then preserved in the refrigerator till staining (Harris and Ukaejiofo, 1969; Bøyum, 1964).

2.3. Preparation of tissue suspension

Fresh tissues specimen were prepared according to Tribukait (Tribukait et al., 1975), as stated below:

1 The specimens were washed with EDTA buffer, 3.029 g of 0.1M tris, 1.022 g of 0.07M sodium chloride, and 0.47 g of 0.005M EDTA.
2 They were dissolved in 250ml of distilled water and pH was adjusted at 7.5 by using 1N HCL.
3 Then, the cell suspension was centrifuged at 1800rpm for 10 min, whereupon supernatant was aspirated.
4 After the centrifugation and the aspiration of the supernatant, the cell is fixed in ice-cold 96–100% ethanol in approximately 1ml for each sample.

Table 1. Biochemical investigation for patients (include liver function, kidney function and Complete Blood Count (CBC) all in reference ranges.

Patient no	Creatinine (mg/dl)	Albumin (g/dl)	SGPT (u/l)	SGOT (u/l)	Bilirubin (mg/dl)	ALP (IU/I)	CBC								
							WBC (k/ul)	RBC (m/ul)	HGB (g/dl)	HCT (%)	MCV (fl)	MCH (pg)	PLT (k/ul)	MCHC (g/dl)	NEUT (%)
1	0.7	4.1	20	25	0.4	57 (ng/ml)	9.8	4.4	11.3	36.5	82	25.4	261	31	49.5
2	1	3.8	36	29	0.4	95 (ng/ml)	9.5	5.1	12.7	40.2	78	24.8	197.9	31.7	42.7
3	0.8	4.2	12	12	0.4	116	8.5	5.1	14.6	46.2	88.9	28.1	152	31.6	66.2
4	1	4.6	38	26.5	0.5	150	9.1	4.9	13.8	40.8	83.3	28.2	93	33.8	63.4
5	0.77	3.04	27.4	34.4	0.47	333.8	25	3.2	9	27.6	85.3	27.9	279	32.6	71.5
6	0.8	4.4	27	13	0.7	96 (ng/ml)	6.5	4.8	11	37	76.9	23	263	29.9	54.3
7	0.7	3.9	15	20	0.4	74 (ng/ml)	12.1	4.7	12.6	40	85.8	27	269.5	31.5	34.8
8	0.77	4.5	25.8	17	0.54	121.4	5.1	3.9	11.7	33.4	85.7	30	228	34.9	58
9	0.6	4	13	35	0.3	41 (ng/ml)	7.3	4.06	11.4	34.8	85.6	28.1	167	32.8	73.3
10	0.9	4.5	10	16	1.2	69 (ng/ml)	5.8	4.1	11.6	35.2	85.8	28.2	159.9	32.8	41.2
11	0.76	4.49	36.9	38.8	1	164	5	4.5	13.7	39.3	86.5	30.1	240.6	34.88	58.4
12	1.62	3.7	61.6	126.8	0.5	691.9	11.9	3.5	10.7	33.4	94.7	30.2	229	31.9	70.2
13	0.93	3.3	22.2	29.6	0.54	126.3	12	4.3	10.7	33.9	78.4	24.7	201	31.6	75.9
14	0.88	4.19	17.16	17.5	0.43	188.3	10.4	5.5	12.9	41.9	76.2	23.5	312	30.8	57.4
15	1.3	4.04	16.4	20.5	0.6	249.8	5.9	4.1	11.6	36.4	87.7	28	186	31.9	50
16	0.96	4.4	9	18.5	0.82	170	6.8	4.2	11.9	38.2	90.3	28.2	136	31.2	61.9
17	2.2	4.28	14.9	9.9	0.83	184.1	5.7	3.8	10.9	33.3	86.03	28.4	225.5	32.9	60.5
18	0.94	4.15	26.4	34.3	0.38	232.4	7.3	4.3	10.6	35.2	80.6	24.2	240	30	62.4
19	1.09	4.4	24.4	15.3	0.6	206.8	4.7	3.9	11.2	34.9	88.7	28.5	157	32	59
20	1.18	4.71	45.7	30.5	0.85	115.3	7.5	4.33	10.9	34	78.8	25.2	162	32	59.3
21	1.09	4.5	32	22.7	0.7	208.3	6.1	4.5	12.3	36.6	81	27.3	145	33.7	37.2
22	1.07	4.4	3.6	14.7	0.46	146.7	7.7	4.3	13.2	39.9	90.8	30	182	33	57.3
23	1.1	4.9	34	28.3	1.2	141.5	10	5	14.6	45.6	88.8	28.4	244	32	77
24	1.2	4.4	14.5	20.4	0.74	181	7.5	4.8	12.9	39.2	80.6	26.5	203	32.9	68.2
25	0.8	4.1	8	13	0.6	85 (ng/ml)	16	4.4	12.6	38.5	86.7	28.4	375	32.8	70

2.4. Staining

Specimens were stained with anti-PD-L1, anti-CD20, and anti-CD8. They were incubated in dark for 15 min at 25 °C, were fixed with paraformaldehyde, and their intensity was measured by FC.

2.5. Immunohistochemistry

The immunohistochemical procedures were done according to Abdo et al., 2013. The serial sections were dewaxed, hydrated, and immersed in antigen retrieval (EDTA solution, pH 8). They were then treated with hydrogen peroxide 0.3% and protein block followed by incubation with PD-L1/CD274 polyclonal antibody (Product # PA5-28115) at a dilution of 1:100, Invitrogen, Zymed San Francisco CA (USA).

The slides were rinsed three times with PBS and incubated with anti-rabbit IgG secondary antibodies (EnVision + System HRP; Dako) for 30 min at room temperature. Slides were visualized with di-aminobenzidine commercial kits (Liquid DAB + Substrate Chromogen System; Dako), and finally were counterstained with Mayer's haematoxylin. As a negative control procedure, the primary antibody was replaced by normal serum (Abdo et al., 2013).

2.6. PD-L1, CD8 and CD20 gene expression

In this investigation, PDL-1 gene expression was applied as an early predictor marker for breast cancer (BC) *via* Quantitative RT-PCR. Each of the blood and tissue samples included internal control for calibration. TRIzols Reagent (15596026, Life Technologies, USA) was applied for total RNA purification from blood samples according to manufacturer protocol. Then, 1μg of total RNA was reverse-transcribed into single-stranded complementary DNA by utilizing QuantiTects Reverse Transcription Kit (Qiagen, USA) using a random primer hexamer in a two-step RT-PCR reaction. Finally, Real-time PCR was performed using Rotor-GeneQ (Qiagen, USA) with β-actin as a house-keeping gene. cDNA amplicons were amplified *via* Maximas SYBR Green/Fluorescein qPCR Master Mix through specific primers with thermal cycling conditions as follows; denaturation for 8 min at 94 °C followed by 40 cycles of 30s denaturing at 94 °C, 45 s annealing at 56 °C and 50 extension at 72 °C with a final extension of 7 min at 72 °C. Relative expression of the target gene was calculated using $2^{-\Delta\Delta ct}$.

2.7. Evaluation of PD-L1, CD8 and CD20 protein expression levels

To compare PD-L1, CD8, and CD20 protein expression levels in tissue and blood sample that included internal control, Western blotting

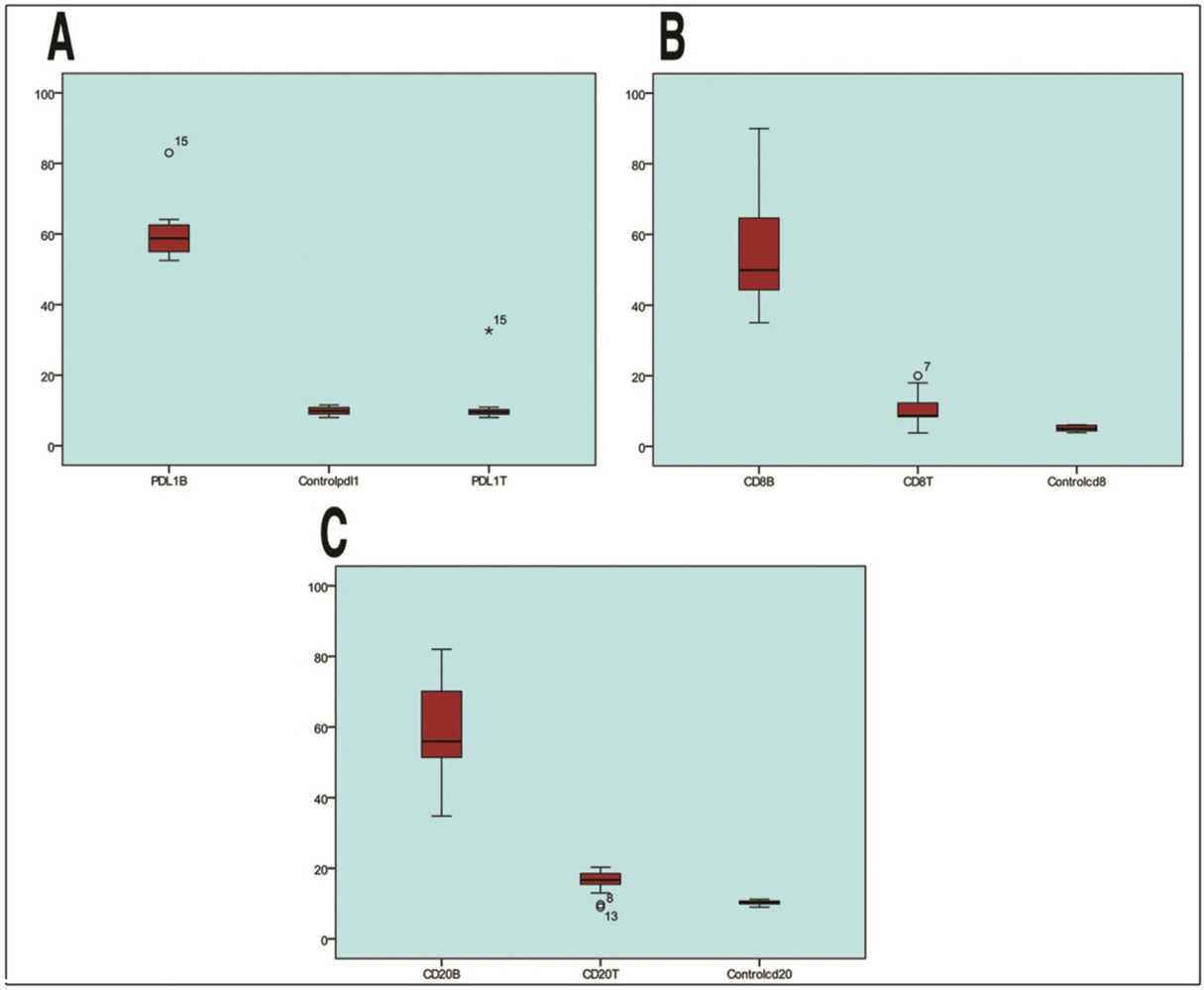

Figure 1. (A) Box plots with different expression of PDL1in blood (PDL1B), tissues (PDL1T), and in control samples (controlPDL1) showing high level of PD-L1 in blood than in control with M±SD (59.5 ± 7.71 and 9.8 ± 1.02, $p<0.005$). (B) different expression of CD8 in blood (CD8B), tissues (CD8T), and in control samples (ControlCD8) showing a significant increase in blood with M±SD (56.3 ± 13.5) than in tissues sample (10.53 ± 3.45, $P < 0.005$) and higher than in control (5.12 ± 0.82, $P < 0.005$). (C) different expression of CD20 in blood (CD20B), tissues (CD20T), and in control samples (ControlCD20) showing extreme significant of CD20 in blood than in control. (57.4 ± 12.8and10.3 ± 0.58,$p<0.005$).

techniques were employed. Total soluble protein for all samples was purified through TRIZOl reagent (15596026, Life Technologies, USA) according to manufacturer protocol. Then, Electrophoresed proteins on SDS-PAGE (12% of them was performed according to Laemmli, 1970) were transferred to a Hybond™ nylon membrane (GE Healthcare) *via* TE62 Standard Transfer Tank with Cooling Chamber (Hoefer Inc.) and were incubated for 1 h at room temperature with Anti- CD20primary antibody (abcam, USA, ab78237) and Anti- CD8 (abcam, USA,ab217344) and Anti-PD-L-1 (ab213524) and Anti- βactin primary antibody (abcam, ab228001). Additionally, β-actin was applied as a housekeeping protein.

2.8. Statistical analysis

Mean values and standard deviations were calculated according to conventional methods. The data were collected and analyzed using Statistical Package of Social Science (SPSS) Version 18. In addition, independent t-test was performed where $P < 0.05$ was considered significant.

3. Results

Our study was carried out to evaluate the possibility of using PD-L1, CD8 and CD20 as early predictive breast cancer biomarkers for Egyptian females. To achieve this goal, FC, quantitative PCR (qPCR), Western blotting and IHC techniques were employed to compare PD-L1, CD20, and CD8 in tissues and blood of healthy and breast cancer samples.

Blood and tissues samples of Egyptian female patients were stained with anti CD8, PD-L1, and CD20 and measured by flow cytometry. Then, Immunohistochemistry (IHC), Western Blot, and qPCR were performed for tracking PD-L1 expression levels.

3.1. Evaluation of PD-L1, CD8, and CD20 for female blood and tissue samples

As shown in Figure 1A and Table 2, female patients' blood samples reflected a significant increase in PD-L1 expression level compared with control blood samples (59.5 ± 7.71 and 9.8 ± 1.02, $p^{<}0.005$). Furthermore, a positive significant correlation was detected between disease subtypes and PD-L1 levels in tissue samples (Figure 2A). On contrary, the PD-L1 level has not been influenced significantly by the patients' age variation. Nevertheless, CD20 level could be dependent on as an early predictive biomarker for breast cancer as a result of extremely significant differences for blood samples compared with control samples. (57.4 ± 12.8and10.3 ± 0.58,$p^{<}0.005$) (Figure 1 C) (Table 2).

Supporting the successful findings of PD-L1, CD20 as early prediction biomarkers for breast cancer, significant superiority was detected for CD8 (Table 2, Figure 1B) Level in patients' blood samples (56.3 ± 13.5) rather than in tissues sample (10.53 ± 3.45, $P < 0.005$) and higher than control (5.12 ± 0.82, $P < 0.005$).

For tissue samples, a highly significant correlation was detected between PD-L1 levels and advanced breast cancer subtypes. A dramatic increase in PD-L1 levels and a slight increase in CD20 levels were

Table 2. Descriptive statistics of markers in blood (B), control (con) and tissue (T) represented by mean ± SD. ($p<0.005$).

Statistics	PDL1			CD20			CD8		
	B	con	T	B	con	T	B	con	T
M±SD	59.5 ± 7.71	9.8 ± 1.02	13.36 ± 15.62	57.42 ± 12.80	10.3 ± 0.58	16.25 ± 2.79	56.35 ± 13.58	5.12 ± 0.82	10.53 ± 3.45
Max/Min	83/37	11.5/7	83/4.4	82/34.8	11.2/9	20.3/9	90/35	6.1/3.9	20/3.8
T-sig	15.7			11.5			15.6		
P-value	<0.005								

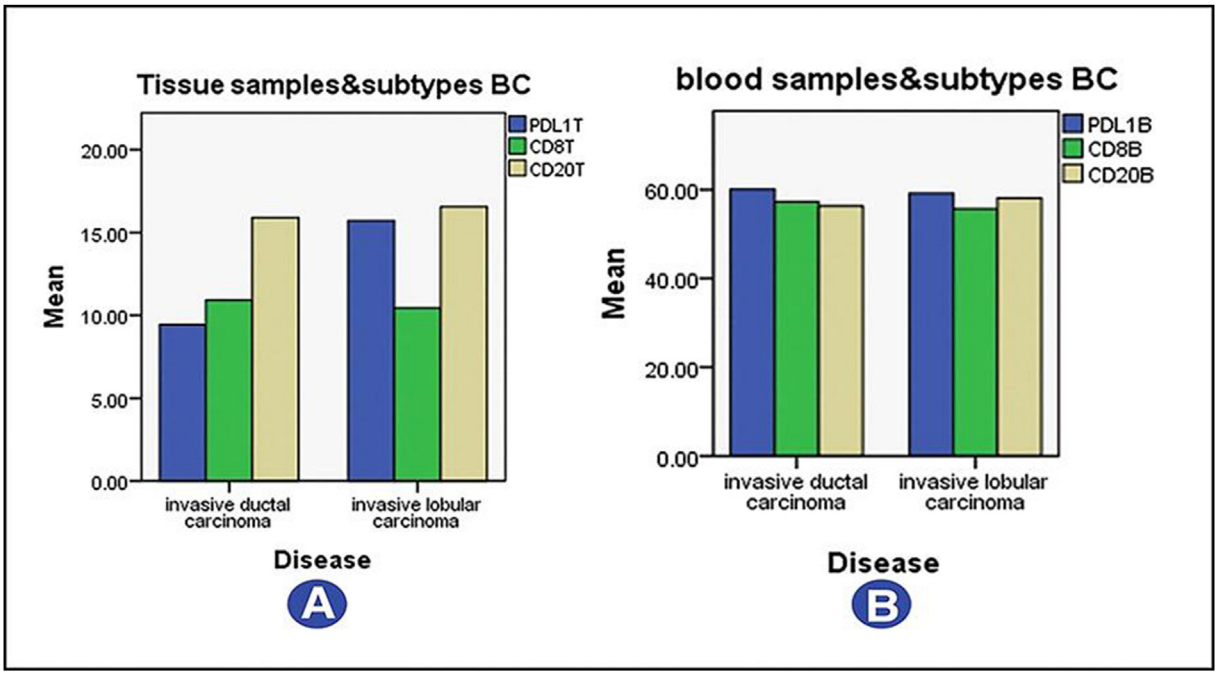

Figure 2. Histogram represents correlation between levels of PDL1, CD8, and CD20, in tissue (A) and blood samples (B), with sub types of breast cancer showing: in tissue (A), a dramatic increase of PD-L1 levels in Invasive lobular carcinoma (ILC) than invasive ductal carcinoma (IDC). In blood (B): a slight increase of PD-L1 and CD8 in IDC.

obtained in Invasive lobular carcinoma compared to invasive ductal carcinoma (Figure 2A).

3.2. Flow cytometry results

Results for the flow cytometry technique of female blood samples indicated a similar correlation between PD-L1, CD8, and CD20 levels on one side, and control samples and advanced breast cancer subtypes on the other (Figure 3). PD-L1 expression levels were arranged in descending order for IDC, and ILC breast cancer subtypes (83% and 45.7%) which were more than 12 folds of the control levels at their highest level. Furthermore, a positive correlation was observed between CD20 expression level and breast cancer subtypes. ILC and IDC breast cancer subtypes expressed in 78.2%, 62.5% of CD20 level were 7:9 folds the level of control. Nevertheless, CD8 expression level is high with 74.2% and 67.7% that distinguished IDC and ILC, respectively (almost 15 folds than control).

PD-L1, CD8, and CD20 expression levels in tissues samples were higher in ILC (34.4%, 30.2% and 35.1%) compared to their levels in IDC (16%, 13.5% and 12.5%, respectively) (Figure 4). However, CD8 levels in tissues sample were 35.1%, 13.5% for IDC and ILC, respectively (Figure 4).

3.3. Immunohistochemistry (IHC) results

Immunohistochemistry (IHC) findings reflected a corresponding increase in PD-L1 (PD-L1 IHC. X200) expression levels with the developing breast cancer stages (Figure 5 A, B, C, D, E and F). Usual ductal hyperplasia case and early-stage adenocarcinoma case remarked with negative expression of PD-L1.

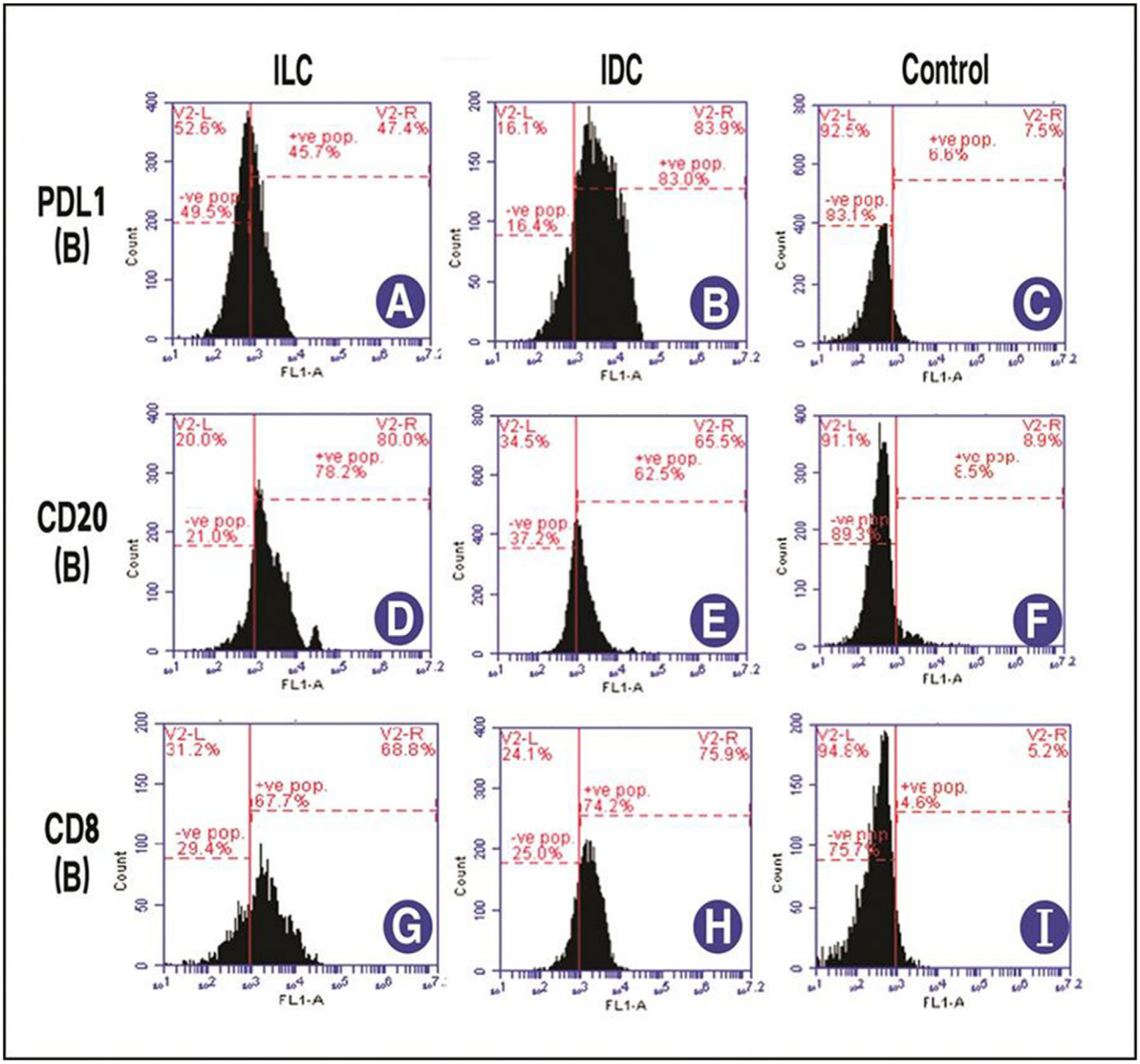

Figure 3. Flow cytometry histogram (selected randomly) of PDL1, CD20, and CD8 for blood samples in breast cancer subtypes ILC, IDC and control samples showing:
A, B, and C (45.7% ILC, 83% IDC, and 6.6% control) for PD-L1
D, E, and F (78.2% ILC, 62.5% IDC, and 8.5% control) for CD20
G, H, and I (67.7% ILC, 74.2% IDC, and 4.6 %control) for CD8.

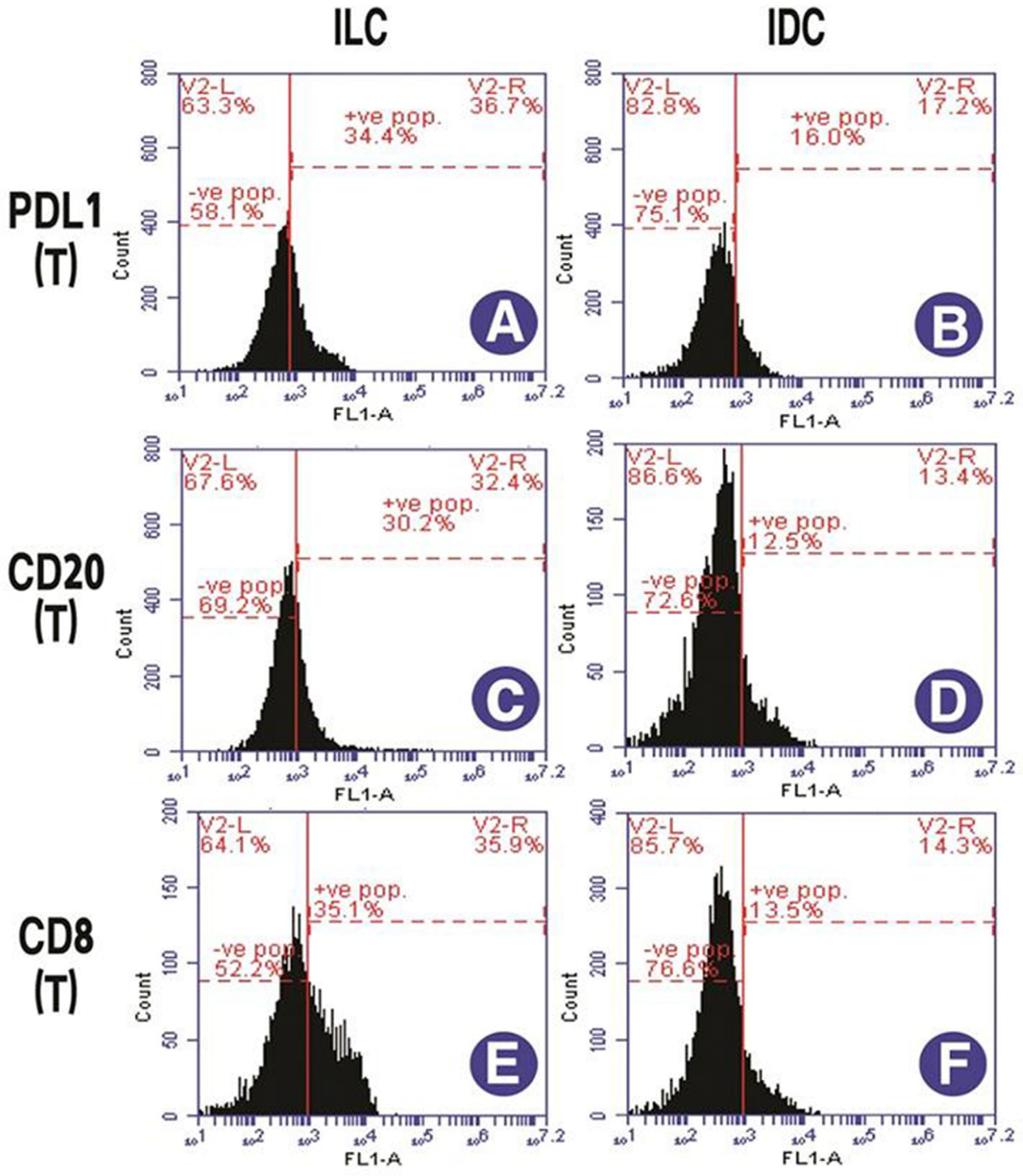

Figure 4. Flow cytometry histogram of PDL1, CD20, and CD8 for tissue samples in breast cancer subtypes ILC and IDC showing higher level of PDL1, CD8 and CD20 in ILC (A, C, E) (34.4%, 30.2% and 35.1%) than in IDC (B, D, F) (16%, 12.5% and 13.5%).

Mild expression of PD-L1 was abundant in the inflammatory cells and it was few in interstitial cells (arrow in Figure 5C and D). While, it was abundant in the inflammatory cells (ar in Figure 5D), and few immunostaining in neoplastic cells (arrow in Figure 5C and D). It is characterized as a moderate stage of adenocarcinoma case. Interestingly, late-stage adenocarcinoma case cause remarkable expression of PD-L1 within neoplastic glandular cells (arrows in Figure 5 E and F) that distinguished late-stage adenocarcinoma case.

3.4. Gene expression

This investigation was carried out to evaluate validity of employing Programmed death-ligand 1 (PDL-1) gene for early prediction of cancer diagnosis. Thus, qPCR method was used to detect PDL-1 gene expression among twenty blood sample patients. According to Figure 6 and compared with PDL-1 gene expression for control sample, all twenty patients reflected varied PDL-1 gene expression. Based on the findings of

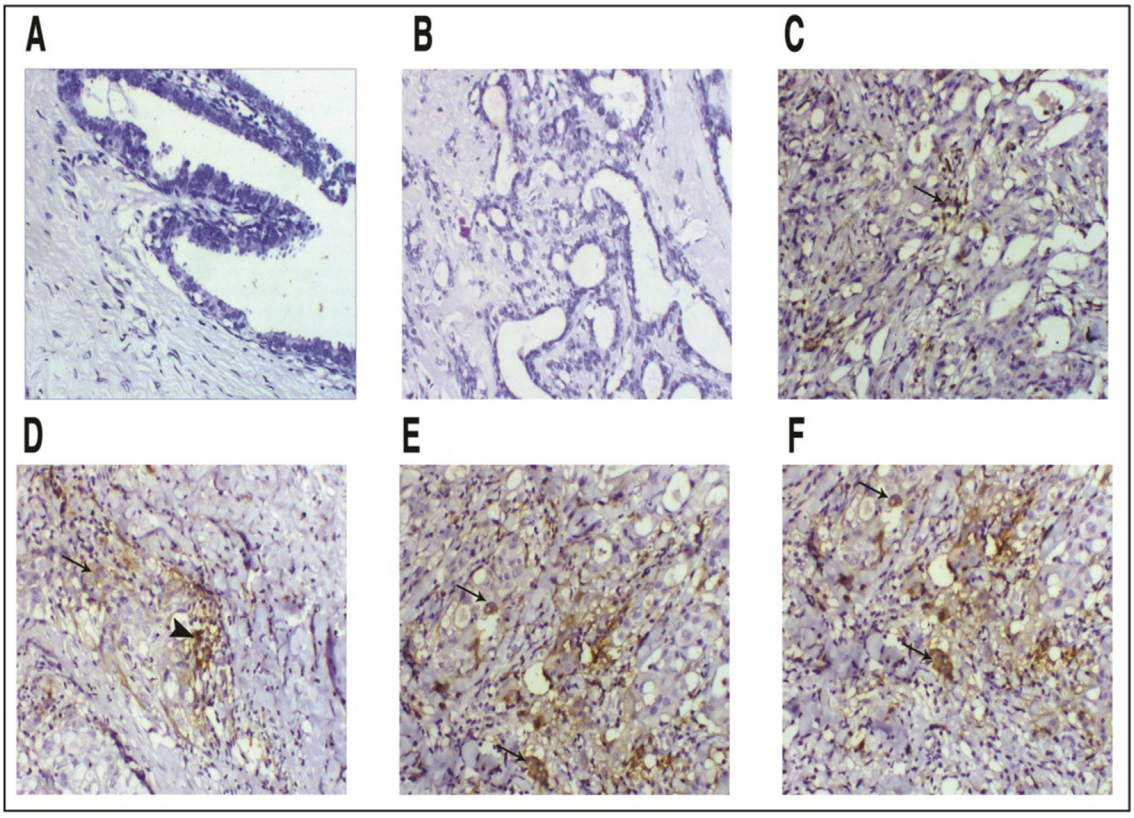

Figure 5. Different developed Breast cancer tissue samples, (A) of usual ductal hyperplasia case, (B) early stage adenocarcinoma case, (C) moderate stage of adenocarcinoma case, (D) moderate stage adenocarcinoma case, (E) late stage adenocarcinoma case, (F) late stage adenocarcinoma case.

this study, the variation of PDL-1 gene expression among patients indicated the effectiveness of using PDL-1 gene expression as an early prediction procedure for cancer diagnosis.

3.5. Evaluation of PD-L1, CD8 and CD20 protein expression levels

For evaluating applied PD-L1, CD8, and CD20 protein expression levels as early predictors, specific antibodies were used. Compared with control, blood and tissue samples expressed 1.6 and 1.2 folds of CD20 protein signal. While, CD8 protein expressions in blood and tissue samples were 1.4 and 1.1 folds of controls, respectively. PDL-1 expression level superiority (1.3 folds) was recorded for blood sample and tissue sample and reflected 1 fold compared with control (Figure 7).

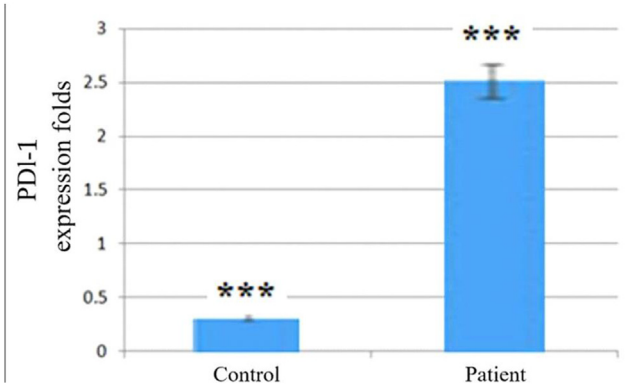

Figure 6. PDl-1 gene expression level (means $p < 0.001$) for BC patients.

4. Discussion

BC is divided into molecular subtypes according to the absence or the presence of estrogen receptor (ER + ve, ER-ve), progesterone receptor (PR + ve, PR-ve), and human epidermal growth factor 2(HER2+ve, HER2-ve), and triple-negative (TNBC) that has no receptors. Estrogen and progesterone act as fuel that induces the metastasis of the disease (Reis-Filho and Pusztai, 2011; Goldhirsch et al., 2011). Anti-estrogen treatment (*e.g.* Tamoxifen) is considered the first-line therapy for BC patients who have (ER + ve) for metastatic BC and after mastectomy. Although it is non-toxic therapy and more effective in postmenopausal patients, most patients relapsed (Osborne et al., 2000; Dieci et al., 2019).

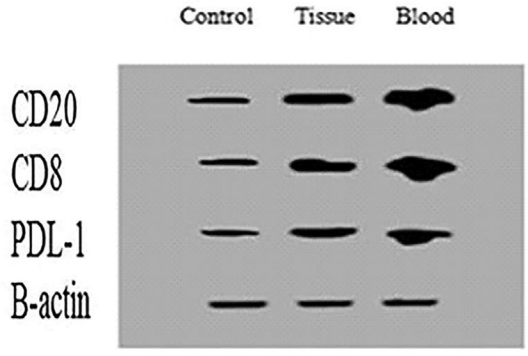

Figure 7. CD20, CD8,PD-L1 and β-actin protein expression level for tissue and blood samples. Further data is found in supplementary files attcheched: Electrophoresed proteins on SDS-PAGE, and Wester blots of CD20, CD8, PD-L1 and β-actin proteins.

One major fact of failure of classical therapies (hormonal therapy, chemotherapy, and targeted therapies) which especially targeted the cancer cell, is the development of secondary resistance due to the adaptable capacity and high mutagenic of cancer cells making the tumor response temporary. For that reason, researchers must distinguish exact biomarkers of breast cancer and potential helpful target for the treatment of the disease to enhance patients' life (Zou et al., 2016; Knappskog and Lønning, 2012).

The CD8 molecule is a heterodimer of α and β chains linked covalently by a disulfide bond. It is expressed on the surface of T-cell as either an αβ heterodimer or a homodimer (Murphy et al., 2008; Gao and Jakobsen, 2000). CD20 is a transmembrane protein expressed on B cells but not plasma cells and regulates B cell differentiation and proliferation. CD8 and CD20 represent an independent prognostic factors in breast cancer (Arias-Pulido et al., 2018b).

PD-1 and PDL-1 pertain to immune checkpoint proteins that serve as co-inhibitory factors and can minimize or halt the development of response of T-cell. Under normal conditions, the immune system implements a series of steps described as the cancer immunity cycle that causes anticancer immune response and cancer cell lysis. The PD-1/PDL-1 pathway act as an adaptive immune resistance mechanism that is applied by cancer cells responding to endogenous immune anti-tumour activity (Pardoll, 2012; Chen and Mellman, 2013; Plaks, 2018).

PD-L1 expression has been barely studied in breast cancer only with IDC as it is the most frequent type of cancer in the Middle East (prognostic studies) (Posabella et al., 2015; Muenst et al., 2014; Al-Kuraya et al., 2005). Accumulating evidence demonstrated that PD-1/PD-L1 pathway blockade has resulted in sustainable clinical responses and long-term remission in both solid tumours (including breast) and hematologic malignancies (Gentzler et al., 2016). The findings of current studies show that the high expression of PD-L1 is related to increased clinical activity among patients with different types of tumour (metastatic melanoma, non-small cell lung cancer, and renal cell carcinoma). These patients were treated with PD-1/PD-L1 pathway blockade (Gandini et al., 2016). Recently, Nobel Prize was awarded to James P. Alison and Tasuku Honjo in Medicine and Physiology because of their findings concerning cancer immunotherapy by suppression of negative immune regulation using a mixing of anti-CTLA-4 and anti-PDL-1 that have been proven for the treatment of several forms of cancer (Darling, 2019). Moreover, in March 2019, the FDA approved the first immunotherapy regimens (Tecentriq) in patients with TNBC and express PD-L1 (Soare and Soare, 2019).

In this manner, overexpress PD-L1that must be screened in patients is a central question faced by researchers attempting to develop anti-PD-1/PD-L1 therapies. In this study, PD-L1 expression was investigated in different subtypes and the result shows a significant increase in PD-L1, CD20, and CD8 expression compared to control ($p < 0.005$) that proves the presence of systemic state of immune tolerance (Weber et al., 2017). The high-level PD-L1 tissue samples (Figure 2A) increase in patients with ILC and could benefit from treatment targeting the PD-1/PD-L1 pathway. In other words, immunotherapy is an effective treatment for ILC (Thompson et al., 2017). There was no significant correlation between the ages with the three markers as reported by previous studies (Ghebeh et al., 2006; Thompson et al., 2004). IHC technique is a valuable tool used in diagnostic pathology, tracking markers, and cancer research (Shojaeian et al., 2018). IHC is considered an integral technique in BC as it is used in classifying BC into subtypes that guide treatment decisions (Zaha, 2014). PD-L1expression is expressed diversely in tumour cells and it is more predominant among more aggressive tumours with a higher histological grade. IHC studies showed marked expression of PD-L1 in late-stage breast cancer (Figure 5 E and F) in contrast with (Figure 5A and B) a negative expression in the early-stages of the disease and mild expression in the moderate stage (Figure 5 C and D).

Results of this study are in accordance with many studies clarifying that PD-L1 expression was proven to be related to a variety of adverse features as higher grade and in agreement with Karink T et al. (Karnik et al., 2018), who used three different antibodies of PDL-1 in BC and obtain significant results indicating high expression of PDL-1 with disease stage. Also, higher level of CD8 was observed, compared to CD20 (Figure 4) and this is in agreement with a previous study (Tawfik et al., 2018).

Most of the previous studies of BC are concerned with prognosis and follow up, but this study is concerned with early diagnosis of the disease, where it can be easily treated. Patients were identified with high PDL-1 could benefit from cancer immunotherapy. This was in accordance with previous reports. They proved that the PD-1/PD-L1 pathway is involved in breast cancer subtypes such as relapse and metastasis (Wang et al., 2007; Charo et al., 2005; Mittendorf et al., 2014). They showed a strong correlation between PD-L1 expression and the presence of a pre-existing immune-active microenvironment that was inhibited by PD-L and firmly was supported by the use of anti-PD-1/PD-L1 therapy in BC patients (Herbst et al., 2014; Arias-Pulido et al., 2018a). Also, there was 100% agreement for expression of PD-L1 not only in tumour cells but also in tumour-infiltrating immune cells (Tawfik et al., 2018). The final finding was the potential benefits of assessing PD-L1 expression as a predictive marker in breast cancer immunotherapy (Baptista et al., 2016; Soliman et al., 2014). The reported results were consistent with the results of this study. There were some limitations in this study such as small size of sample.

The findings showed that using Programmed death-ligand 1(PDL-1) gene expression as an early prediction tool of breast cancer (BC) diagnosis was supported by Jiang et al. (2019) who indicated a significant difference for PD-1/PD-L1 expression levels among three molecular subtypes. They were correlated with clinical therapy and cleared implication of PD-1/PD-L1 in clinical diagnosis. The findings added by Stovgaard et al. (2019) who detected real variation for gPD-L1 expression levels with high correlation to prognosis both in the adjuvant and neoadjuvant chemotherapy. PD-L1, CD8 and CD20 protein expression levels as predictor for breast cancer was supported by Plaks (Plaks, 2018) who showed noticeable heterogeneity at single-cell level of PD-L1 expression in BC tumour cells against tumour cells using western markers at low cell numbers. Furthermore, the findings of this study were in agreement with the findings of Rom-Jurek et al. (2018) who explained that PD-L1 expression level was varied amongst breast cancer cell lines. Interestingly, cytotoxic treatments and other extrinsic parameters differentially affect the expression.

5. Conclusion

This study suggested that PDL-1, CD20, and CD8 were performed as predictive markers in the early diagnosis of breast cancer. PD-L1 expression is a promising biomarker and helpful to clinicians aiming to select the appropriate immunotherapy for breast cancer. Although this work provides analytic validity, larger future studies with more effort will be needed to prove clinical utility. Using qPCR and western blotting for evaluating PDL-1, CD20, and CD8 gene and protein expression levels indicated successful applying of these markers as early predictor tools for breast cancer diagnosis procedures.

Declarations

Author contribution statement

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The authors declare no conflict of interest.

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