RESEARCH ARTICLE

Editorial Process: Submission:09/28/2024 Acceptance:01/16/2025

Immunohistochemical Expression of *PD-L1* and *CTLA-4* in Triple Negative Breast Cancer and Their Prognostic Associations

Nehal Moaz Mehrez[1]*, Dina Abdallah Ibrahim[1], Maha Esmail El-Zaafarany[2], Dina A El-Tantawy[1], Azza Abdel-Aziz[1]

Abstract

Objective: Programmed Death-Ligand 1 (*PD-L1*) and Cytotoxic T Lymphocyte -Associated Antigen-4 (*CTLA-4*) are presently considered as prognostic markers and therapeutic targets in numerous human malignancies. The goal of this study was to determine whether *PD-L1* and *CTLA-4* might be used to predict patients' survival in Triple Negative Breast Cancer (TNBC). **Methods:** This retrospective cohort study analyzed 100 primary TNBC cases that had surgical resection at the Oncology Center of Mansoura University (OCMU), Faculty of Medicine, Egypt. Clinicopathological data and survival outcomes were collected, and immunohistochemistry (IHC) was performed for *PD-L1* and *CTLA-4* expression. **Result:** In 29% of TNBCs, *PD-L1* was expressed. *PD-L1* positivity was significantly associated with high tumor grade (P=0.007). *PD-L1* did not, however, significantly associate with survival. *CTLA-4* was expressed in 45% of TNBCs. *CTLA-4* expression was significantly associated with lymph node metastasis (P=0.009), distant metastasis (P=0.001) and advanced TNM stage (P=0.001). In TNBC, multivariate analysis identified *CTLA-4* expression as an independent prognostic predictor for both disease-free survival (P=0.002) and overall survival (P=0.003). **Conclusion:** The selection of patients for immunotherapy and checkpoint-blockade treatment may be guided by *CTLA-4*, an independent prognostic factor for the overall survival and disease-free survival of TNBC patients.

Keywords: *PD-L1*- *CTLA-4*- TNBC- Survival- Immunotherapy

Asian Pac J Cancer Prev, **26 (1)**, 319-326

Introduction

Breast cancer is the most common malignancy in females and is the most often diagnosed cancer in the great majority of countries [1]. In 2020, an estimated 2.3 million female breast cancer cases were diagnosed worldwide, and around 685,000 females died because of the disease [2].

About 15–20% of all cases of breast cancer are Triple Negative Breast Cancer (TNBC), which is characterized by the lack of Estrogen Receptor (ER), Progesterone Receptor (PR), and Human Epidermal Growth Factor Receptor 2 (HER2) expression by immunohistochemical (IHC) analysis [3]. Because of the absence of *HER2* expression and targetable hormone receptors, TNBC is often accompanied by a poor prognosis and a restricted selection of treatment routes [4]. These features highlight the significance of studying the pathogenesis and available treatments for TNBC.

Breast cancer was not formerly considered to be immunogenic. Nonetheless, in comparison with other subtypes of breast cancer, TNBC is associated with greater immune cell activity and abundance, which suggests that immunotherapy more particularly, the use of immune checkpoint inhibitors (ICIs) treatment may be beneficial [5].

Immune checkpoints, that are co-stimulators or co-inhibitors, firmly regulate the activation of T-cells [6]. Immune checkpoints frequently implicated in cancer pathogenesis are Programmed Death Protein 1 (*PD-1*), Programmed Death-Ligand 1 (*PD-L1*) and Cytotoxic T Lymphocyte -Associated Antigen-4 (*CTLA-4*) [7].

The reduction of cytotoxic T-cell activity and proliferation is the outcome of the interaction between *PD-1* and *PD-L1*, which also encourages the differentiation of regulatory T-cells and contributes to immune cell dysfunction [8]. T-cell immunological responses are inhibited by *CTLA-4*, an immunosuppressive cytokine belonging to the immunoglobulin superfamily [9]. Tumor cells in cancer patients have the ability to evade immune surveillance, takeover certain immunological checkpoint

*[1]Department of Pathology, Faculty of Medicine, Mansoura University, Mansoura, Egypt. [2]Medical Oncology Unit, Internal Medicine Department, Professor of Medical Oncology, Faculty of Medicine, Mansoura University, Mansoura, Egypt. *For Correspondence: dr_nehalmoaz@hotmail.co.uk*

pathways, and withstand the cytotoxic effects of host T cells [10]. Consequently, ICIs fortify the immune system's anti-tumor reactions and augment its capacity to eradicate tumor cells [11]. Therefore, immunotherapy continues to develop its applications in various solid tumors and represents a promising treatment strategy for TNBC [12].

This study aimed to evaluate the immunohistochemical expression of *CTLA-4* and *PD-L1* in TNBC tissue samples and explore the association between these markers and other clinicopathological variables as well as patient outcomes.

Materials and Methods

One hundred formalin-fixed, paraffin-embedded (FFPE) tissue blocks from TNBC patients were examined in this retrospective cohort research. The patients were diagnosed at the Pathology Laboratory of the Oncology Centre at Mansoura University (OCMU), Faculty of Medicine, Mansoura University, Egypt, between January 2015 and December 2020 and had not received preoperative chemotherapy nor radiation. Clinicopathological data for these 100 cases were collected retrospectively from the OCMU's pathology database. The data included patient age, tumor size, nodal involvement, local recurrence, distant metastasis, and tumor stage, in accordance with the American Joint Committee on Cancer's (AJCC) revised eighth version [13]. The histological type and grade of the tumor were identified by reviewing slides stained with hematoxylin and eosin (H&E).

The patients' follow-up periods ranged from 2 to 107 months, with a median of 46 months. Data on follow-up were gathered from patient health records and through interviews conducted by phone with either the patients or their relatives. The length of follow-up in months, the presence or absence of recurrence (whether local recurrence or distant metastasis), disease-related mortality, disease-free survival (DFS), which was determined from the date of surgery to documented relapse, and overall survival (OS), which was defined as the interval between surgery and the last follow-up or disease-specific death, were among the important follow-up data.

Tissue Microarray Construction

The tissue microarray blocks (TMA) were made manually using a validated technique [14]. Five TMA blocks were produced, each containing three representative cores from the 100 cases under study. Additionally, multiple cores from normal tissues (such as the appendix, gallbladder, placenta, and tonsil) were incorporated into each block based on a pre-designed map. These normal tissue cores served as orientation markers and as negative and positive controls for the immunohistochemistry (IHC) staining markers, with tonsil tissue being the positive control for both *PD-L1* and *CTLA-4*.

Immunohistochemistry

According to the user's handbook standardized protocol preprogrammed into the autostainer software, IHC was carried out using Autostainer Link 48, utilizing its optimized reagents with pharmDx kits EnVisionTM FLEX Visualization Systems (Link code K8000) and EnVision FLEX Hematoxylin (Link code K8008). Using an ordinary light microscope, two examining pathologists independently and semi-quantitatively interpreted the IHC results. Each antibody was then scored according to its most suitable particular scoring technique or system. The combined positive score (CPS) was used for *PD-L1*, and the Anti-*PD-L1* (QR001) Rabbit Monoclonal primary antibody (Quartett, Berlin, Germany, 1:100, Ready to use) was used. To calculate CPS, divide the number of *PD-L1* positive tumor cells, lymphocytes and histiocytes by the total number of vital tumor cells, then multiply the result by 100. CPS $\geq$ 1 is considered positive [15]. Anti-*CTLA-4* (F8) Mouse Monoclonal antibody (Medaysis, San Francisco, USA, 1:100, Ready to use) was used and any cytoplasmic positivity either of neoplastic cells and/or tumor- infiltrating lymphocytes (TILs) was recorded for *CTLA-4*, and a cut-off value of 10% was used [16].

Statistical analysis

The statistical analyses were conducted using SPSS 25.0 (IBM Corporation, New York, USA). The Pearson chi-square ($\chi 2$) test was used to examine the association between *PD-L1* and *CTLA-4* expression and clinicopathological factors. When more than 20% of the cells had counts below 5, the Fisher Exact Test (FET) was used as a correction for the $\chi 2$ test. Kaplan-Meier curves were created to evaluate the relationship between *PD-L1* and *CTLA-4* and patient survival, and the log-rank test was used to compare the two groups statistically. In order to determine the factors impacting DFS and OS for the multivariate analysis, Cox regression analysis was performed, along with the computation of hazard ratios.

Ethical considerations

The Institutional Research Board (IRB) of the Mansoura University Faculty of Medicine in Egypt gave the study approval (Code Number: MDP.21.11.88, 2021). To preserve anonymity and secrecy, the pathology code numbers of the paraffin blocks were used in place of the patients' names. All procedures followed the current revision of the Helsinki Declaration of medical research involving human subjects [17]. The donor blocks were also put back into the archive for use in any upcoming research or patient-related projects.

Results

Along with the previously stated *PD-L1* and *CTLA-4* IHC assessment criteria, 29% of TNBCs were *PD-L1* positive and 71% were *PD-L1* negative (Figure 1), 45% of TNBCs were *CTLA-4* positive (Figure 2), and 55% were *CTLA-4* negative. As shown in Table 1, *PD-L1* demonstrated a significant association with tumor grade, at which grade 3 tumors showed higher expression of *PD-L1* (P=0.007). *PD-L1* expression and other clinico-pathological characteristics, such as patient age, tumor size, lymph node metastasis, distant metastasis, TNM stage, and local recurrence, were not shown to be associated, though. *CTLA-4* expression and axillary lymph node metastasis were significantly associated;

Table 1. Associations between the Expression of *PDL-1, CTLA-4*, and Different Clinicopathological Parameters

Clinicopathological parameters	*PDL-1* expression		Test of significance	*CTLA-4* expression		Test of significance
	Negative n=71 (%)	Positive n=29 (%)		Negative n=55 (%)	Positive n=45 (%)	
Age (years)			p=0.435			
≤ 50 years	33 (46.5)	11 (37.9)		24 (43.6)	20 (44.4)	p=0.935
> 50 years	38 (53.5)	18 (62.1)		31 (56.4)	25 (55.6)	
T stage			p=0.203			
T1	9 (12.7)	4 (13.8)		7 (12.7)	6 (13.3)	p=0.621
T2	52 (73.2)	18(62.1)		41 (74.5)	29 (64.4)	
T3	7 (9.9)	7 (24.1)		6 (10.9)	8 (17.8)	
T4	3(4.2)	0		1 (1.8)	2 (4.4)	
Lymph node metastasis (N)						
Negative (N0)	26 (36.6)	10 (34.5)	p=0.840	26 (47.3)	10 (22.2)	p=0.009*
Positive (N1,N2,N3)	45 (63.4)	19 (65.5)		29 (52.7)	35 (77.8)	
M stage (Metastasis)						
M0 (absent)	46 (64.8)	22 (75.9)	p=0.281	46 (83.6)	21 (46.7)	p=0.001*
M1 (present)	25 (35.2)	7 (24.1)		9 (16.4)	24 (53.3)	
TNM stage	6 (8.5)	0	P=0.211	5 (9.1)	1 (2.2)	p=0.001*
I II	26 (36.6)	14 (48.3)		31 (56.4)	9 (20)	
III	14 (19.7)	8 (27.6)		11 (20)	11 (24.4)	
IV	25 (35.2)	7 (24.1)		8 (14.5)	24 (53.3)	
Tumor grade						
2	27 (38.0)	3 (10.3)	p=0.007*	20 (36.4)	10 (22.2)	p=0.125
3	44 (62.0)	26 (89.7)		35 (63.6)	35 (77.8)	
Local recurrence						
Absent	59 (83.1)	21 (72.4)	p=0.225	45 (81.8)	35 (77.8)	p=0.615
Present	12 (16.9)	8 (27.6)		10 (18.2)	10 (22.2)	

P, Probability value; *, statistically significant (P<0.05); *PDL-1*, Programmed death-ligand 1; *CTLA-4*, cytotoxic T lymphocyte-associated antigen-4

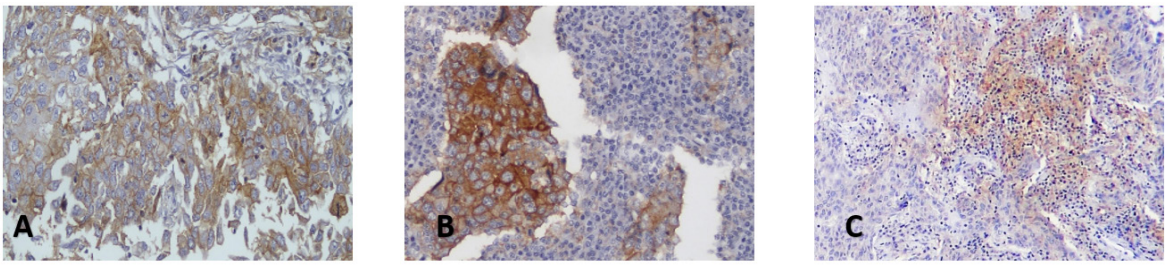

Figure 1. Immunohistochemical Staining of *PDL-1* in Various Cases of TNBC: *PD-L1* Membranous Staining in Tumor Cells (A). *PD-L1* Membranous Staining in Tumor Cells and Negative Staining in TILs (B). *PD-L1* Cytoplasmic Staining in TILs and Negative Staining in Tumor Cells (C), (DAB X200).

patients with positive lymph nodes had increased *CTLA-4* expression (P=0.009). The expression of *CTLA-4* was shown to be significantly positively associated with distant metastasis (P=0.001). Additionally, a significant association was found between the TNM stage and *CTLA-4* expression, with increased *CTLA-4* expression seen in stage III and IV tumors (P=0.001).While no observed significant associations between *CTLA-4* and other clinicopathological features.

The median period for DFS was 54 months (95% Confidence Interval [CI], 45-71.45). In 41% of cases, there was a disease relapse. Table 2 displays the results of univariate analysis, which showed that lower DFS periods were associated with younger patient age (P=0.027), nodal metastasis presence (P=0.001), distant metastasis presence (P<0.001), higher TNM stages (P<0.001), local recurrence (P=0.001), and *CTLA-4* expression (P=0.001) (Figure 3c). A statistically insignificant association (P=0.602) was discovered between *PD-L1* expression and DFS (Figure 3a). To determine the effect of the parameters strongly linked with DFS (from the univariate analysis) on the occurrence of an early recurrence in patients

Table 2. Univariate and Multivariate Survival Analysis of the Disease-Free Survival (DFS) and Overall Survival (OS) in triple negative breast cancer.

Clinicopathological parameters	Disease-free survival				Overall survival			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	Median DFS time (min-max) / months	Log-rank test	Hazard ratio (95% CI)	P value	Median OS time (min-max) per months	Log-rank test	Hazard ratio (95% CI)	P value
Age (years)	50(15.44-64.56)	P=0.027*	1	0.382	62(34.26-89.74)	P=0.105		
≤ 50 years	73.18(60.55-85.83)		1.45 (0.632-3.31)		32 (19.78-44.22)			
> 50 years								
T stage	56.9 (36.35-77.49)	P=0.076			84 (45.17-122.83)	P=0.03*	1	0.667
T1	66.99 (56.35-77.64)				42 (23.65-60.35)		1.24 (0.473-3.22)	0.159
T2	31.39 (20.87-41.92)				22(11-33)		2.22 (0.732-6.75)	0.297
T3	18.5 (15.56-21.44)				34 (0.821-78.81)		2.62 (0.429-15.96)	
T4								
Lymph node metastasis (N)	86 (74-97)	P=0.001*	1	0.001*	72 (72-72)	P=0.005*	1	0.821
Negative	31 (21.78-40.9)		3.82 (1.67-8.74)		34 (21.81-46.7)		1.17 (0.303-4.51)	
Positive								
M stage (Metastasis)	86.07 (76.71-95.43)	P <0.001*	1	0.001*	64.82 (54.55-75.1)	P=0.007*	1	0.003*
M0	24.81 (18.22-1.4)		12.3 (5.94-25.42)		40 (28.79-51.21)		2.16 (1.31-3.56)	
M1								
TNM stage	87 (71.4-104)	P <0.001*	1	0.381	89.2 (89.2-89.2)	P=0.001*	2.75 (0.279-26.99)	0.386
I	96.36 (88.18-104.55)		0.350 (0.03-3.66)	0.312	67.9 (67.9-67.9)		4.03 (0.356-45.64)	0.26
II	59.06 (35.81-82.31)		3.32 (0.325-33.80)	0.889	26 (11.06-40.94)		Undefined	0.868
III	22.39 (17.49-27.29)		Undefined		37 (24.80-49.19)		1	
IV								
Tumor grade	70.6 (55.12-86.08)	P=0.258			62 (10.52-113.48)	P=0. 228		
2	58.06 (47.01-69.11)				41 (30.88-51.11)			
3								
Local recurrence	71.25 (61.1681.33)	P=0.001*			41 (21.23-60.77)	P=0.581		
Absent	31.44 (21.24-41.65)				46 (3.76-88.25)			
Present								
PDL-1 expression	54 (54.0-65.0)	P=0.602			46 (25.98-66.02)	P=0.703		
Negative	60 (43.67-69.61)				42 (31.50-52.49)			
Positive								
CTLA-4 expression	76.19 (65.31-87.07)	P=0.001*	1	0.002*	62 (56.28-77.61)	P=0.002*	1	0.003*
Negative	34.68 (26.22-43.14)		2.80 (1.48-5.30)		34 (23.05-44.95)		2.17 (1.31-3.59)	
Positive								

P, Probability value; *, statistically significant (P<0.05); statistically highly significant (if P ≤ .001); *PDL-1*, Programmed death-ligand 1; *CTLA-4*, cytotoxic T lymphocyte-associated antigen-4

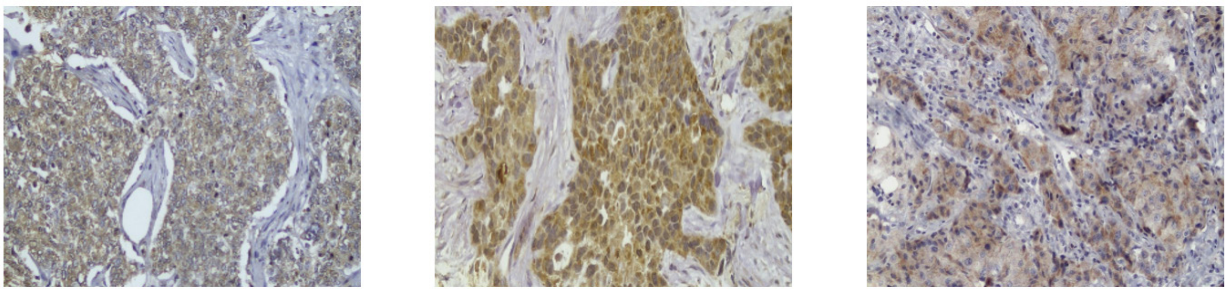

Figure 2. Immunohistochemical Cytoplasmic Staining of *CTLA-4* in Tumor Cells in Various Cases of TNBC (DAB x200).

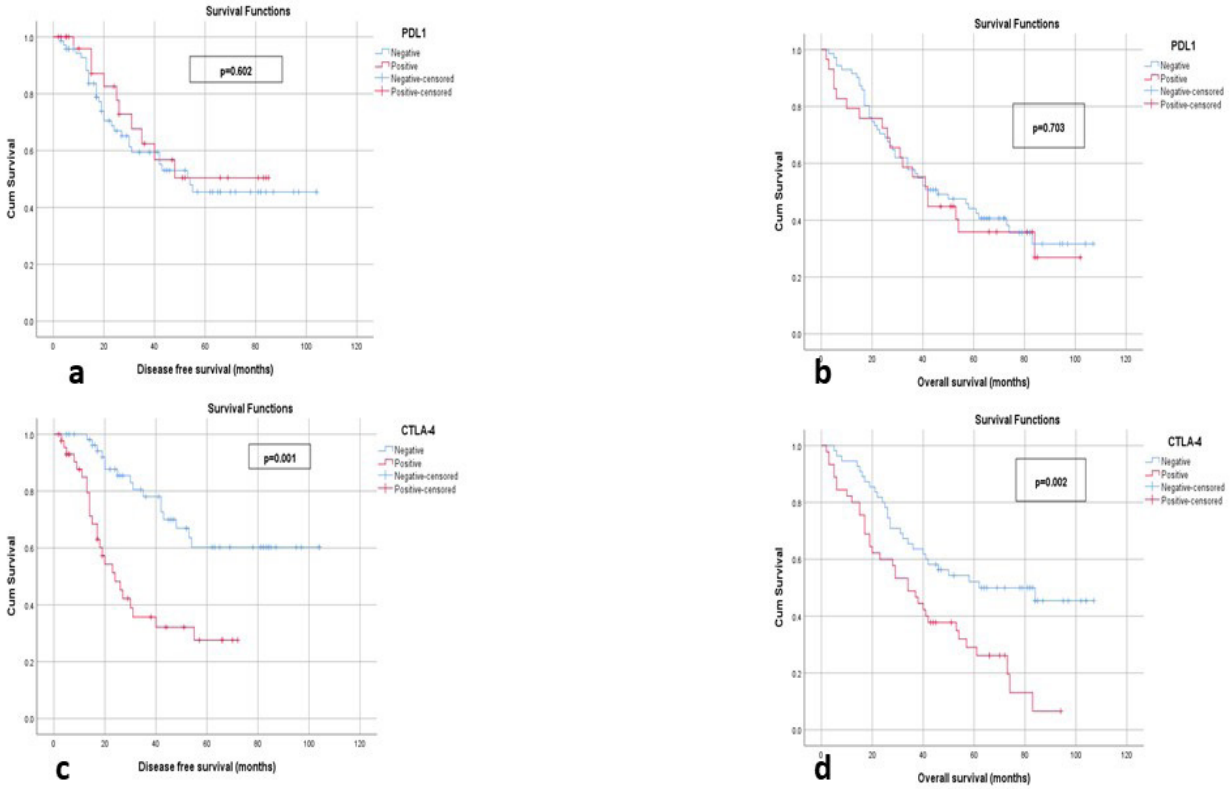

Figure 3. Kaplan-Meir Survival Curves for patients with triple negative breast cancer stratified by *PDL-1* expression and *CTLA-4* expression. No statistically significant associations between *PDL-1* and neither DFS (a; log rank; P=0.602) nor OS (b; log rank; P=0.703). Significantly lower DFS (c; log-rank; p= 0.001) and OS (d; log-rank; p=0.002) in patients with positive expression of *CTLA-4* compared to patients with negative expression for *CTLA-4*.

with TNBC, a multivariate Cox regression analysis was performed.

The aforementioned analysis revealed that the existence of lymph node metastasis was an independent predictor of a reduced DFS (Hazard Ratio [HR] = 3.82, 95.0% CI: from 1.67 to 8.74 with P=0.001). Also, it was shown that distant metastasis was recognized as an independent prognostic factor for reduced DFS (HR = 12.3 with 95.0% CI ranged from 5.94 to 25.42 with P=0.001). Additionally, it was found that positive expression of *CTLA-4* was considered as an independent predictor for lower DFS (HR = 2.80 with 95.0% CI ranged from 1.48 to 5.30 with P=0.002).

The median OS of TNBC patients in this study was 42 (95% CI, 27.34-56.66) months. During the follow-up period of this study, 52% of patients died due to disease-related factors. Univariate survival analysis, as shown in Table 2, revealed significant associations between shorter OS and factors like larger tumor size (P=0.03), nodal metastasis presence (P=0.005), distant metastasis presence (P=0.007), higher TNM stages (P=0.001), and *CTLA-4* expression (P=0.002) (Figure 3d). On the other hand, *PD-L1* expression and OS did not significantly associate (P=0.703) (Figure 3b). The results of multivariate analysis showed that in TNBC patients, *CTLA-4* positivity (P=0.003) and distant metastasis (P=0.003) were both independent predictors of shorter overall survival.

Discussion

Immune checkpoint blockade is rapidly gaining recognition as a leading strategy in tumor treatment.

Varity of cancer types, such as melanoma, bladder, lung, kidney, head, and neck cancers, have shown significant advantages from checkpoint blocking. These advantages in breast cancer, however, are still debatable [10]. The most widely accepted forms of immunotherapy target the *PD-1* receptor, its ligand *PD-L1*, and *CTLA-4* [18]. Consequently, in 100 TNBC patients, this cohort research sought to evaluate the expression of *CTLA-4* and *PD-L1* and investigate any possible associations with prognostic variables and survival outcomes. The frequency of *PD-L1* expression in the studied cases was 29%; that is close to Zhu et al. (2019), who reported *PD-L1* expression in 22% of their studied cases [19]. However, studies by Qin et al. [20] and Sukumar et al. [21] reported higher rates of *PD-L1* expression (61.5% & 80% respectively). Additionally, this study revealed *CTLA-4* positive expression in 45% of TNBCs. This finding is close to that of Kassardjian et al. (2018), who reported overexpression of *CTLA-4* in >50% of their studied cases [22]. On the contrary, Cabioglu et al. [23] and Vardas et al. [24] reported positive expression of *CTLA-4* in 82% and 36% of their cases, respectively. These discrepancies in *PD-L1* and *CTLA-4* expression can be linked to variations in the interpretation of staining patterns, the methodologies used for scoring, the cutoff values established, and the diverse monoclonal antibodies employed by the researchers.

Our study demonstrated a significant association between *PD-L1* expression and tumor grade, which is in line with findings published by Qin et al. [25]. Nevertheless, we were unable to find any associations between *PD-L1* expression and other clinico-pathological characteristics, aligning with the results observed by Constantinou et al. [26] and Botti et al. [27] studies. In other studies, many clinicopathological variables were significantly associated with *PD-L1* expression, such as larger tumor size, the distant metastasis, and the nodal metastasis [19, 25, 28].

Regarding *CTLA-4* expression, this study has revealed statistically significant associations between *CTLA-4* and both axillary lymph node metastasis and advanced tumor stage. These results align with those from several other studies that also reported such significant associations [29, 30]. Additionally, we identified a statistically significant association between *CTLA-4* and distant metastasis (P=0.001). Furthermore, research conducted by Abbasov et al. [31] demonstrated a statistically significant association between *CTLA-4* and T stage, indicating that tumors classified as T3 and T4 exhibited higher levels of *CTLA-4* expression. Conversely, a study by Lan et al. [32] indicated that in patients with breast cancer, neither interstitial *CTLA-4* nor tumor *CTLA-4* expression was linked to any clinical parameters.

With respect to the relationship between *PD-L1* IHC expression and patient outcomes, our study found no association between *PD-L1* expression and either DFS or OS in TNBC patients. These results are largely in line with the findings published by Cabioglu et al.[23] and Constantinou et al. [26]. However, several other studies have indicated a statistically significant association between *PD-L1* expression and both reduced DFS and poorer OS [25, 33]. In contrast, research by Parvathareddy et al. [28] found a statistically significant association between *PD-L1* expression and both longer DFS and improved OS.

About the association between patient outcomes and *CTLA-4* IHC expression, our study identified a statistically significant relationship between *CTLA-4* expression and both reduced DFS and poor OS in the univariate analysis. Additionally, multivariate Cox regression analysis indicated that positive *CTLA-4* expression was an independent prognostic factor for lower DFS and poor OS in TNBC patients. These findings align with the research conducted by Stovgaard et al. [18], which reported similar associations. Supporting our observations, studies by Yu et al. [34] and Lu et al. [35] found that higher *CTLA-4* expression was linked to lower overall survival rates among individuals with breast cancer. However, conflicting data from studies by Fang et al. [10], Cabioglu et al. [23], and Bagbudar et al. [36] showed that in patients with breast cancer, high *CTLA-4* expression was linked to a longer DFS and a better OS. This disparity might be explained by differences in the primary antibodies utilized, scoring systems, follow-up periods, as well as genetic and ethnic differences among study populations.

In conclusion, our study demonstrated that for patients with triple-negative breast cancer (TNBC), *CTLA-4* expression is an independent predictor of worse overall survival (OS) and decreased disease-free survival (DFS). Consequently, in TNBC, *CTLA-4* appears to be a promising prognostic and therapeutic target, potentially aiding in the selection of patients for immunotherapy. On the contrary, *PD-L1* expression had no prognostic associations in TNBC.

Author Contribution Statement

All authors contributed equally, all authors reviewed the results and approved the final version of the manuscript..

Acknowledgements

None.

Ethical Declaration

This study was conducted upon approval of the committed Institutional Research Board (IRB) at Faculty of Medicine, Mansoura University, Egypt (Code Number: MDP.21.11.88, 2021). The study was processed under the ethical standards of the Helsinki declaration.

Approval

This study wasn't approved by any scientific body and isn't part of an approved student thesis.

Data Availability

The datasets are available from the corresponding author upon request.

Conflict of Interest

The authors declare that there are no conflicts of interest to disclose.

References

1. Enan ET, Abd El Hafez A, Hussin E, Hany HSEDI. Immunohistochemical Expression of Caspase1 and Epidermal Growth Factor Receptor in Invasive Breast Carcinoma and Their Biological and Prognostic Associations. Asian Pac J Cancer Prev. 2024;25(7):2529-37. https://doi.org/10.31557/APJCP.2024.25.7.2529.
2. Arnold M, Morgan E, Rumgay H, Mafra A, Singh D, Laversanne M, et al. Current and future burden of breast cancer: Global statistics for 2020 and 2040. Breast. 2022;66:15-23. https://doi.org/10.1016/j.breast.2022.08.010.
3. Yin L, Duan JJ, Bian XW, Yu SC. Triple-negative breast cancer molecular subtyping and treatment progress. Breast Cancer Res. 2020;22(1):61. https://doi.org/10.1186/s13058-020-01296-5.
4. Borri F, Granaglia A. Pathology of triple negative breast cancer. Semin Cancer Biol. 2021;72:136-45. https://doi.org/10.1016/j.semcancer.2020.06.005.
5. Liu Z, Li M, Jiang Z, Wang X. A Comprehensive Immunologic Portrait of Triple-Negative Breast Cancer. Transl Oncol. 2018;11(2):311-29. https://doi.org/10.1016/j.tranon.2018.01.011.
6. Chen L. Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity. Nat Rev Immunol. 2004;4(5):336-47. https://doi.org/10.1038/nri1349.
7. Caserta S, Innao V, Musolino C, Allegra A. Immune checkpoint inhibitors in multiple myeloma: A review of the literature. Pathol Res Pract. 2020;216(10):153114. https://doi.org/10.1016/j.prp.2020.153114.
8. Han Y, Liu D, Li L. *PD-1*/*PD-L1* pathway: current researches in cancer. Am J Cancer Res. 2020;10(3):727-42.
9. Babteen NA, Fawzy MS, Alelwani W, Alharbi RA, Alruwetei AM, Toraih EA, et al. Signal peptide missense variant in cancer-brake gene CTLA4 and breast cancer outcomes. Gene. 2020;737:144435. https://doi.org/10.1016/j.gene.2020.144435.
10. Fang J, Chen F, Liu D, Gu F, Chen Z, Wang Y. Prognostic value of immune checkpoint molecules in breast cancer. Biosci Rep. 2020;40(7):BSR20201054. https://doi.org/10.1042/BSR20201054.
11. Farshbafnadi M, Pastaki Khoshbin A, Rezaei N. Immune checkpoint inhibitors for triple-negative breast cancer: From immunological mechanisms to clinical evidence. Int Immunopharmacol. 2021;98:107876. https://doi.org/10.1016/j.intimp.2021.107876.
12. Ribas A, Wolchok JD. Cancer immunotherapy using checkpoint blockade. Science. 2018 Mar 23;359(6382):1350-55. doi: 10.1126/science.aar4060.
13. Koh J, Kim MJ. Introduction of a New Staging System of Breast Cancer for Radiologists: An Emphasis on the Prognostic Stage. Korean J Radiol. 2019;20(1):69-82. https://doi.org/10.3348/kjr.2018.0231.
14. Shebl AM, Zalata KR, Amin MM, El-Hawary AK. An inexpensive method of small paraffin tissue microarrays using mechanical pencil tips. Diagn Pathol. 2011;6:117. https://doi.org/10.1186/1746-1596-6-117.
15. Li JJ, Tsang JY, Tse GM. Tumor Microenvironment in Breast Cancer-Updates on Therapeutic Implications and Pathologic Assessment. Cancers (Basel). 2021;13(16):4233. https://doi.org/10.3390/cancers13164233.
16. Amer BS, Amer RS, Awad RA, Darwish SA, Bedeer AE. Association of CTLA4 and *PD-L1* immunohistchemical expression with various clinicopathological parameters and survival in non–small-cell lung cancer. Cancer Care Res Online. 2022;6(4):1-2. https://doi.org/10.21608/jcbr.2022.168536.1282.
17. World Medical Association. World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. JAMA. 2013;310(20):2191-4. https://doi.org/10.1001/jama.2013.281053.
18. Stovgaard ES, Nielsen D, Hogdall E, Balslev E. Triple negative breast cancer - prognostic role of immune-related factors: a systematic review. Acta Oncol. 2018;57(1):74-82. https://doi.org/10.1080/0284186X.2017.1400180.
19. Zhu X, Zhang Q, Wang D, Liu C, Han B, Yang JM. Expression of *PD-L1* Attenuates the Positive Impacts of High-level Tumor-infiltrating Lymphocytes on Prognosis of Triple-negative Breast Cancer. Cancer Biol Ther. 2019;20(8):1105-12. https://doi.org/10.1080/15384047.2019.1595282.
20. Qin G, Wang X, Ye S, Li Y, Chen M, Wang S, et al. NPM1 upregulates the transcription of *PD-L1* and suppresses T cell activity in triple-negative breast cancer. Nat Commun. 2020;11(1):1669. https://doi.org/10.1038/s41467-020-15364-z.
21. Sukumar J, Gast K, Quiroga D, Lustberg M, Williams N. Triple-negative breast cancer: promising prognostic biomarkers currently in development. Expert Rev Anticancer Ther. 2021;21(2):135-48. https://doi.org/10.1080/14737140.2021.1840984.
22. Kassardjian A, Shintaku PI, Moatamed NA. Expression of immune checkpoint regulators, cytotoxic T lymphocyte antigen 4 (*CTLA-4*) and programmed death-ligand 1 (*PD-L1*), in female breast carcinomas. PLoS One. 2018;13(4):e0195958. https://doi.org/10.1371/journal.pone.0195958.
23. Cabioglu N, Onder S, Oner G, Karatay H, Tukenmez M, Muslumanoglu M, et al. TIM3 expression on TILs is associated with poor response to neoadjuvant chemotherapy in patients with locally advanced triple-negative breast cancer. BMC Cancer. 2021;21(1):357. https://doi.org/10.1186/s12885-021-08054-6.
24. Vardas V, Tolios A, Christopoulou A, Georgoulias V, Xagara A, Koinis F, et al. Immune Checkpoint and EMT-Related Molecules in Circulating Tumor Cells (CTCs) from Triple Negative Breast Cancer Patients and Their Clinical Impact. Cancers (Basel). 2023;15(7):1974. https://doi.org/10.3390/cancers15071974.
25. Qin T, Zeng YD, Qin G, Xu F, Lu JB, Fang WF, et al. High *PD-L1* expression was associated with poor prognosis in 870 Chinese patients with breast cancer. Oncotarget. 2015;6(32):33972-81. https://doi.org/10.18632/oncotarget.5583.
26. Constantinou C, Papadopoulos S, Karyda E, Alexopoulos A, Agnanti N, Batistatou A, et al. Expression and Clinical Significance of Claudin-7, PDL-1, PTEN, c-Kit, c-Met, c-Myc, ALK, CK5/6, CK17, p53, EGFR, Ki67, p63 in Triple-negative Breast Cancer-A Single Centre Prospective Observational Study. In Vivo. 2018;32(2):303-11. https://doi.org/10.21873/invivo.11238.
27. Botti G, Collina F, Scognamiglio G, Rao F, Peluso V, De Cecio R, et al. Programmed Death Ligand 1 (*PD-L1*) Tumor Expression Is Associated with a Better Prognosis and Diabetic Disease in Triple Negative Breast Cancer Patients. Int J Mol Sci. 2017;18(2):459. https://doi.org/10.3390/ijms18020459.
28. Parvathareddy SK, Siraj AK, Ahmed SO, Ghazwani LO, Aldughaither SM, Al-Dayel F, et al. *PD-L1* Protein Expression in Middle Eastern Breast Cancer Predicts Favorable Outcome in Triple-Negative Breast Cancer. Cells. 2021;10(2):229. https://doi.org/10.3390/cells10020229.
29. Mao H, Zhang L, Yang Y, Zuo W, Bi Y, Gao W, et al. New insights of *CTLA-4* into its biological function in breast

cancer. Curr Cancer Drug Targets. 2010;10(7):728-36. https://doi.org/10.2174/156800910793605811.
30. Holah NS. The Clinical Value of VDR and CTLA 4 in Evaluating the Prognosis of Invasive Duct Carcinoma of Egyptian Patients and their Benefit as a Target Therapy. Asian Pac J Cancer Prev. 2021;22(4):1183-94. https://doi.org/10.31557/APJCP.2021.22.4.1183.
31. Abbasov A, Aktas Cetin E, Cabioglu N, Mollavelioglu B, Onder S, Emiroglu S, et al. Differential Expression of Novel Immune Checkpoint Receptors on Tumor Infiltrating Lymphocytes in Patients with Locally Advanced Breast Cancer after Neoadjuvant Chemotherapy. Neoplasma. 2021;68(5):1079-90. https://doi.org/10.4149/neo_2021_210127N141.
32. Lan G, Li J, Wen Q, Lin L, Chen L, Chen L, et al. Cytotoxic T lymphocyte associated antigen 4 expression predicts poor prognosis in luminal B HER2-negative breast cancer. Oncol Lett. 2018;15(4):5093-97. https://doi.org/10.3892/ol.2018.7991.
33. Tomioka N, Azuma M, Ikarashi M, Yamamoto M, Sato M, Watanabe KI, et al. The therapeutic candidate for immune checkpoint inhibitors elucidated by the status of tumor-infiltrating lymphocytes (TILs) and programmed death ligand 1 (*PD-L1*) expression in triple negative breast cancer (TNBC). Breast Cancer. 2018;25(1):34-42. https://doi.org/10.1007/s12282-017-0781-0.
34. Yu H, Yang J, Jiao S, Li Y, Zhang W, Wang J. Cytotoxic T lymphocyte antigen 4 expression in human breast cancer: implications for prognosis. Cancer Immunol Immunother. 2015;64(7):853-60. https://doi.org/10.1007/s00262-015-1696-2.
35. Lu L, Bai Y, Wang Z. Elevated T cell activation score is associated with improved survival of breast cancer. Breast Cancer Res Treat. 2017;164(3):689-96. https://doi.org/10.1007/s10549-017-4281-x.
36. Bagbudar S, Karanlik H, Cabioglu N, Bayram A, Ibis K, Aydin E, et al. Prognostic Implications of Immune Infiltrates in the Breast Cancer Microenvironment: The Role of Expressions of *CTLA-4*, *PD-1*, and LAG-3. Appl Immunohistochem Mol Morphol. 2022;30(2):99-107. https://doi.org/10.1097/PAI.0000000000000978.