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PD-1 Gene Expression as a Possible Diagnostic Biomarker for Breast Cancer

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Abstract

Programmed cell death protein-1(PD-1) is a cell surface protein that can regulate immune response by promoting self-tolerance by suppressing T-cell inflammatory activity. This study aimed to investigate the etiological role of *PD-1* gene expression as a biomarker for breast cancer detection and its relationship with clinical characteristics. Eighty peripheral blood samples (fifty blood samples were collected from breast cancer patients and thirty from healthy women as a control group) were randomly recruited to screen the *PD-1* gene expression using Quantitative Real Time Polymerase Chain reaction (RT-qPCR). The results showed that the *PD-1* gene had a fold change of 8.946 (indicating high expression) and was detected in 80% of patients with tumor grade II. The fold change in PD-1 gene expression ranged from 8 to 10 (high expression) across all tumor stages. 80% of patients were positive for estrogen receptor, with a fold change of 8.571 (high expression), compared to the *PD-1* gene control group, which showed a significant association ($P \leq 0.05$) with estrogen receptor status. *PD-1* gene expression showed a significantly elevated fold change (9.14, $p \leq 0.01$) in 64% and 68% of positive breast cancer patients for progesterone receptor and human epidermal growth factor receptor 2 (Her-2), respectively, when compared with the control, a highly significant association depending on the progesterone and human epidermal growth factor receptor 2 status. *PD-1* gene expression can serve as an important biomarker and therapeutic target in breast cancer.

Keywords: *PD-1* gene, Iraqi Breast Cancer, Hormone receptor status, Immune response

التعبير الجيني لجينPD1 كعلامة تشخيصية محتملة لسرطان الثدي

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الخلاصة

بروتين موت الخلية المبرمج −1 (*PD−1*) هو بروتين سطح الخلية الذي يمكنه تنظيم الاستجابة المناعية من خلال تعزيز تحمل الذات عن طريق قمع النشاط الالتهابي للخلايا التائية. هدفت هذه الدراسة للتحري عن دور التعبير الجيني لجين *PD−1*كمؤشر حيوي للكشف عن سرطان الثدي وعلاقته بالخصائص السريرية. تضمنت الدراسة جمع ثمانين عينة دم محيطية (50 من إناث سرطان الثدي و 30 من نساء سليمات كمجموعة تحكم) بشكل عشوائي لفحص تعبير جين *PD−1* باستخدام تقنية الوقت الحقيقي لتفاعل البلمرة المتسلسل الكمي qRT−PCR. أظهرت النتائج أن التعبير العالي (8.946) لجين *PD−1* وجد في 80٪ من المرضى المصابين بالدرجة الثانية من الورم. تراوح مدى التعبير الجيني لجين *PD−1* بين (8 −10) في جميع مراحل مرضى الورم، و 80٪ من المرضى كانوا حاملين مستقبلات هرمون الاستروجين الإيجابية الذين لديهم تعبير عالي لجين *PD−1* (8.571) مما يشير إلى ارتباط معنوي كبير ($P \geq 0.05$) بحالة مستقبلات هرمون الاستروجين. تضاعف التعبير لجين *PD−*1 كان مرتفعًا (9.14) عند مستوى احتمالية $p \geq 0.01$ مع ارتباط معنوي عالي في 64% و68% من مرضى سرطان الثدي الحاملين لمستقبلات البروجسترون و عامل نمو البشرة البشري 2 الموجبة على التوالي بالمقارنة مع مجموعة السيطرة مع وجود ارتباط معنوي كبير اعتمادًا على حالة هرموني البروجسترون ومستقبل عامل نمو البشرة البشري 2. يمكن استخدام التعبير عن جين *PD−1* كعلامة حيوية مهمة وهدف علاجي لعلاج سرطان الثدي.

1. Introduction:

Breast cancer is the most common cancer in women of all races and ethnicities, with around 2.3 million new cases every year. It represents one in eight cancer cases in both females and males and a quarter of all cancers in females, with 70% mortality occurring in the poor population, according to the published report by the World Health Organization [1]. Breast carcinoma is regarded as the highest rate among the population in Iraq [2]. More than 40% of breast cancer cases in Iraq are discovered in middle-aged women with advanced stages of the disease [3-5].

The activating of self-tolerance process via inhibiting of T-cell inflammatory activity is carried by a cell surface receptor protein called programmed cell death-1 (PD-1) and prevents autoimmune diseases, but it can also prevent the immune response from fighting cancer cells, the PD1 protein also called cluster of differentiation 279 (CD279) acts as a regulator of the immune response [6], and act as a checkpoint through the first mechanism, it promotes apoptosis of antigen-specific T-cell (Ag- specific T cell) in lymph nodes, the other mechanism is reducing the apoptosis in regulatory T-cells (anti-inflammatory and suppressive T-cells) [7].

The *PDCD-1* gene encodes PD-1 protein. PD-1 is a cell-surface receptor belonging to the immunoglobulin family; it is expressed on T cells and pro-B cells. Two ligands bind to PD-1 (PD-L1 and PD-L2). The PD-1 protein acts as a negative regulator of the immune response, and it's composed of 288 amino acids. It is a member of the T-cell regulators family (CD28/CTLA-4) [10]. PD-1/PD-L1 binding inhibits the effector functions of CD8+ T cells during viral infection, such as activation and expansion. The inhibition caused by the binding of the receptor with its ligand, so the intracellular tail, which contains two phosphorylation sites located in an immunoreceptor tyrosine-based inhibitory motif, suggests that *PD-1* negatively regulates T-cell receptor TCR signals [8,9]. Another study provides evidence that

both human PD-1 and B and T lymphocyte attenuator (BTLA) bind to the Src homology-2 protein tyrosine phosphatase (SHP), also known as nonreceptor tyrosine phosphatases (PTP) SHP1 and SHP2, in a bivalently, parallel mode, involving both SH2 domains of SHP1 or SHP2 [10].

Inhibition of antitumor activity can be achieved through PD-L1 expression on tumor cells, which binds to PD-1 on effector T cells [11], thereby mediating tolerance and providing immune homeostasis, leading to reduced survival in patients with many types of cancer [12]. Upregulation of *PD-1* upon monocyte activation, causing interaction with its ligand PD-L1, induces the production of IL-10, which inhibits CD4 T cell function [13].
Overexpression of *PD-1* on the cell surface of CD8+ T cells indicates T-cell exhaustion (e.g., in chronic infection or cancer). Increasing *PD-L1* gene expression has been reported in several cancers; thus, the role of *PD-1* in cancer-related immune evasion is well established [14]. This study aimed to demonstrate the etiological role of the *PD-1* gene in Iraqi breast cancer women and its relation with clinical characteristics by investigation of *PD-1*gene expression using the qPCR technique.

2. Materials and methods:

2.1 Patients' blood samples

The study involved eighty blood samples, including fifty samples collected from Iraqi female breast cancer patients who underwent treatment at the Medicine City Hospital in Baghdad and private laboratories from February to October 2023. Thirty blood samples were collected from healthy female donors as a control. All patients undergoing chemotherapy or radiotherapy were excluded. The research was conducted in accordance with the Ethics Committee of the Ministry of Health and Environment in Iraq and the University of Baghdad. Participation in this study was voluntary, and informed consent was obtained from all patients. Data for the Tumor Node Metastasis (TNM) staging system for breast cancer, classification, and hormonal status for breast cancer females were collected from patients' archives who participated in this study. Five ml of peripheral blood was collected from each patient in EDTA-containing tubes, and the plasma was separated and stored at -20 °C.

2.2. PD-1 gene expression analyses

Total cellular RNA was isolated from blood samples using TRIzol Reagent (Thermo Scientific, USA). The Quantus Fluorometer was used to detect the concentration of extracted RNA, thereby assessing the quality of the samples for qRT-PCR. For one µl of RNA, 199 µl of diluted QuantyFlour Dye was mixed. After 5 min of incubation at room temperature in the dark, RNA concentrations were measured. The RNA concentration in the samples ranges from 18 to 29 ng/µL. Based on the initial measurements, all samples were standardized to a final concentration of 1.8 ng/µl by dilution in nuclease-free water, and 1 µl of this template was used in the subsequent reaction. One-Step RT-qPCR, as described by Ali and Mahood [15], was used in this study; the total volume of reaction was 10 µl, containing (5 µl qPCR Master Mix, 0.25 x RT mix, 0.25 $MgCl_2$, and 0.5 µM for each (forward and reverse) primer, 2.5 µl nuclease-free water, and 1 µl of RNA. Detection of *PD-1*gene expression level carried by RT-qPCR technique using (Mic qPCR Cycler, Bio Molecular System, Australia). The *GAPDH* gene was used as a housekeeping gene. Table 1 shows that the primer pairs were used to detect *PD-1* expression levels using a (MicqPCR Cycler, BioMolecular System, Australia) for the qRT-PCR technique. The Glyceraldehyde phosphate dehydrogenase (GAPDH) was used (as an internal control) [16]. Primers were ordered from Macrogen (Korea). The qRT-PCR was done with the following program: the first cycle included (reverse transcriptase enzyme activation at 37 °C for 15 min, initial denaturation at 95°C for 10 min), followed by 40 cycles of 95 °C for 20 sec, 60 °C for 20 sec, and 72°C for 20 sec.

Relative quantification data for *PD-1* gene expression in blood samples collected from breast cancer patients compared to the control group were calculated using the Pfaffl method (2^ΔΔCt). (fold change $2^{-\Delta\Delta Ct}$), ΔΔCT =ΔCT Treated - ΔCT Control, ΔCT =CT gene - CT House Keeping gene.

Table 1: Primer sequences used for *PD-1* gene expression

Primer Name	Sequences (5`→3`)	References
PD1-F	5`-TAGAGAAGTTTCAGGGAAGG-3`	Ghorbani *et al*., [16]
PD1-R	5`-ATGTGTAAAGGTGGAGGG-3`	
GAPDH- F	5`-GAAGGTGAAGGTCGGAGTC- 3`	Rostkowska-Nadolska *et al*., [17]
GAPDH-R	5`-GAAGATGGTGATGGGATTC-3`	

2.3. Statistical Analysis:

The statistical analysis was achieved by the Statistical Analysis System- SAS [18], for detecting differences in the studied parameter factors. The LSD test was applied to compare the means of characteristic patients. The Chi-square test was applied for comparisons between percentages of groups; the *p*-values ($p \leq 0.05$ and $p \leq 0.01$) were considered significant.

3. Results:

3.1 Characteristics of Patients

Table 2 refers to the clinical characteristics of female breast cancer patients in the current study. The mean age was 40.7 years with a standard deviation (SD) of 0.85 and 35.86 years with an SD of 0.95 years for the case and control groups, respectively. out of 50 patient samples, 38 (76%) were older than fifty years, and 12 (24%) were equal to or less than fifty years. The age range was (30-70) years, with a significant association ($p \leq 0.01$) with breast cancer.

A high rate of 92% (46/50) of patients who participated in this study had invasive ductal carcinoma (IDC), referring to a highly significant association of breast cancer with IDC tumor type, with $p \leq 0.01$. The clinical evaluation of cancer involves determining its grade, which refers to the degree of cellular atypia observed microscopically and its stage, which defines the anatomical progression of the disease

The results of this study depend on the grade shown; most of the patients (80%) were diagnosed with grade II of breast cancer. A highly significant difference ($P \leq 0.01$) was identified. Stage III was the most prevalent stage among all patients, observed in 36% (18/50).

Table 2: Clinical characteristics of females with breast malignancies

Characteristics	No.	%	Chi-Square	*P*- value
Total No.	50	100		
Age > 50	38	76	12.68 **	0.0001
Age ≤ 50	12	24		
Tumor Type				
IDC	46	92	14.27 **	0.0001
ILC	4	8		
Grade				
I	2	4	12.94 **	0.0001
II	40	80		
III	8	16		
Stage				
IA,IB	8	16	7.16 **	0.0084
IIA,IIB	14	28		
IIIA,IIIC	18	36		
IV	10	20		
TNM				
1	16	32	11.41 **	0.0001
2	24	48		
3	8	16		
4	2	4		
LNM				
LNM +ve	40	80	13.00 **	0.0001
LNM –ve	10	20		

Invasive Lobular Carcinoma **(ILC)**, Invasive Ductal Carcinoma **(IDC)**, Tumor Node Metastasis **(TNM)**, Lymph Node Metastasis **(LNM)**. Highly Significant. ** (P≤0.01),

The TNM staging system is a method for classifying the anatomical extent of cancer's spread. The three key components of this global system are: T refers to Tumor, N refers to Nodes, and M (Metastasis). Depending on TNM staging, 80% (40/50) of patients had positive lymph nodes, and 20% (10/50) were negative.

Hormonal status showed positive status for estrogen (ER) and progesterone (PR) hormones in 64% of women with breast cancer. A significant proportion of patients (72%) exhibited negative HER2 protein status; a highly significant relation ($p \leq 0.01$) was seen for the hormones, as shown in Table 3.

Table 3:Hormonal status of patients with breast malignancies

Characteristics	No.	%	Chi-Square	P- value
Estrogen (ER)				
ER +ve	32	64	9.31 **	0.0037
ER –ve	18	36		
Progesterone(PR)				
PR +ve	32	64	9.31 **	0.0037
PR –ve	18	36		
Her-2				
Her-2 +ve	14	28	12.28 **	0.0001
Her-2 -ve	36	72		
Highly Significant, ** ($P \leq 0.01$).				

Estrogen: ER, Progesterone: PR, Human epidermal receptor: Her-2

3.2. The relation of PD-1 expression level with the clinical characteristics of breast cancer patients

The study aimed to investigate the expression levels of the *PD-1* gene in blood samples from patients with breast cancer compared to those from healthy individuals, serving as a control group. The qPCR products were pure and homogeneous, as indicated by the melting curves of the *PD-1* gene, which showed sharply defined peaks (Figure 1). High fold change expression of the *PD-1* gene (12.8) in breast cancer patients compared to 5.7 in the control group (Figure 2).

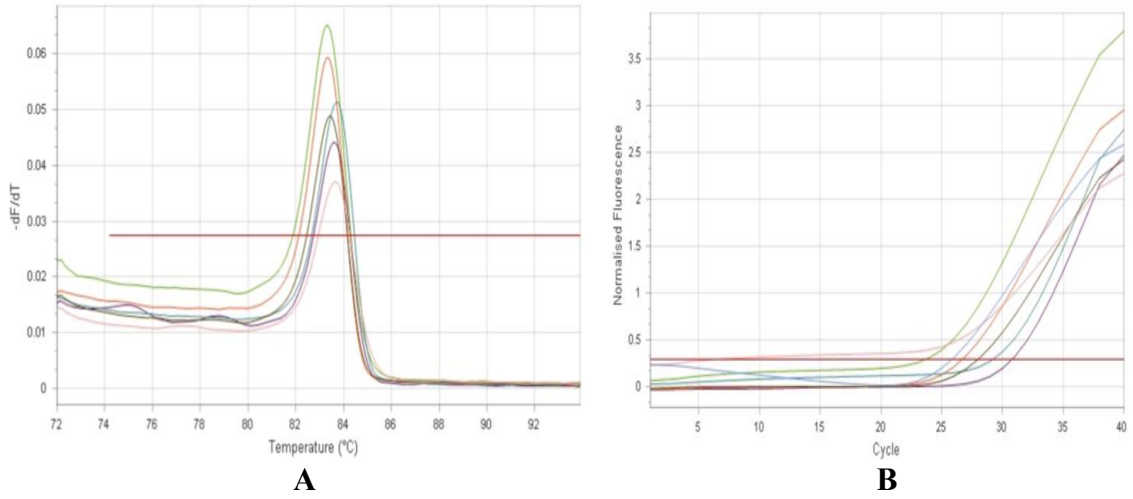


Figure 1: A: Melting curves B: temperature curve of qPCR amplification for *PD-1* gene.

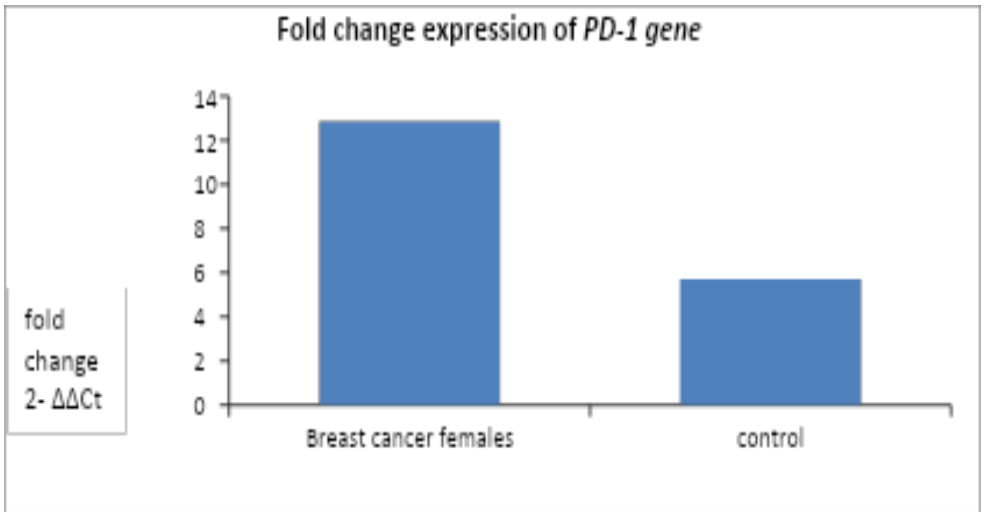


Figure 2: Fold change in *PD-1* gene expression in breast cancer females compared to the control group.

The results in Table 4 indicate the fold change in *PD-1* gene expression in breast cancer patients and its relationship with clinical characteristics. An average *PD-1* fold change of 8.098 was observed in 76% of patients under 50 years, and 14.99 in 24% of patients over 50 years. A highly significant difference ($p \leq 0.01$) was found in PD-1 fold expression levels by age compared with control samples (5.714). Regarding tumor type, the *PD-1* gene showed a 14.48-fold increase in 92% (46/50) of IDC patients and a 5.061-fold increase in 8% (4/50) of invasive lobular carcinoma ductal patients (ILC) compared to the fold change in the control samples. A highly significant difference ($p \leq 0.01$) has been observed depending on tumor type. A high fold change expression of *PD-1* 8.946 was found in 80 %

Table 4: The relation *PD-1* expression level to clinical characteristics of breast cancer patients

Characteristics	No.	%	Relative fold change expression of the *PD-1* gene	LSD value	*P*- value
Age					
< 5o	38	76	8.098	2.074 **	0.0026
≥50	12	24	14.99		
Tumor type					
IDC	46	92	14.48	2.821 **	0.0001
ILC	4	8	5.061		
Grade					
I	2	4			
II	40	80	8.946	1.945**	0.0001
III	8	16	5.577		
Stage					
IA,IB	8	16	8.002	1.673 **	0.0093
IIA,IIB	14	28	8.642		
IIIA,IIIC	18	36	7.81		
IV	10	20	10.01		
TNM					
1	16	32	11.16	2.306 **	0.0001
2	24	48	8.477		
3	10	20			
LNM					
LNM +ve	40	80	8.692	1.873 **	0.0001
LNM -ve	10	20	4.849		
Control	30	100	5.714	--	--

Invasive Lobular Carcinoma **(ILC**), Invasive Ductal Carcinoma **(IDC)**, Tumor Node Metastasis (**TNM)**, Lymph Node Metastasis **(LNM)**. Highly significant, ** (P≤0.01).

(40/50) of patients with tumor grade II compared to a fold change of 5.57 in patients with tumor grade III. The PD-1 gene showed 8-10-fold expression in all tumor stages (high expression). Other features, such as tumor stage, TNM, tumor size, and LNM, are summarized in Table 4.

3.3. The relation of PD-1gene expression level to the hormonal status of women with breast cancer

The current results demonstrated that a majority of patients (64%, 32/50) were estrogen receptor-positive and exhibited an 8.57-fold increase in *PD-1* gene expression (high expression), indicating a significant association ($P \leq 0.05$) of *PD-1* gene expression with estrogen status. Folding expression of *PD-1* gene level was elevated 9.145 and 9.27 in 64% (32/50) and 68 % (34/50) of progesterone and Her-2 positive breast cancer patients, respectively, (high gene expression) when compared with the control group, a highly significant association of *PD-1* gene ($P \leq 0.01$) depending on the progesterone and Her-2/status (Table 5).

Table 5: The relation of *PD-1* gene expression level to the hormonal status of breast cancer patients

Hormone *P* – value	No.	%	Relative fold change expression of the *PD-1* gene	LSD value	*P*-Vale
Estrogen(ER)					
ER -ve	18	36	6.726	1.276 *	0.0296
ER +ve	32	64	8.571		
Progesterone(PR)					
PR-ve	18	36	6.279	1.861 **	0.0063
PR +ve	32	64	9.145		
Her-2(H)					
H-ve	16	32	5.682	2.074 **	0.0007
H+ve	34	68	9.272		
Control	30	100	5.714	---	---
Significant, * (P≤0.05). Highly Significant, ** (P≤0.01).					

4. Discussion

In the current study, the fold change in *PD-1* gene expression was investigated using qRT-PCR in peripheral blood samples from Iraqi patients with breast cancer, and its relation to clinical parameters was assessed. The results showed an increase in breast cancer risk as women aged, about 80% of breast cancer patients were diagnosed at 45 years of age or older, and about 43% of patients were between 40 and 50 years. The two-hit hypothesis of carcinogenesis may explain the findings of this study. This theory suggests that while an individual may inherit or acquire a single functional defect in one allele of a tumor suppressor gene, malignancy progresses only when the second allele is inactivated[19]. Our data suggests that environmental stressors and lifestyle factors—such as pollutants and smoking—may have served as the 'second hit' in this cohort. This biallelic inactivation could explain the significant molecular dysregulation observed, specifically the 8.571-fold increase in PD-1 expression, which reflects a breakdown in cellular immune checkpoints.

Regarding the tumor stage, stage III was predominant, possibly influenced by hormonal status. Sixty-four % of patients were positive for ER and PR, while 70% of patients were known to be positive for HER-2. Some breast cancer develops due to hormonal problems, including the ER+PR+ receptor, which induces an uncontrolled growth of breast cells. Hormone receptor positivity accounts for approximately two-thirds of all breast cancer cases [20].

PD-1 gene expression and its association with several clinical-pathological parameters were studied to determine its etiological role in breast cancer pathogenesis. The results revealed that all clinical features showed an upregulation of *PD-1* gene fold expression, ranging from 8-14 in patients compared to controls, with a highly significant ($P \leq 0.01$) fold change in PD-1 expression dependent on characteristics, such as age, IDC, grade II, stage II, T3NM, and LNM of the tumor. This finding is consistent with the studies performed by [21,22]. *PD-1,* an immunosuppressive molecule, is expressed in peripheral blood T cells and is linked to the prognosis of patients, including those with invasive ductal carcinoma [23,24].

The current study suggests that *PD-1* gene expression was higher in patients with more advanced breast cancer, with fold changes of 8.9-10, depending on grade and stage of breast cancer, respectively. The current findings are consistent with previous results published by Sun

et al.,[25]. They suggest that the prognostic value of *PD-1*(+) immune cell subsets correlate with shorter patient survival. They conclude that targeting the PD-1 pathway could be a practical approach to treating breast cancer patients.

Another study proposes that *PD-1* gene expression in the peripheral blood T-cell population can serve as a new prognostic marker in solid tumors [26]. After tumor antigens activate T cells, the *PD-1* gene is highly expressed, leading to T-cell exhaustion and inhibiting their antitumor activity. The *PD1/ PD-L1* expression was detected in 465 Chinese patients with triple-negative breast cancer (TNBC). Additionally, the prognostic effect of PD-L1 / TILs in 465 Chinese patients with triple-negative breast cancer (TNBC) was investigated; the results show that the expression status of pre-treatment TILs/PD-L1 represents an important tool to optimize the selection of patients with TNBC for immunotherapy and management of disease [27].

The results showed a high *PD-1* change in folding gene expression depending on positive hormonal status, with values of 8.5, 9.14, and 9.27 for ER, PR, and Her-2, respectively, compared with the 5.7 in the control group, exhibiting a highly significant correlation ($P \leq 0.01$). Our results are inconsistent with Zawlik *et al.,*[28], who found that TNBC points to a trend towards the significance of higher expression of *PD-1* and *PD-L1*; a higher level of *PD-L1* expression is also found in aggressive breast cancer subtypes (triple-negative and HER2). High levels of ER decrease the expression of *PD-1/PD-L1* and CD8+ T cell infiltration by inhibiting the IL-17 signal transduction and Th17 cell infiltration in breast cancer females [39].

This finding confirmed the role of the PD-1 protein in cancer progression and decreased antitumor activity. The overexpression of PD-1 on CD8+ T cells and PD-L1 and PD-1 on TNBC cells may benefit from targeted immune therapies to a greater degree than other subtypes of patients [30]. Other reports suggest that detection of *PD-1* gene expression may serve as an early prognostic factor for breast cancer [31]. However, the limitation of the current study is the small number of patients; therefore, further studies with larger patient numbers are needed to confirm the present results about the prevalence of *PD-1* gene expression in breast cancer patients and the possibility of its use as a biomarker for the diagnosis of breast cancer.

5. Conclusion

According to the present results, a high *PD-1* gene expression, especially in advanced-stage patients with (PR+ve and Her-2+ve) hormonal status in breast cancer, may be used as an important biomarker and therapeutic target for breast cancer; other studies are needed to confirm the current data.

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Competing interest

The authors declare that they have no conflicts of interest

Ethics approval and consent to participate

Authors attest to the approval of ethical considerations. The study received ethical clearance from the local ethical council at the University of Baghdad, and the study protocol was approved by the Ministry of Health / Cancer Council under their letter 659 dated 6/5/2019

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