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Research article

Expression of immune checkpoints (PD-L1 and IDO) and tumour-infiltrating lymphocytes in breast cancer

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ABSTRACT

Background: Breast cancer (BC) has become the most common cancer globally in 2020 as well as in the United Arab Emirates. The breast tumor microenvironment is composed of various immune cell types, including lymphocytes. Tumour-infiltrating lymphocytes (TILs) play a crucial role in tumor eradication and progression. Further, immune checkpoint markers such as programmed death receptor ligand 1 (PD-L1) and indoleamine-2,3-dioxygenase (IDO) have been associated with tumor evasion from the immune system. In this study, we aimed to explore the status of TILs, PD-L1 and IDO as well as to investigate their association with the clinicopathological parameters.

Materials and methods: A total of 59 patients diagnosed with primary infiltrating BC were selected, after which tissue sections were stained to identify TILs along with immunohistochemical staining of PD-L1 and IDO. Moreover, in-silico tools were used to assess the expression of PD-L1, IDO and CD3ε in various molecular subtypes of BC.

Results: It was found that the percentage of TILs correlated with estrogen receptor (ER) and progesterone receptor (PR) expression. This was supported by the finding that most of the triple-negative breast cancer (TNBC) cases belonged to the group with a high percentage of TILs (h-TILs). Similarly, the expression of PD-L1 and IDO was correlated with the ER and PR, whereas TNBC cases showed a high expression of PD-L1 and IDO. This goes in line with the in-silico findings where the TNBC group showed the highest expression of PD-L1 and IDO as well as the T cell marker CD3ε.

Conclusion: This study highlighted a possible link between the immunosuppressive markers PD-L1 and IDO with TILs density in the BC microenvironment.

1. Introduction

Breast cancer (BC) has been rated as the highest cancer diagnosed according to the 2020 global cancer registry and the first leading cause of death in females [1]. Noteworthy, regardless of ethnicity and gender, BC was the most commonly diagnosed cancer in the UAE according to its 2017 cancer registry [2].

The tumor microenvironment is composed of multiple other cell types, including fibroblasts and immune cells. Within the immune cell population, there are lymphocytes such as T, B and natural killer cells, as well as antigen-presenting cells such as macrophages and dendritic cells. Despite the anti-tumor activity of the immune system, the neoplastic cells may grow progressively unrecognized by escaping the immune response and developing into immune-resistant cancerous cells [3, 4, 5].

Tumour-infiltrating lymphocytes (TILs) are known to be one of the main components of the tumor microenvironment [6]. TILs comprise lymphocytes residing within the tumor nests and dispersed in the stroma

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between cancer cells, known as intratumoral and stromal TILs, respectively [7, 8].

One of the extensively studied markers in cancers is the immune checkpoint inhibitor, the programmed death receptor-ligand 1(PD-L1). PD-L1 exerts its function by inducing peripheral tolerance and limiting T-cell repertoire expansion. Such characteristics can be adapted by PD-L1 positive tumours to exploit and prevent elimination by immune cells [9, 10]. In BC, PD-L1 expression has been associated with better prognosis in addition to higher TILs [11].

An additional immunosuppressive enzyme that has been explored in BC is the indoleamine-2,3-dioxygenase (IDO). It is a tryptophan catabolic enzyme that is expressed throughout fetal life for protection from maternal T lymphocyte destruction. Although IDO expression is suppressed in normal physiological conditions in adults, it was found to be expressed during inflammatory settings and in different tumours. IDO expression has been linked to tumour evasion by dampening the antigen-specific immune response. In addition, it promotes tumour neovascularization via releasing different cytokines like IFN-γ and IL-6 [12, 13, 14]. In this study, we aimed to explore the status of TILs, PD-L1 and IDO in our cohort and their association with clinicopathological parameters.

2. Subjects, materials and methods

2.1. Clinical characteristics and histological examination of patients

This study included a total of 59 patients diagnosed with primary infiltrating BC surgically treated at Sharjah Breast Care Centre, University Hospital Sharjah (SBCC-UHS) between May 2013 and January 2021. Complete clinicopathological and immunohistochemical data were retrieved for all specimens. The study was conducted according to the principles of the Declaration of Helsinki and approved by UHS Ethical and Research Committee (Ref. No.: UHS-HERC-01-28012019).

The demographic data and the clinicopathological characteristics of the patients are summarized in Table 1. The mean age of the included patients was 50 ± 13.31 years at the time of diagnosis. All the patients were classified into four molecular subtypes: luminal A, luminal B (HER2−/HER2+), HER2-overexpressing and triple-negative breast cancer (TNBC). Sections were cut from the formalin-fixed paraffin-embedded (FFPE) BC blocks and stained by hematoxylin and eosin (H&E), which were examined microscopically, as previously described [15].

Table 1. Demographic and clinical characteristics of study subjects (N = 59).

		N	
Age (years)	Minimum	18	
	Maximum	86	
	Mean (SD)	50 (13.31)	
		N	%
Location	Right	28	47.5
	left	31	52.5
Diagnosis	IDC	44	74.6
	ILC	5	8.5
	Mixed	4	6.8
	Medullary	6	10.2
DCIS	Absent	25	42.4
	Present	34	57.6
Grade Nottingham	Grade 1	8	13.6
	Grade 2	19	32.2
	Grade 3	32	54.2
Tumor Size	≤2.5	28	50.9
	2.51 - ≤ 5	20	36.4
	>5	7	12.7
Lymph Node	Absent	27	45.8
	Present	32	54.2
Lymphovascular involvement	Absent	37	62.7
	Present	22	37.3
ER	Negative	14	23.7
	Positive	45	76.3
PR	Negative	20	33.9
	Positive	39	66.1
Her2 overexpression	Negative	41	69.5
	Positive	18	30.5
Ki-67	<14%	16	27.1
	≥14%	43	72.9
Molecular Subtypes	Luminal A	15	25.4
	Luminal B/Her2 Negative	15	25.4
	Luminal B/Her2 Positive	16	27.1
	Her2 over-expression	2	3.4
	Triple Negative (TNBC)	11	18.6

DCIS: ductal carcinoma in situ, ER: estrogen receptor, Her2: epidermal growth receptor 2, IDC: invasive ductal carcinoma, ILC: infiltrating lobular carcinoma, PR: progesterone receptor, SD: standard deviation, TNBC: triple negative breast cancer.

2.2. Assessment of stromal TILs

The assessment of the TILs was performed according to the recommendations of the TILs Working Group 2014 [7]. The percentage of stromal TILs was calculated as a semi-quantitative parameter with a cut-off value of 50% [16]. The percentage of stromal TILs was determined by dividing the area occupied by mononuclear inflammatory cells over total intratumoral stromal area. All mononuclear cells within the borders of the invasive tumour (including lymphocytes and plasma cells) were scored [7].

2.3. Immunohistochemistry staining and assessment

Immunohistochemical staining was performed using the Dako Autostainer Link 48 (DAKO, Glostrup, Denmark). The FFPE BC tissues were sectioned in a 4 μm thickness, air dried for 10 min and baked at 60 °C in the oven for 1 h before use. Deparaffinization, rehydration and antigen retrieval were performed in the Dako PT link pre-treatment system. Antigen retrieval was done using EnVision FLEX Target Retrieval reagents. The incubation for the slides was done for 1 h at a pH of 9.0 at 65°C, followed by 20 min of retrieval at 97 °C. The surface of each slide was covered with wash buffer to avoid drying. The automated staining protocol consisted of the application of Envision Flex Peroxidase-Blocking solution (DAKO, Glostrup, Denmark) for 5 min, followed by incubation of the primary antibodies: (PD-L1 (E1L3N) XP Rabbit monoclonal antibody (mAb) and IDO (D5J4E) rabbit mAb, Cell Signaling Technology, USA) for 20 min at the optimal dilution (1:400 for both), followed by incubation with peroxidase-labeled polymer (Envision Flex/HRP; DAKO, Glostrup, Denmark) for 20 min. This was followed by the application of substrate chromogen-FLEX DAB (3,3′-diaminobenzidine tetrahydrochloride) for 10 min. The sections were rinsed following each step using the Envision Flex wash buffer (DAKO, Glostrup, Denmark). After the last wash step, the slides were counterstained using hematoxylin, dehydrated and mounted. In each run, negative and positive controls were included based on the manufacturers' instructions. The expression of the PD-L1 and IDO was assessed according to their cut-off values: >1% [16, 17].

2.4. In silico analysis of expression of PD-L1and IDO in BC patients

The UALCAN tool (http://ualcan.path.uab.edu/index.html) was used to assess the expression of CD3ε, PD-L1 and IDO in primary BC cases (n = 719) with various molecular subtypes: luminal, HER2 positive and TNBC.

Further analysis was performed to assess the expression of these markers among the various molecular subtypes of BC.

2.5. Statistical analysis

Statistical analysis was performed using the SPSS 27 (IBM, Armonk, NY, USA) software package. Descriptive univariate analyses were conducted using frequencies and percentages for categorical variables as well as means, medians, and standard deviations for scale variables. The Chi-square test was implemented to assess the relationship between categorical variables. The normality of continuous variables was tested visually using the Q-Q plots and statistically using the Kolmogorov-Smirnov test. Differences in the means of normally distributed continuous variables were analyzed using the independent t-test and ANOVA test for two independent or multiple samples, respectively. Non-parametric tests, including Mann–Whitney or Kruskal–Wallis tests, were used for skewed continuous outcomes. The level of significance was set at 5%. Therefore, a p-value below 0.05 was regarded as statistically significant.

3. Results

3.1. Assessment of TILs in BC patients and its association with clinicopathological parameters

TILs were observed with varying percentages in all the included 59 BC patients. Based on the cut-off value of 50%, patients were divided into 2 groups: high TILs (h-TILs, n = 46) and low TILs (l-TILs, n = 11). The association between the percentage of TILs in the BC microenvironment and the different pathological characteristics of the patients showed several significant findings. First, TILs and ER showed an inverse relationship, where h-TILs were reported in 53.8% of patients with negative ER expression compared to 9.1% among patients with positive ER ($p = 0.001$). Likewise, TILs and PR expression showed an inverse relationship where h-TILs' infiltration was reported in 47.4% of patients with negative PR expression compared to 5.3% among patients with positive PR expression ($p < 0.0005$). Furthermore, this was highlighted in the categorization of BC cases according to the different molecular subtypes, where 63.6% of TNBC cases were in the h-TILs group, compared to 8.7% with other molecular subtypes ($p < 0.0005$). Therefore, a higher lymphocytic infiltration was found in the hormonal negative BC cases in this study. Regarding tumor grade, all the cases with grade 1 and grade 2 tumors had l-TILs, while all cases in the h-TILs group were BC cases with grade 3 tumors ($p = 0.003$, Table 2).

3.2. Expression of PD-L1 and IDO in BC patients

Immunohistochemistry staining of PD-L1 (Figure 1A) and IDO (Figure 1B) showed positive expression on the tumor cells of most BC tissues. Upon association with the pathological parameters, PD-L1 showed a significant correlation with negative expression of ER and PR. PD-L1 was found to be expressed in 84.6% of the patients with negative ER compared to 28.6% of patients with positive ER ($p < 0.0005$). Similarly, PD-L1 was found to be expressed in patients negative for PR with 73.7% of patients with negative PR compared to 25.0% of the patients who were positive for PR ($p = 0.001$, Table 3). Out of all the different BC molecular subtypes assessed, 90% of TNBC cases expressed PD-L1, while only 31.1% of the cases with other subtypes showed positive PD-L1 expression (Figure 2A).

The assessment of intra-tumoral IDO expression showed that 84.6% of negative ER cases were positive for IDO, while IDO was only found in 25.6% of ER-positive cases ($p < 0.0005$). Moreover, 70% of PR negative cases expressed IDO; however, only 22.2% of PR positive cases showed a positive IDO expression ($p < 0.0005$). This was further confirmed upon molecular subtype assessment, where all TNBC cases were positive for intra-tumoral IDO, and only 24.4% of other subtypes were positive for IDO expression ($p < 0.0005$, Figure 2B).

3.3. Association of TILs with PD-L1 and IDO expression

Since PD-L1 and IDO are crucial players in the immune evasion by tumor cells, it was essential to investigate the association between TILs and PD-L1 as well as IDO expression. Interestingly, a direct association was observed in patients between TILs and PD-L1 expression. In the h-TILs group, 90.9% of cases were positive for PD-L1, while 28.6% of patients were positive for PD-L1 in the l-TILs group ($p < 0.0005$, Figure 3A). Also, a similar observation was made for IDO expression, which was reported in 81.8% of the h-TILs group compared to 30.2% in the l-TILs group ($p = 0.004$, Figure 3B).

Furthermore, there was a direct association between PD-L1 and IDO expressions in our cohort. As shown in Figure 4, positive PD-L1 expression was reported in 66.7% of IDO-positive cases compared to 28.1% in the IDO-negative group ($p = 0.006$).

3.4. In silico expression of PD-L1, IDO and CD3ε in BC patients

In order to further evaluate the expression of PD-L1 and IDO in a bigger BC cohort, *in silico* tools were utilized. As shown in Figure 5A, PD-L1 was found to be significantly higher in the triple-negative group compared to the luminal subtype ($p = 0.024$). Similarly, IDO expression was upregulated in the triple negative subgroup in comparison to HER2 positive as well as luminal BC subtypes ($p = 0.0089$ and $p = 0.00072$, respectively, Figure 5B). Interestingly, the well-established marker for T lymphocytes, CD3ε, was found to be expressed in BC patients with a significant further increase in the triple-negative compared to the luminal subtype ($p = 0.035$, Figure 5C).

4. Discussion

The tumor microenvironment in BC comprises various cell types, including immune lymphocytic cells, including TILs. The degree of infiltrating immune cells, in addition to the morphological variations such as grade and histological type, showed an impact on the response of BC patients to treatment and overall survival [18].

In this study, we report the presence of TILs in varying expression patterns in BC patients based on the hormonal receptor status. For instance, most patients with negative ER expression fell into the h-TILs group. This observation is in line with other studies that reported a clear association between low ER expression and higher lymphocytic infiltrate, impacting the patients' survival [19, 20]. Regarding the other hormonal

Table 2. Association between TILs and hormone receptor status, tumor grade and molecular subtypes [%(N)].

	ER		PR		Nottingham Grade			Molecular subtypes	
	Negative	Positive	Negative	Positive	G1	G2	G3	Others	TNBC
l-TILs	46.2% (6)	90.9% (40)	52.6% (10)	94.7% (36)	100% (7)	100% (19)	64.5% (20)	91.3% (42)	36.4% (4)
h-TILs	53.8% (7)	9.1% (4)	47.4% (9)	5.3% (2)	0% (0)	0% (0)	35.5% (11)	8.7% (4)	63.6% (7)
p-value	p = 0.001		<0.0005		p = 0.003			<0.0005	

ER: estrogen receptor, PR: progesterone receptor, TILs: tumor infiltrating lymphocytes, TNBC: triple negative breast cancer.

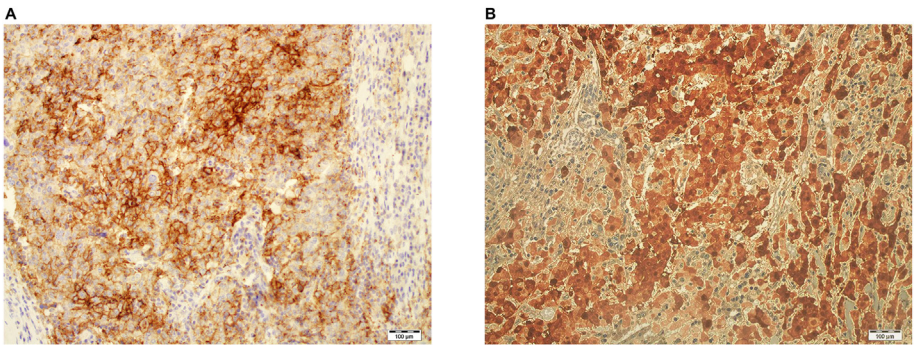

Figure 1. Immunohistochemical staining of PD-L1 and IDO in breast cancer patients. Representative images of (A) PD-L1 and (B) IDO expression in infiltrating mammary carcinoma. The PD-L1 and IDO expression cut-off was > 1%.

Table 3. Association of IDO and PD-L1 with the hormone receptor expression status [%(N)].

		IDO			PD-L1		
		Negative	Positive	Total	Negative	Positive	Total
ER	Negative	15.4% (2)	84.6% (11)	23.2% (13)	15.4% (2)	84.6% (11)	23.6% (13)
	Positive	74.4% (32)	25.6% (11)	76.8% (43)	71.4% (30)	28.6% (12)	76.4% (42)
	p-value	<0.0005			<0.0005		
		Negative	Positive	Total	Negative	Positive	Total
PR	Negative	30% (6)	70.0% (14)	35.6% (20)	26.3% (5)	73.7% (14)	34.5% (19)
	Positive	77.8% (28)	22.2% (8)	64.3% (36)	75.0% (27)	25.0% (9)	65.0% (36)
	p-value	<0.0005			0.001		

ER: estrogen receptor, IDO: indoleamine-2,3-dioxygenase, PD-L1: programmed death receptor ligand 1, PR: progesterone receptor.

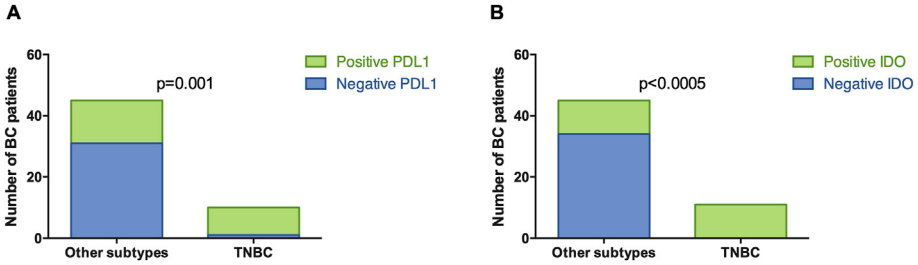

Figure 2. PD-L1 and IDO expression in different breast cancer molecular subtypes. The breast cancer patients with triple-negative (TNBC) molecular subtype were compared to the other subtypes according to the expression of (A) PD-L1 and (B) IDO. The comparison was done using the Chi-square test with a p-value <0.05, considered statistically significant.

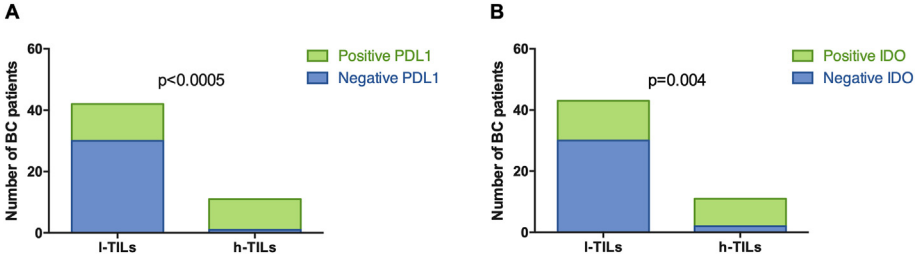

Figure 3. Association between tumour-infiltrating lymphocytes (TILs) and the expression of PD-L1 and IDO in breast cancer. The breast cancer patients presenting with more than 50% of TILs (high-TILs; h-TILs) were compared to the low-TILs (l-TILs) group according to the expression of (A) PD-L1 and (B) IDO. The comparison was done using the Chi-square test with a p-value <0.05 considered statistically significant.

receptor PR, a previous study by Miyoshi et al. indicated an association with the proportion of TILs in BC patients that do not show any recurrence [21], which is consistent with our data.

Another supporting finding was the association between TILs and the molecular subtypes of BC. It was found that those patients with a high percentage of TILs presented with the TNBC subtype. This further emphasizes the association of the presence of TILs along with hormonal receptor expression and molecular subtypes of BC. Previous studies have indicated that the incremental increase in TILs improved disease-free survival and overall survival, resulting in a better clinical outcome

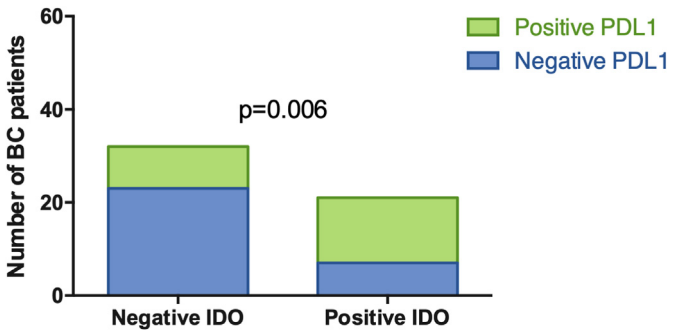

Figure 4. Association between PD-L1 and IDO in breast cancer patients. Most patients expressing positive IDO showed a significant positive expression of PD-L1. The comparison was done using the Chi-square test with a p-value <0.05 considered statistically significant.

[8, 11]. Additionally, Denkert et al. reported the role of TILs in the neoadjuvant chemotherapy setting, where 40% of patients with h-TILs achieved a complete pathological response [22].

Another crucial factor is the histological grade of BC patients that was linked to the immunological filtrate within the tumor and to patient clinical outcome irrespective of other factors including hormonal receptor status, tumour size, lymph node metastasis or BC molecular subtype [23]. This is in agreement with our findings, where all cases with histological grade 3 showed a higher lymphocytic infiltration when compared to patients with grades 1 and 2. This indicates that a higher immunological infiltrate is reported to be present in BC patients with a higher grade.

In this report, PD-L1 showed a significant correlation with negative expression of ER and PR hormonal receptors. Also, there was a direct association between the high presence of TILs and PD-L1 expression, which is similar to previous findings in BC as well as other cancer types [24, 25, 26]. The differential expression of PD-L1 in the molecular subtypes of BC was confirmed in a larger in silico cohort that showed the highest expression in TNBC. IDO expression in different malignancies has been linked to immune escape and tumor outgrowth. Several studies reported its expression to be variable in the molecular subtypes, with a prominent expression in the TNBC subtype [13, 27, 28, 29]. Likewise, IDO was highly expressed in TNBC patients, whether the included 59 cases by IHC or the 719 larger in silico cohort. Furthermore, ER and PR receptors' negativity showed an independent association with an increase in IDO expression. Previous studies explored the expression of IDO in basal-like as well as TNBC and its association with hormonal receptors [30, 31]. Additionally, IDO^{+} BC tissues were found to have an increased TILs density, with similar findings observed in our study [31].

Expressions of PD-L1 and IDO were found to be linked in BC, which could be attributed to diminished T-cell immune responses. Co-expression was found among different solid cancers harboring mismatch repair gene defects, especially in lower gastrointestinal tumors [27, 32]. In our study, there was a direct association between PD-L1 and IDO expressions, where 66.7 % of IDO^{+} BC cases were also positive for PD-L1. A direct correlation between PD-L1 and IDO was reported in a previous study investigating primary and metastatic BC [27]. These markers might contribute mechanistically to tumor evasion from immune cells, specifically T cells. Looking at the TNBC subtype, a high density of TILs was observed along with a high expression of PD-L1 and IDO, thus indicating an association between these markers. Interestingly, our in-silico analysis revealed an increase in CD3ε marker in TNBC compared to other subtypes, which further supports the recruitment and inhibition of T cells by BC.

In conclusion, this study explored the status of TILs in BC patients and their association with the clinicopathological parameters. Additionally, this study highlighted a possible link between the immunosuppressive markers PD-L1 and IDO with TILs density in the BC microenvironment.

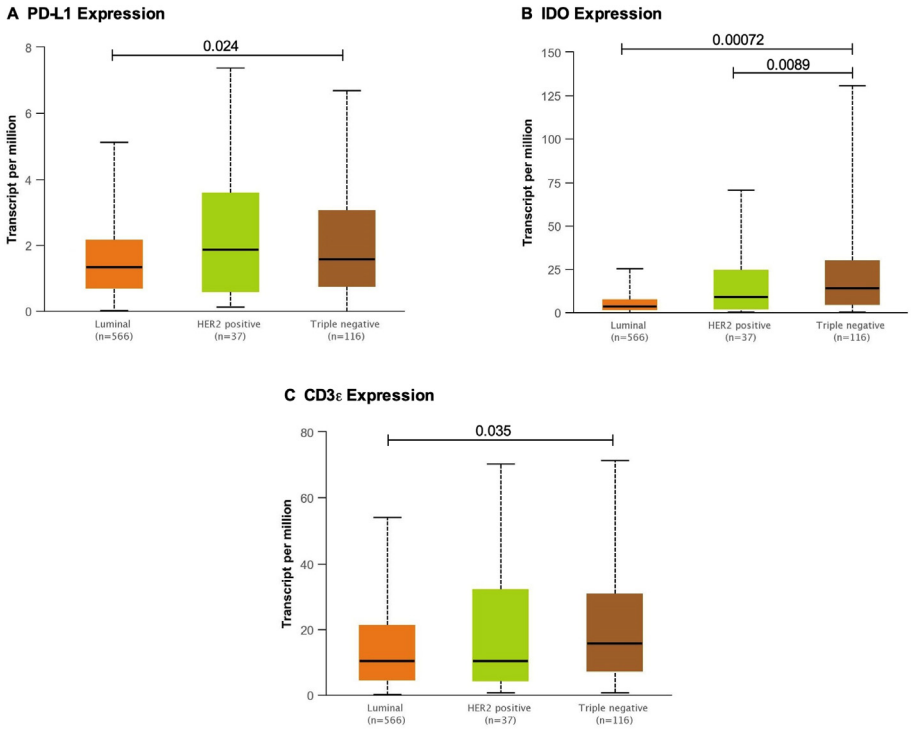

Figure 5. In silico analysis of PD-L1, IDO and CD3ε expression in various molecular subtypes of breast cancer. Differential expression of (A) PD-L1, (B) IDO, (C) CD3ε in 719 BC patients with luminal subtypes (n = 566), HER2 positive (n = 37), and triple-negative (TNBC, n = 116) subtypes. The comparison was done using the Student's t-test, considering unequal variance with a p-value <0.05 considered statistically significant.

Declarations

Author contribution statement

Noura Alkhayyal: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Noha M. Elemam: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Amal Hussein; Majd Jundi: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Sulaman Magdub; Azzam A. Maghazachi: Contributed reagents, materials, analysis tools or data.

Iman M. Talaat: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Riyad Bendardaf: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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