Cancer Cell International

RESEARCH Open Access

High *CTLA-4 gene* expression is an independent good prognosis factor in breast cancer patients, especially in the HER2-enriched subtype

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Abstract

Background Breast cancer (BC) is the most common cancer in women and the leading cause of cancer-related death worldwide. This heterogeneous disease has been historically considered a non-immunogenic type of cancer. However, recent advances in immunotherapy have increased the interest in knowing the role of the immune checkpoints (IC) and other immune regulation pathways in this neoplasia.

Methods In this retrospective study, we evaluated the correlation of mRNA expression of *CTLA-4*, *PDCD1 (PD1)*, *CD274 (PD-L1)*, *PDCD1LG2 (PD-L2)*, *CD276 (B7-H3)*, *JAK2*, *and FOXO1* with clinicopathological factors and BC patient's outcome by real-time quantitative polymerase chain reaction (qPCR).

Results Our results showed that immunoregulatory gene expression depends on BC immunophenotype being *CTLA-4* and *PDCD1* (PD1) overexpressed on triple-negative/*basal-like* (TN/BL) and luminal B/HER2-positive phenotypes, respectively, and *CD276* (B7-H3), *JAK2* and *FOXO1* associated with both luminal A and luminal B/HER2-negative tumors. In addition, we found that these genes can also be related to aggressive and non-aggressive clinicopathological characteristics in BC. Finally, survival analysis showed that *CTLA-4* expression levels emerge as a significant independent factor of good prognosis in BC patients, especially in the HER2-enriched subtype.

Conclusion Considering all these data, we can conclude that the expression of immunoregulatory genes depends on tumor phenotype and has potential clinical implications in BC patients.

Keywords Breast carcinoma, Immune checkpoints, mRNA expression, CTLA-4, HER2-enriched

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Introduction

Breast cancer (BC) is the most common female cancer in both developed and developing countries, representing the leading cause of cancer-related deaths among women worldwide [1]. This neoplasia is a heterogeneous disease of subgroups with different molecular features and clinical outcomes. Classically, BC has been classified into four major molecular subtypes based on the immunohistochemistry expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2): luminal A (ER+/PR+/HER2-), luminal B (ER+/PR+/HER2-/+), HER2-enriched (ER-/PR-/HER2+) and triple-negative/basal-like (TN/BL) (ER-/PR-/HER2-) [2–4]. Although significant progress has been made in the diagnosis and treatment, clinical outcomes still depend on the patient and the intrinsic tumor subtype. Therefore, new therapeutic and prognosis biomarkers are needed to develop novel targeted therapies and improve patient survival.

Under normal conditions, the immune system recognizes and eliminates malignant cells through an active anti-neoplastic response, inhibiting carcinogenesis and maintaining cellular homeostasis [5]. However, during neoplastic transformation, malignant cells develop different strategies to avoid attacks from the immune cells. One of these strategies is the expression of immune checkpoints (IC), a set of inhibitory proteins that physiologically restrict the immune response to maintain immune homeostasis and self-tolerance, protecting host tissues from unnecessary damage and developing autoimmune disorders due to excessive inflammation. However, cancer cells can exploit these pathways to evade immune surveillance and avoid immune-mediated destruction [6]. IC molecules and the pathways that regulate the immune response have emerged as promising therapeutic targets for different neoplasia, including BC.

Historically, BC has been considered a non-immunogenic type of cancer. Nevertheless, recent breakthroughs with immune checkpoint inhibitors (ICI) in other cancers, coupled with the association of tumor-infiltrating lymphocytes (TILs) [7, 8] and immune gene signatures with better clinical outcomes in BC [9, 10], have led to the development of clinical trials to evaluate the efficacy of ICI, particularly on the TN/BL subtype. Some trials have yielded promising results, while others have demonstrated limited efficacy. For example, the IMpassion130 trial demonstrated the effectiveness of combining atezolizumab with nab-paclitaxel in locally advanced or metastatic TN/BL BC patients that expressed programmed cell death ligand-1 (PD-L1) on immune cells, improving the progression-free survival (PFS) and overall survival (OS) compared to the placebo group [11–14]. Similarly, the KEYNOTE-355 trial reported improved PFS in previously untreated patients with locally recurrent inoperable or metastatic TN/BL BC that expressed PD-L1 when treated with pembrolizumab combined with chemotherapy [15]. Promising results were also found in the KEYNOTE-522 trial, which showed an increase in event-free survival (EFS) in previously untreated stage II or III TN/BL BC who received pembrolizumab in combination with neoadjuvant chemotherapy [16]. However, not all the clinical trials have been positive. The IMpassion131 trial did not improve PFS or OS in patients treated with atezolizumab plus paclitaxel compared to paclitaxel alone [17]. Based on these results, atezolizumab plus nab-paclitaxel and pembrolizumab in combination with chemotherapy were approved by the Food and Drug Administration (FDA) for the treatment of metastatic TN/BL BC.

Several IC pathways have already been documented. The most extensively studied pathways involve the interactions between cytotoxic T lymphocyte antigen 4 (CTLA-4) and CD80/CD86 and the binding of programmed cell death protein 1 (PD-1) to PD-L1/PD-L2 on neoplastic cells. Numerous promising IC molecules have recently been identified, including inhibitory ligands belonging to the B7 proteins family, such as B7-H3 [18]. These proteins inhibit cell proliferation, cytokine production, and cytotoxic activity of effector T cells, leading to immune evasion [19, 20]. Recent studies have also demonstrated the intrinsic expression of CTLA-4 and PD-1 in different neoplasm and cancer cell lines [21–23], suggesting that these ICs may be involved in other functions beyond the immune system regulation. PD-L1 and PD-L2 not only modulate the immune response but also participate in tumor-intrinsic functions such as neoplastic cell proliferation, migration, and invasion [24, 25], epithelial-to-mesenchymal transition (EMT) regulation [25, 26], stem cell properties acquisition [26–28], apoptosis inhibition, and chemotherapy resistance [29]. At the same time, B7-H3 has also been associated with some of these non-immunological functions, such as cell invasion and migration [30–33], regulation of angiogenesis [34], and cellular glucose metabolism [35–37], the EMT process and the maintenance of stem cell properties [38–40] and resistance to apoptosis [41] and antineoplastic treatments [37, 42–44].

Together with IC molecules, JAK2 and FOXO1 modulate the active anti-neoplastic response. Thus, JAK2 is a tyrosine kinase protein that, along with signal transducer and activator of transcription (STATs) proteins, plays a crucial role in initiating the transcription of various target genes involved in multiple cellular processes [45]. Its expression in neoplastic cells has been associated with cell proliferation, migration and invasion, angiogenesis, treatment and apoptosis resistance, and maintenance of stemness and EMT characteristics, which have been linked to poor prognosis and clinicopathological features

[46–50]. On the other hand, FOXO1 is a transcription factor belonging to the FOXO subfamily, which also participates in the transcription of various genes related to multiple immunological processes [51]. These processes include dendritic cell migration [52], production of inflammatory factors [53], B lymphocytes, and T regulatory cell maturation [54], as well as the formation and functioning of memory T cells [55].

Additionally, the expression of FOXO1 in neoplastic cells is an independent factor of good prognosis, restricting the migration and invasion of neoplastic cells by inhibiting the EMT process [56, 57]. Consequently, many authors widely recognize it as a tumor suppressor protein. Nevertheless, conflicting data exists, as depending on the tumor microenvironment (TME), it may also function as an oncogene, promoting cell proliferation and drug resistance in several cancer types [58, 59].

All the above data suggest that the expression of different IC molecules and pathways related to immune response regulation may play distinct roles in BC. Therefore, this has raised our interest in investigating clinicopathological and prognostic value of *CTLA-4*, *PDCD1*, *CD274*, *PDCD1LG2*, *CD276*, *JAK2*, and *FOXO1* genes in BC patients. We hypothesize that their expression in BC depends on tumor phenotype and may have clinical implications in patients with this neoplasia. To confirm these questions, we conducted a retrospective study on a large clinical series of BC patients ($n=250$) previously stratified by immunophenotypes, and mRNA expression results were correlated with clinicopathological factors and prognosis.

Methods

Patients and tumor samples

We studied a retrospective cohort of 250 non-consecutive patients with primary invasive BC diagnosed between 1994 and 2021. Samples and data from patients included were provided by the Dr. Balmis University General Hospital (DBUGH) and the HUB-ICO-IDIBELL Biobanks (PT17/0015/0024), both integrated into the Spanish National Biobanks Network. Samples were processed following standard operating procedures with the appropriate approval of the Ethics and Scientific Committees. Eligibility criteria were histological diagnosis of invasive BC, stages I-III, and follow-up data at least one year after surgical treatment. Patients with tumors in stage IV or treated with neoadjuvant therapy and samples with insufficient tissue or low quality/quantity RNA were excluded.

The clinical and pathological variables were: patient age, tumor size, histological grade according to Scarff-Bloom-Richardson classification modified by Elston and Ellis [60], vascular invasion, necrosis, lymph-node status, cell proliferation index determined by Ki-67 staining, stromal tumor-infiltrating lymphocytes (sTIL) percentage, hormone receptor (HR) status, defined as ER and/or PR positive/negative, and immunophenotypes. Patient follow-up data were obtained from clinical reports. OS was defined as the time from surgery to patient death, and disease-free survival (DFS) as the time from surgery to local and/or distant recurrence.

The study followed the Declaration of Helsinki, and the Institutional Ethics Committees approved the project (ethics code PI2019/058 and PI2020-242). Patient data were anonymized, and informed consent was obtained from all participants in this study. The manuscript has been written following the reporting recommendations for tumor marker prognostic studies (REMARK) criteria [61] and the MIQE guide for the publication of results obtained by real-time polymerase chain reaction (qPCR) [62].

RNA isolation and complementary DNA synthesis

Three 1-mm thick punches of formalin-fixed paraffin-embedded (FFPE) tissue (≥4 mm deep) from preselected areas of each tumor sample, with at least 30% tumor cell content, and normal breast tissue from reduction mammoplasties were used for the RNA extraction. Briefly, the punches were manually cut into smaller pieces with a scalpel, deparaffinized using mineral oil (Ref. M5940, Sigma-Aldrich, Darmstadt, Germany), and further disrupted using proteinase K (Ref. 19131, Qiagen. Hilden, Germany). The RNA isolation was later carried out with the RNeasy FFPE Kit (Ref. 73604, Qiagen, Hilden, Germany) following the manufacturer's instructions incorporating DNase I treatment to yield DNA-free RNA. RNase inhibitor (Ref.: N8080119, Applied Biosystems, Forster City, CA, USA) was added to avoid sample degradation. NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure RNA concentration and purity concerning 260/230 nm and 260/280 nm ratios. RNA samples were also subjected to a second quality control using the TapeStation 4200 system (Agilent Technologies, Waldbronn, Germany). Apart from the concentration, this equipment also provides two values to evaluate the quality of the samples: the RINe, which measures RNA integrity based on a numerical range from 1 (totally degraded RNA) to 10 (intact RNA), and the DV200, defined as the percentage of RNA fragments above 200 nucleotides for the total number of fragments. Since it has been shown that RINe values of FFPE tumor tissue samples are not a sensitive measure of RNA quality, only DV200 values were used as a quality criterion in this study. Thus, and following Illumina's recommendations, all those samples that obtained a DV200 value equal to or higher than 30% were included [63]. Finally, the isolated RNA was stored at −80 °C until its reverse transcription.

Reverse transcription reactions were performed from 2 µg of total RNA using random hexanucleotides and the High-Capacity cDNA Reverse Transcription Kit (Ref.: 4368814, Thermo Fisher Scientific) following the manufacturer's instructions. The synthesized cDNA was then diluted to a final concentration of 20 ng/µl and stored at -20 °C until its analysis.

Gene expression analysis

qPCR was performed using a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific) according to the manufacturer´s instructions. We used TaqMan Fast Universal PCR Master Mix (Ref.: A44360, Thermo Fisher Scientific) and assays based on hydrolysis probes (TaqMan® Gene Expression Assays, Thermo Fisher Scientific) for specific cDNA retrotranscripted from mRNA since these assays do not detect genomic DNA. Gene expression was performed for *CTLA-4* (Hs00175480_m1), *PDCD1* (Hs00169472_m1), *CD274* (Hs00204257_m1), *PDCD1LG2* (Hs00228839_m1), *CD276* (Hs00987207_m1), *JAK2* (Hs01078136_m1) and *FOXO1* (Hs01054576_m1). We used ACTB (Hs99999903_m1) and PUM1 (Hs00472881) to normalize gene expression as reference genes. As reference calibrator samples, we used a pool of RNA from normal breast tissue obtained from breast reduction of healthy patients. No template controls were included in each reaction, and all experiments were done by duplicates. Relative changes in gene expression were calculated as the fold change by the $2^{-\Delta\Delta Ct}$ method [64] and were analyzed using the 7500 software v2.0.6 (Thermo Fisher Scientific).

Statistical analyses

For the study, a relative quantification (RQ) value of 1.5 was taken into account as a cut-off point to differentiate between normal-low expression (RQ < 1.5) versus overexpression (RQ ≥ 1.5), as described in previous studies [65, 66]. All statistical analyses were performed using the SPSS version-23 statistical software package (SPSS Inc, Chicago, IL, USA). The Kolmogorov–Smirnov test was used to identify the distribution of quantitative variables. These variables were represented by the average ± standard deviation or the median and interquartile range (25-75th), depending on whether they followed a parametric or non-parametric distribution. The frequency percentage of each group defined qualitative variables. Chi-square or Fisher tests were used to measure the association between qualitative variables. To evaluate the magnitude of each of these associations, the Odds Ratio (OR) was also calculated, with a 95% confidence interval (CI). The variables with more than two categories were analyzed using logistic regressions to complete the bivariate analysis. Survival curves were plotted using the Kaplan–Meier method and compared using the log-rank tests. Univariate and multivariate survival analyses were performed using Cox's proportional hazard model. Multivariate analysis included all molecular and clinicopathological variables of significant value in univariate analysis. In all cases, p-values < 0.05 were considered statistically significant, while p-values > 0.05 but ≤ 0.15 were interpreted as trends toward significance.

Results

Patients and tumor characteristics

Our clinical series included 250 patients diagnosed with invasive BC. Patients were predominantly older than 50 years (64.8%; 162/250), with a mean age of 58 (range 30–94) years. Tumors were predominantly invasive ductal carcinoma (90.4%; 226/250), larger than 20 mm (50.4%; 126/250), with histological grade 3 (58.8%; 147/250), low sTIL infiltration (92.4%; 230/250) and high proliferative rate (52.4%; 131/250). Vascular invasion and necrosis were present in 36.8% (92/250) and 23.2% (58/250) tumors, respectively. Lymph nodes were positive in 53.2% (133/250) cases. Regarding the immunophenotype, all molecular subtypes were equally represented (20%; 50/250). However, looking at the HR expression, luminal tumors were the most abundant (60%; 150/250) (Table 1).

PDCD1 (PD1), *CD274* (PD-L1), and *CD276* (B7-H3) were overexpressed in the majority of BC tumors

Tumor samples were classified according to the RQ value into two groups: overexpression (RQ ≥ 1.5) and normal-low expression (RQ < 1.5). Based on this, we found that *CTLA-4* was overexpressed in 113 (45.2%), *PDCD1* (PD1) in 142 (56.8%), *CD274* (PD-L1) in 175 (70%), *PDCD1LG2* (PD-L2) in 123 (49.2%), *CD276* (B7-H3) in 179 (71.6%), *JAK2* in 98 (39.2%) and *FOXO1* in 79 (31.6%) samples (Fig. 1). When grouping the immune checkpoint genes, we observed that at least one was overexpressed in most cases analyzed (95.2%). Interestingly, simultaneous overexpression of five or four immune checkpoint genes was observed in 38 (15.2%) and 67 (26.8%) samples. Tumors with only one overexpressed immune checkpoint gene accounted for 16.8% (42/250). As for *JAK2* and *FOXO1*, it was observed that approximately half of the cases (123/250; 49.2%) were either normal or underexpressed at the same time, whereas only 20% (50/250) were characterized by overexpression of both genes.

CTLA-4, *PDCD1* (PD1), *CD274* (PD-L1), and *PDCD1LG2* (PD-L2)mRNA overexpression was associated with unfavorable clinicopathological variables

We evaluated the distribution of clinicopathological variables based on the mRNA expression levels of each gene. These results were compiled in Tables 2, 3, 4 and 5 and Tables S1-S4 in supplementary materials. Our

Table 1 Descriptive analysis of the clinicopathological variables and patient characteristics. *sTIL, stromal tumor-infiltrating lymphocytes; HR, hormone receptor*

Clinicopathological variables	Patients ($n=250$) % (n)
Age	
≤ 50 years old	35.2% (88)
> 50 years old	64.8% (162)
Tumor size	
≤ 20 mm	49.6% (124)
> 20 mm	50.4% (126)
Histological Grade	
1	6.8% (17)
2	34.4% (86)
3	58.8% (147)
Histological Type	
Invasive ductal carcinoma	90.4% (226)
Invasive lobular carcinoma	9.6% (24)
Vascular Invasion	
Present	36.8% (92)
Absent	63.2.% (158)
Necrosis	
Present	23.2% (58)
Absent	76.8% (192)
Ki67 Index	
Low (< 15%)	26.4% (66)
Intermediate (15-19%)	21.2% (53)
High (> 19%)	52.4% (131)
% sTIL	
Low (< 30%)	92% (230)
High (≥ 30%)	8% (20)
Molecular Subtypes	
Luminal A-like	20% (50)
Luminal B/HER2-negative	20% (50)
Luminal B/HER2-positive	20% (50)
HER2-enriched	20% (50)
Triple-negative	20% (50)
Lymph Nodes Status	
Positive	53.2% (133)
Negative	46.8% (117)
HR Status	
Negative	40% (100)
Positive	60% (150)

results showed a significant association between *CTLA-4* overexpression and HR-negative tumors ($p=0.022$). In addition, high levels of *CTLA-4* and *CD274* (PD-L1) were related to histological grade 3 and positive lymph nodes samples, respectively, but only as a trend ($p>0.05$). We also observed high sTIL density in tumors with *CTLA-4*, *PDCD1* (PD1), and *CD274* (PD-L1) overexpression ($p<0.012$), whereas middle/high proliferation rates were significantly related to *CTLA-4*, *PDCD1* (PD1), *PDCD1LG2* (PD-L2) and *JAK2* overexpression ($p<0.040$). Conversely, opposite results were obtained for

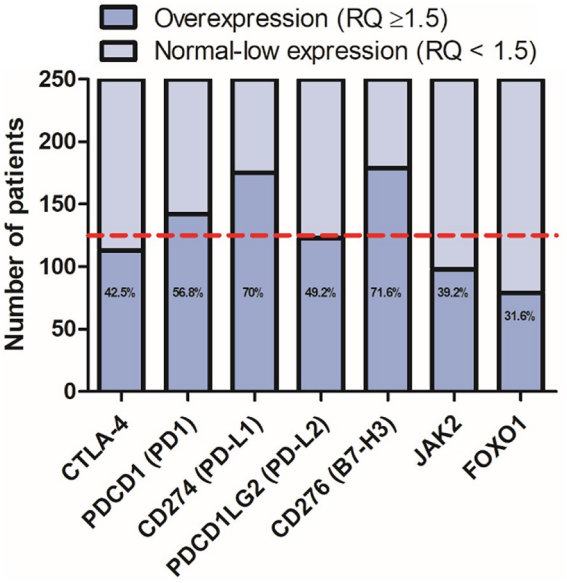

Fig. 1 Percentage of BC patients overexpressing immunoregulatory genes. *RQ, relative quantification value*

CD276 ($p=0.026$ and 0.048, respectively). Furthermore, we found *JAK2* and *FOXO1* overexpression in HR-positive tumors ($p<0.008$). The increase in *FOXO1* expression was also associated with tumors smaller than 20 mm ($p=0.016$), with histologic grade 2 ($p=0.002$), low/intermediate proliferative rate ($p<0.004$), and absence of lymphovascular invasion ($p=0.004$). A trend toward significance was observed between *FOXO1* expression and low sTIL density ($p=0.096$), and negative lymph node status ($p=0.055$).

Regarding the intrinsic BC subtypes, high *CTLA-4* and *PDCD1* (PD1) mRNA levels were correlated with TN/BL and luminal B/HER2-positive phenotypes, respectively (both $p=0.046$), whereas *CD276* (B7-H3), *JAK2* and *FOXO1* overexpression were significantly associated with luminal A and luminal B/HER2-negative tumors ($p<0.024$). A trend towards significance was also observed between *PDCD1* (PD1) overexpression and luminal B/HER2-negative and TN/BL tumors (both $p=0.072$). No significant associations were found for *CD274* (PD-L1) and *PDCD1LG2* (PD-L2).

CTLA-4 overexpression is an independent favorable prognostic factor for DFS

The prognostic value of several clinicopathological and molecular variables was evaluated with univariate and multivariate analyses. Median follow-up was 66 months (range 12–281 months), and the average OS and DFS were 47 (range 1-269) and 38 months (range 1-132), respectively. Of our patients, 25 (10%) had a relapse and/or distant metastasis, whereas 38 (15.2%) died of the disease or other causes at the last follow-up.

Table 2 Correlation between *CTLA-4* and *PDCD1* (PD1) mRNA expression and clinical-pathological variables. Chi-Square test; *logistic regression

	Patients (*n* = 250)	**CTLA-4 ≤ 1,50 *(n = 137) % (n)***	**CTLA-4 > 1,50 *(n = 113) % (n)***	**OR (CI95)**	***P***	**PDCD1 ≤ 1,50 *(n = 108) % (n)***	**PDCD1 > 1,50 *(n = 142) % (n)***	**OR (CI95)**	***P***
Histological grade									
1	17	70.6% (12)	29.4% (5)	1		47.1% (8)	52.9% (9)	1	
2	86	67.4% (58)	32.6% (28)	1.2 (0.4–3.6)	0.800*	43% (37)	57% (49)	1.2 (0.4–3.3)	0.759*
3	147	45.6% (67)	54.4% (80)	2.9 (1.0-8.5)	0.059*	42.9% (63)	57.1% (84)	1.2 (0.4–3.2)	0.741*
Histological type									
Invasive ductal carcinoma	226	53.5% (121)	46.5% (105)	0.6 (0.2–1.4)	0.219	44.7% (101)	55.3% (125)	2.0 (0.8–4.9)	0.144
Invasive lobular carcinoma	24	66.7% (16)	33.3% (8)			29.2% (7)	70.8% (17)		
Ki67 Index									
Low (< 15%)	66	71.2% (47)	28.8% (19)	1		53% (35)	47% (31)	1	
Intermediate (15-19%)	53	50.9% (27)	49.1% (26)	2.4 (1.1–5.1)	**0.025***	34% (18)	66% (35)	2.2 (1.0-4.6)	**0.039***
High (> 19%)	131	48.1% (63)	51.9% (68)	2.7 (1.4-5.0)	**0.002***	42% (55)	58% (76)	1.6 (0.9–2.8)	0.143*
% sTIL									
Low (< 30%)	230	58.3% (134)	41.7% (96)	7.9 (2.3–27.7)	**< 0.0001**	46.1% (106)	53.9% (124)	7.7 (1.7–33.9)	**0.002**
High (≥ 30%)	20	15% (3)	85% (17)			10% (2)	90% (18)		
Molecular Subtypes									
Luminal A-like	50	64% (32)	36% (18)	1		54% (27)	46% (23)	1	
Luminal B/HER2-	50	66% (33)	34% (17)	0.9 (0.4–2.1)	0.834*	36% (18)	64% (32)	2.1 (0.9–4.7)	0.072*
Luminal B/HER2+	50	52% (36)	48% (24)	1.6 (0.7–3.7)	0.225*	34% (17)	66% (33)	2.3 (1.0-5.1)	**0.046***
HER2-enriched	50	48% (24)	52% (26)	1.9 (0.9–4.3)	0.109*	56% (28)	44% (22)	0.9 (0.4-2.0)	0.841*
Triple-negative	50	44% (22)	56% (28)	2.3 (1.0-5.1)	**0.046***	36% (18)	64% (32)	2.1 (0.9–4.7)	0.072*
HR Status									
Negative	100	46% (46)	54% (54)	0.6 (0.3–0.9)	**0.022**	46% (46)	54% (54)	1.2 (0.7-2.0)	0.466
Positive	150	60.7% (91)	39.3% (59)			41.3% (62)	58.7% (88)		

Univariate analyses showed that patients older than 50 years ($p=0.003$), with larger tumor size ($p<0.0001$), histological grade 3 ($p=0.007$), vascular invasion ($p=0.032$), necrosis ($p<0.0001$), intermediate proliferative rate ($p=0.023$) and HR-negative status ($p=0.001$) showed shorter OS (Fig. 2 and Supplementary Table S5), especially in HER2-enriched and TN/BL phenotypes (both $p<0.033$) (Fig. 3). However, there was no significant association between the molecular variables and OS. We only observed that patients with *FOXO1* overexpressing tumors had a trend to longer OS ($p=0.097$) (Fig. 2 and Supplementary Table S5). Considering these results, a second analysis was performed with gene expression data in each BC phenotype. We performed Kaplan-Meier curves for OS and observed that patients with luminal B/HER2-positive overexpressing *CD274* (PDL1) showed higher OS, but the differences did not reach statistical significance ($p=0.120$) (Figure S1). In contrast, HER2-enriched patients with lower *CTLA-4* expression significantly decreased OS ($p=0.033$) (Fig. 4).

Similarly, DFS was significantly affected by larger tumor size ($p=0.004$), histological grade 3 ($p=0.010$), presence of necrosis ($p<0.0001$), low *CTLA-4* expression ($p=0.037$) and HR-negative status ($p=0.003$) (Fig. 2 and Supplementary Table S5), especially in the HER2-enriched subtype ($p=0.013$) (Fig. 3). Through Kaplan-Meier curves, we also observed that patients whose tumors had low levels of *CTLA-4* showed shorter DFS ($p=0.037$) (Fig. 5). Furthermore, after stratifying the analysis by phenotypes, it was observed that decreased *CTLA-4* expression was also significantly associated with lower DFS in HER2-enriched tumors ($p=0.011$) (Fig. 4).

By multivariate analyses, we demonstrated that older age ($p=0.023$) and the presence of necrosis ($p=0.021$) were independent prognostic factors for OS. Regarding DFS, the presence of necrosis ($p=0.025$), HER2-enriched phenotype ($p=0.033$), and *CTLA-4* expression ($p=0.031$) emerged as significant independent predictors of relapse (Fig. 2 and Supplementary Table S5).

Discussion

Our study extensively analyzed several ICs and immunoregulatory pathways in a clinical series of 250 BC patients. Most tumors overexpressed *CD276* (B7-H3), *CD274* (PD-L1), and *PDCD1* (PD-1), while *CTLA-4*, *PDCD1LG2* (PD-L2), *JAK2*, and *FOXO1* showed lower expression. These results partly agree with Fang et al. [67], who analyzed the gene expression of 50 different ICs in BC compared to healthy tissue. They observed an overexpression of *CD276* (B7-H3) and *PDCD1* (PD-1) and an underexpression of *PDCD1LG2* (PD-L2), which is consistent with our results. However, opposite results were found in *CTLA-4* and *CD274* (PD-L1) expression. These discordances may be due to the lack of a reference or standard cut-off point to define the presence or absence of overexpression. Regarding *JAK2* and *FOXO1* expression, our data agree with those from Liu et al. [68] and Lallemand et al. [69], who demonstrated that both genes were underexpressed. Therefore, our results suggest that the expression profiles of the immunoregulatory genes are complex and highly variable in BC.

Here, we also evaluated the association between gene expression and clinicopathological features and phenotypes. Our results showed that *CTLA-4* was overexpressed in tumors with intermediate-high proliferation index, a high percentage of sTIL, and negative HR status. Similarly, *PDCD1 (PD-1)* overexpression was associated with tumors exhibiting intermediate proliferation and a high sTIL content. Regarding *CD274* (PD-L1) and *PDCD1LG2* (PD-L2), both were associated with a high percentage of sTIL and intermediate proliferation, respectively. *JAK2* overexpression was also associated with an intermediate proliferation index. On the contrary, *CD276* (B7-H3) overexpression was associated with tumors exhibiting low cell proliferation and sTIL content. In contrast, *FOXO1* overexpression correlated with smaller tumors with histological grade 2, no lymphovascular invasion, and low-to-intermediate proliferative index. These findings suggest that *CTLA-4*, *PDCD1* (PD-1), *CD274* (PD-L1), and *JAK2* are related to unfavorable clinicopathological features in BC and that their expression may be influenced by the presence of immune cells in the TME.

After analyzing gene expression across different BC phenotypes, we found significant *CTLA-4* and *PDCD1* (PD-1) overexpression in TN/BL and luminal B/HER2-positive phenotypes, respectively. Additionally, luminal A and B/HER2-negative phenotypes showed elevated *CD276* (B7-H3), *JAK2*, and *FOXO1* levels. However, no significant differences were observed for *CD274* (PD-L1) and *PDCD1LG2* (PD-L2) compared to other phenotypes.

These results are consistent with existing literature in BC. Initial studies by Contardi et al. [70] demonstrated CTLA-4 expression in invasive ductal carcinomas by immunohistochemistry. Subsequently, Mao et al. [71] and Kassardjian et al. [72] revealed elevated levels of *CTLA-4* mRNA and protein in neoplastic tissue, as well as an association with axillary metastases and advanced clinical stages [71]. Similarly, a recent immunohistochemical study associated CTLA-4 expression with lymph node involvement, histological grade 3, larger tumor size, and high proliferation index [73]. However, our clinical series did not fully replicate these results, except for increased Ki-67 expression. There is controversy regarding the relationship between CTLA-4 expression and BC cell proliferation, as reported by Navarrete-Bernal et al. [74]. Recent literature, however, supports our findings, showing *CTLA-4* overexpression in HR-negative tumors [75]

Table 3 Correlation between *CD274* (PD-L1) and *PDCD1LG2* (PD-L2) mRNA expression and clinical-pathological variables. Chi-Square test; *logistic regression

	Patients (*n* = 250)	**CD274 ≤ 1,50 (*n* = 137) % (n)**	**CD274 > 1,50 (*n* = 113) % (n)**	**OR (CI95)**	***P***	**PDCD1LG2 ≤ 1,50 (*n* = 108) % (n)**	**PDCD1LG2 > 1,50 (*n* = 142) % (n)**	**OR (CI95)**	***P***
Age									
≤ 50	88	23.9% (21)	76.1% (67)	0.6 (0.3–1.1)	0.119	55.7% (49)	44.3% (39)	1.4 (0.8–2.3)	0.255
> 50	162	33.3% (54)	66.7% (108)			48.1% (78)	51.9% (84)		
Histological grade									
1	17	23.5% (4)	76.5% (13)	1.3 (0.4–4.4)	0.622*	64.7% (11)	35.3% (6)	1	
2	86	32.6% (28)	67.4% (58)	0.9 (0.5–1.5)	0.597*	58.1% (50)	41.9% (36)	1.3 (0.4–3.9)	0.615*
3	147	29.3% (43)	70.7% (104)	1		44.9% (66)	55.1% (81)	2.3 (0.8–6.4)	0.129*
Ki67 Index									
Low (< 15%)	66	28.8% (19)	71.2% (47)	1.2 (0.6–2.2)	0.639*	62.1% (41)	37.9% (25)	1	
Intermediate (15-19%)	53	26.4% (14)	73.6% (39)	1.3 (0.6–2.7)	0.452*	35.8% (19)	64.2% (34)	2.9 (1.4–6.2)	**0.005***
High (> 19%)	131	32.1% (42)	67.9% (89)	1		51.1% (67)	48.9% (64)	1.6 (0.9–2.9)	0.145*
% sTIL									
Low (< 30%)	230	32.2% (74)	67.8% (156)	9.0 (1.2–68.6)	**0.011**	50.9% (117)	49.1% (113)	1.0 (0.4–2.6)	0.941
High (≥ 30%)	20	5% (1)	95% (19)			50% (10)	50% (10)		
Molecular Subtypes									
Luminal A-like	50	20% (10)	80% (40)	2.1 (0.8–5.1)	0.118*	52% (26)	48% (24)	1.2 (0.5–2.6)	0.688*
Luminal B/HER2-	50	32% (16)	68% (34)	1.1 (0.5–2.5)	0.832*	48% (24)	52% (26)	1.4 (0.6-3.0)	0.424*
Luminal B/HER2+	50	34% (17)	66% (33)	1.0 (0.4–2.3)	1.000*	60% (30)	40% (20)	0.8 (0.4–1.9)	0.685*
HER2-enriched	50	30% (15)	70% (35)	1.2 (0.5–2.8)	0.668*	38% (19)	62% (31)	2.1 (0.9–4.6)	0.073*
Triple-negative	50	34% (17)	66% (33)	1		56% (28)	44% (22)	1	
Lymph Nodes Status									
Positive	133	24.8% (33)	75.2% (100)	1.7 (1.0-2.9)	0.056	48.1% (64)	51.9% (69)	1.3 (0.8–2.1)	0.366
Negative	117	35.9% (42)	64.1% (75)			53.8% (63)	46.2% (54)		

Table 4 Correlation between *CD276 (B7-H3)* and *JAK2* mRNA expression and clinical-pathological variables. Chi-Square test; *logistic regression

	Patients (*n* = 250)	**CD276 ≤ 1,50 (*n* = 71) % (*n*)**	**CD276 > 1,50 (*n* = 179) % (*n*)**	**OR (CI95)**	***P***	**JAK2 ≤ 1,50 (*n* = 152) % (*n*)**	**JAK2 > 1,50 (*n* = 98) % (*n*)**	**OR (CI95)**	***P***
Ki67 Index									
Low (< 15%)	66	21.2% (14)	78.8% (52)	2.0 (1.0–4.0)	**0.048***	57.6% (38)	42.4% (28)	1.6 (0.8–2.9)	0.153*
Intermediate (15-19%)	53	20.8% (11)	79.2% (42)	2.1 (1.0-4.4)	0.059*	47.2% (25)	52.8% (28)	2.4 (1.2–4.6)	**0.009***
High (> 19%)	131	35.1% (46)	64.9% (85)	1		67.9% (89)	32.1% (42)	1	
% sTIL									
Low (< 30%)	230	26.5% (61)	73.5% (169)	0.4 (0.1–0.9)	**0.026**	60.4% (139)	39.6% (91)	0.8 (0.3–2.1)	0.688
High (≥ 30%)	20	50% (10)	50% (10)			65% (13)	35% (7)		
Molecular Subtypes									
Luminal A-like	50	18% (9)	82% (41)	3.0 (1.2–7.6)	**0.018***	54% (27)	46% (23)	2.7 (1.1–6.3)	**0.023***
Luminal B/HER2-	50	18% (9)	82% (41)	3.0 (1.2–7.6)	**0.018***	50% (25)	50% (25)	3.2 (1.3–7.4)	**0.008***
Luminal B/HER2+	50	40% (20)	60% (30)	1.0 (0.4–2.2)	1.000*	58% (29)	42% (21)	2.3 (1.0-5.4)	0.058*
HER2-enriched	50	26% (13)	74% (37)	1.9 (0.8–4.4)	0.139*	66% (33)	34% (17)	1.6 (0.7–3.9)	0.272*
Triple-negative	50	40% (20)	60% (30)	1		76% (38)	24% (12)	1	
HR Status									
Negative	100	33% (33)	67% (67)	1.5 (0.8–2.5)	0.188	71% (71)	29% (29)	2.1 (1.2–3.6)	**0.007**
Positive	150	25.3% (38)	74.7% (112)			54% (81)	46% (69)		

Table 5 Correlation between *FOXO1* mRNA expression and clinical-pathological variables. Chi-Square test; *logistic regression

	Patients (*n* = 250)	**FOXO1 ≤ 1,50 (*n* = 171) % (*n*)**	**FOXO1 > 1,50 (*n* = 79) % (*n*)**	**OR (CI95)**	***P***
Tumor size					
≤ 20 mm	124	61.3% (76)	38.7% (48)	0.5 (0.3–0.9)	**0.016**
> 20 mm	126	75.4% (95)	24.6% (31)		
Histological grade					
1	17	58.8% (10)	41.2% (7)	2.2 (0.8–6.3)	0.128*
2	86	57% (49)	43% (37)	2.4 (1.4–4.3)	**0.002***
3	147	76.2% (112)	23.8% (35)	1	
Vascular invasion					
Present	92	79.3% (73)	20.7% (19)	0.4 (0.2–0.8)	**0.004**
Absent	158	62% (98)	38% (60)		
Ki67 Index					
Low (< 15%)	66	57.6% (38)	42.8% (28)	2.7 (1.4–5.2)	**0.002***
Intermediate (15-19%)	53	56.6% (30)	43.4% (23)	2.8 (1.4–5.6)	**0.003***
High (> 19%)	131	78.6% (103)	21.4% (28)	1	
% sTIL					
Low (< 30%)	230	67% (154)	33% (76)	0.4 (0.1–1.3)	0.096
High (≥ 30%)	20	85% (17)	15% (3)		
Molecular Subtypes					
Luminal A-like	50	50% (25)	50% (25)	5.3 (2.1–13.4)	**0.001***
Luminal B/HER2-	50	56% (28)	44% (22)	4.1 (1.6–10.6)	**0.003***
Luminal B/HER2+	50	70% (35)	30% (15)	2.3 (0.9–5.9)	0.101*
HER2-enriched	50	82% (41)	18% (9)	1.2 (0.4–3.3)	0.790*
Triple-negative	50	84% (42)	16% (8)	1	
Lymph Nodes Status					
Positive	133	73.7% (98)	26.3% (35)	0.6 (0.3-1.0)	0.055
Negative	117	62.4% (73)	37.6% (44)		
HR Status					
Negative	100	83% (83)	17% (17)	3.4 (1.9–6.4)	**< 0.0001**
Positive	150	58.7% (88)	41.3% (62)		

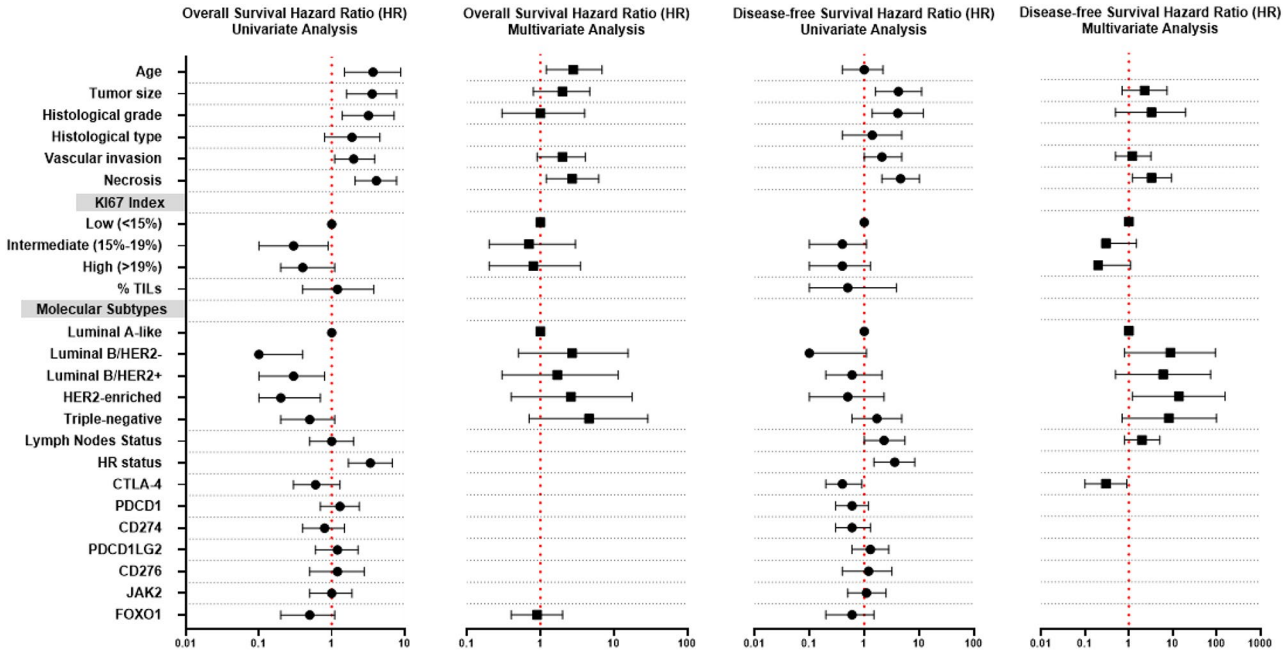

Fig. 2 Univariate and multivariate analysis of OS and DFS for all variables included in the study. **Cox regression*

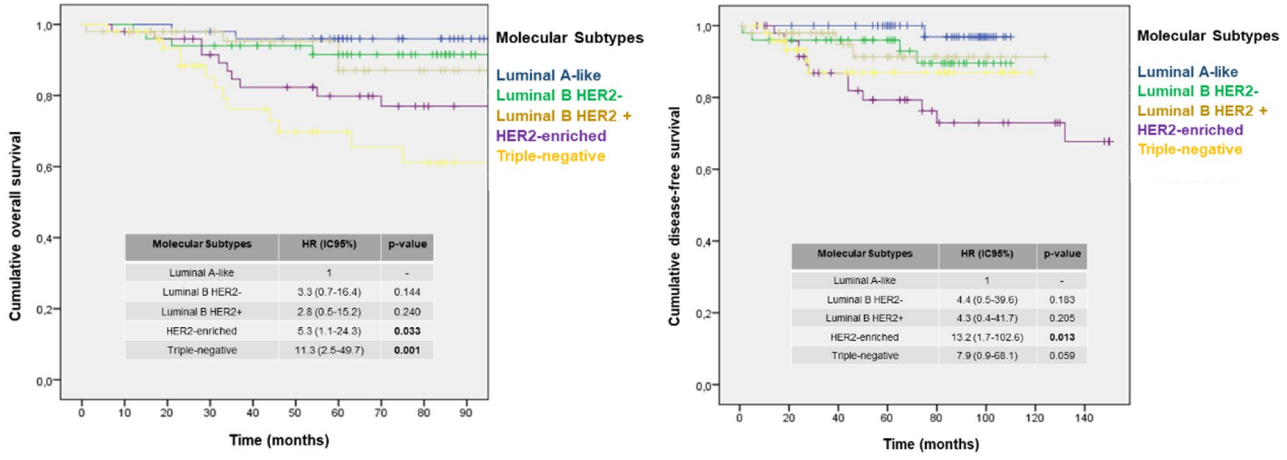

Fig. 3 Survival analysis in BC molecular subtypes for OS and DFS (Kaplan-Meier method; log-rank test)

and the presence of TILs [76], especially intratumoral Treg lymphocytes [77, 78]. These studies agree with the direct association of *CTLA-4* expression with genes involved in leukocyte differentiation and T-cell activation [75]. Nevertheless, Catacchio et al. [79] found no correlation between CTLA-4 levels and the TIL presence (stromal vs. intratumoral), reflecting BC heterogeneity. Our findings are corroborated by our previous in vitro studies, which show intrinsic expression, particularly in TN/BL cell lines [23]. Furthermore, some authors have proposed that CTLA-4 activation in neoplastic cells correlates with cell adhesion, drug metabolism, and ion and amino acid transport [74]. Considering all these data, *CTLA-4* may be a crucial IC in the etiopathogenesis of TN/BL tumors.

Few studies have evaluated *the* significance of *PDCD1 (PD-1)* in BC. They reported elevated *PDCD1* (PD-1) mRNA levels in neoplastic tissue compared to healthy tissue and associations with a lower clinical stage [80, 81], a minimal expression in neoplastic cells without apparent correlation with other clinicopathological characteristics [82], or a relation with histological grade 2–3 [83]. Additionally, the high mRNA expression observed in the peripheral blood of BC patients [80, 84] may reflect an active systemic anti-neoplastic response. Most studies have focused on analyzing its expression in TILs, showing association with larger tumors, higher grade, lymph node involvement, absence of ER, and elevated Ki67 expression [85]. Our results align with those of Ren

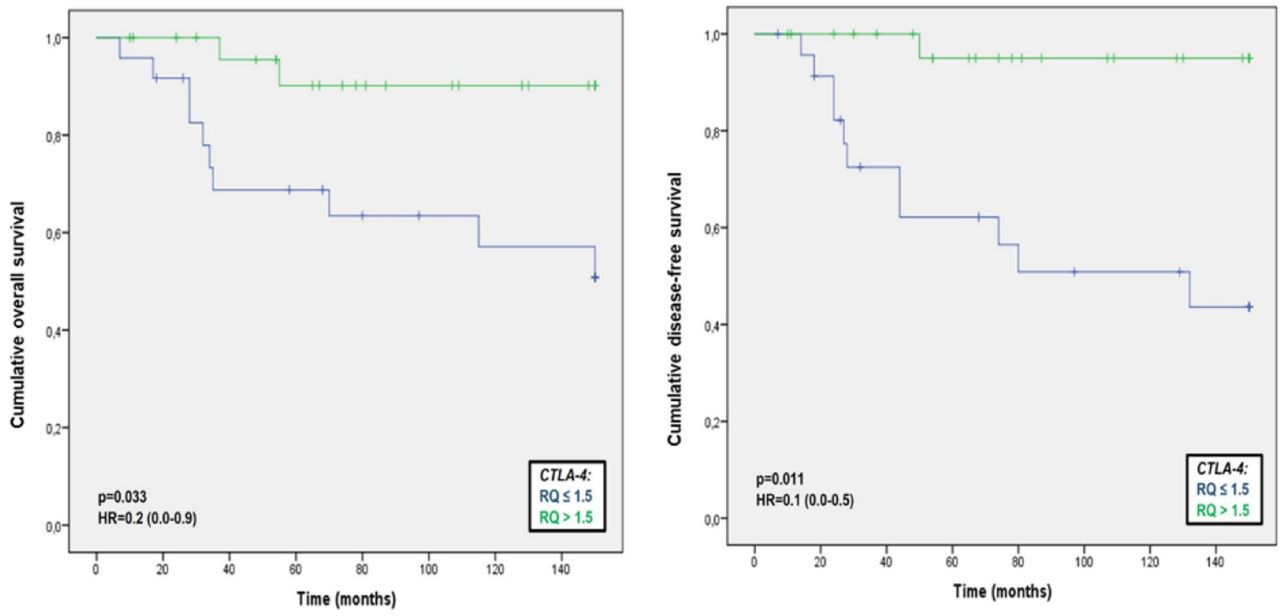

Fig. 4 Kaplan-Meier plot for overall survival (**A**) and disease-free survival rates (**B**) (%) for HER2-enriched patients classified according to tumor *CTLA-4* mRNA expression (Kaplan-Meier method; log-rank test)

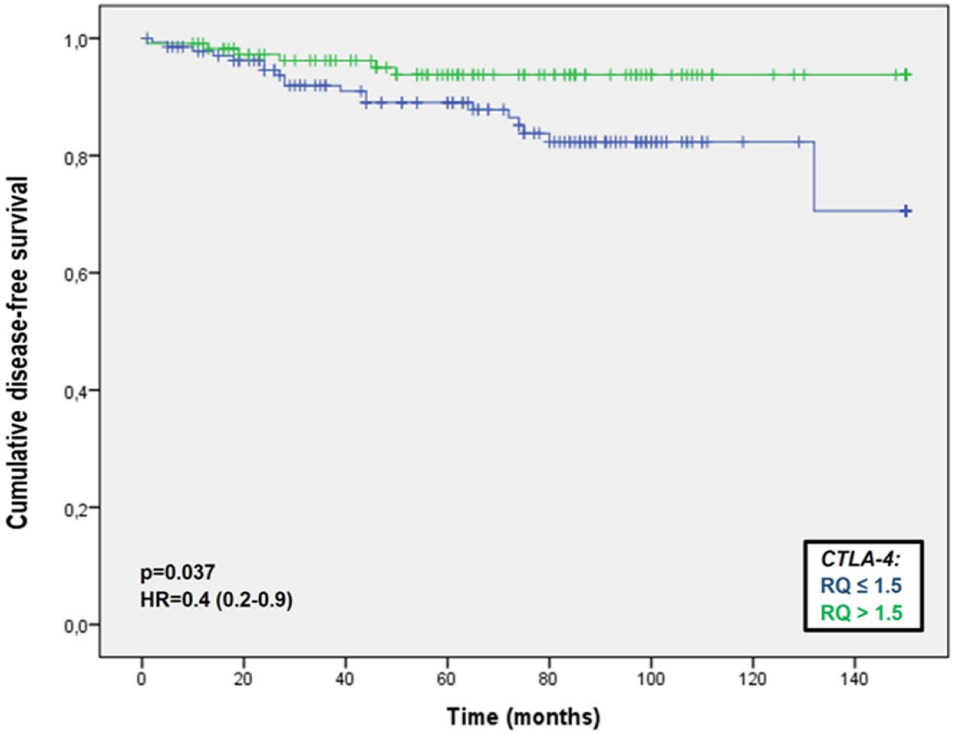

Fig. 5 Kaplan-Meier plot for disease-free survival rate (%) for all patients classified according to tumor *CTLA-4* mRNA expression (Kaplan-Meier method; log-rank test)

et al. [25] and Muenst et al. [85], who observed a direct relationship between increased proliferation, no distant metastasis, and a higher percentage of PD-1-positive TILs in luminal B/HER2-positive tumors. Similarly, Fang et al. [84] reported increased *PDCD1* (PD-1) expression in samples with a higher proportion of PD-1-positive TILs in luminal B/HER2-positive tumors. Interestingly, in peripheral blood samples from patients with PR-positive tumors, *PDCD1* (PD-1) levels were higher than HER2-positive tumors. It is known that hormones induce PD-1

expression in various immune cells, including macrophages, dendritic cells, and B lymphocytes [86]. The relationship between PD-1 expression and TILs in the TME, as described by other authors [79, 87, 88], was expected, given its involvement in lymphocyte activation and leukocyte migration [88]. Indeed, several investigators have demonstrated the association of PD-1 with intratumoral and stromal CD8+T cells, neutrophils, and fibroblasts [79, 88]. Although the intrinsic expression of *PDCD1* (PD-1) in BC has not been extensively studied in clinical series, existing data suggest stability across different BC cell lines [23] and overexpression in HER2-enriched and TN/BL phenotypes [89]. Discrepancies arise regarding its correlation with ER levels, with some studies showing no association [81]. These discrepancies may be due to the limited number of cases analyzed by these authors, as the methodology applied was similar. In addition, it is plausible that our results might be influenced by immune cells within the TME.

Regarding *CD274* (PD-L1) expression, literature is scarce and contradictory. Uhercik et al. [81] found no association between mRNA levels and clinicopathological features. In contrast, Schalper et al. [90] observed an association with a high percentage of TILs, consistent with our findings. Conversely, Sabatier et al. [91] reported overexpression in larger tumors, histological grade 3, HR-negative status, and high Ki-67 expression. PD-L1 protein expression revealed associations with younger age, larger tumor size, and high Ki-67 expression. Furthermore, several researchers noted associations with a high percentage of TILs [79, 92–95]. However, contradictions exist regarding associations with histological grade, lymph node involvement, necrosis, and HR expression [92, 93, 96–98]. These discrepancies may be due to subjective interpretation, differences in statistical cut-off points, or technical problems. Additionally, it is noteworthy that the anti-PD-L1 antibody used in these studies (E1L3N - Cell Signaling Technology) is not FDA-approved for BC.

Currently, the only FDA-approved anti-PD-L1 antibodies as companion tests in metastatic TN/BL BC are SP142 (Ventana/Roche) and 22C3 (Dako/Agilent), which select patients for treatment with atezolizumab and pembrolizumab, respectively. These antibodies are based on different clones, staining protocols, detection platforms, and scoring algorithms, with distinct cut-off points for result interpretation. They also vary in sensitivity, targeting tumor cells (22C3) or immune cells (SP142) and recognizing specific PD-L1 epitopes, resulting in unique staining patterns. Therefore, considering all of these, there are questions about whether the tests can further influence PD-L1-positive cell identification or whether it can be independently selected when making treatment decisions involving PD-1 and PD-L1 inhibitors [99]. Therefore, quantifying gene expression might offer an alternative approach. However, robust studies and clinical trials are needed to validate the use of molecular technology for evaluating PD-L1.

On the other hand, *PDCD1LG2* (PD-L2) has been relatively underexplored in BC compared to *CD274* (PD-L1). Only one study has examined its expression by immunohistochemistry. The authors observed an association with younger patients (<50 years), positive lymph node status, and ER-negative tumors [96]. However, these findings were not observed in our clinical series, likely due to methodological differences, which made the results not comparable.

Previous molecular studies have also highlighted *CD276* (B7-H3) overexpression in neoplastic tissue compared to healthy counterparts, with some expression also observed in stromal cells [100]. Immunohistochemical studies have detected expression in the membrane and cytoplasm of neoplastic cells [100–103]. Additionally, this IC is expressed in circulating neoplastic cells of BC patients, regardless of disease stage [104]. In accordance with our results, Kim et al. [105] found an association with lower sTIL density, mainly CD8+T lymphocytes. However, there are still contradictory results, as other studies showed an association with larger tumor size, lymphovascular invasion, lymph node involvement, and advanced clinical stages [100, 106], correlations with smaller tumor size and low microvascular density [101] or no significant association [102].

Regarding the BC subtype, our data agree with other authors who reported a correlation between HR-positive status and the absence of HER2. However, this association was not significant, probably because of the limited number of cases analyzed [100]. Recently, we reported intrinsic expression of *CD276* (B7-H3) in luminal cell lines [23]. Conversely, Kim et al. [105] reported significant B7-H3 overexpression in the TN/BL phenotype. These discrepancies could be explained by the existence of two isoforms of the protein, potential post-translational modifications, or the ability to bind to different receptors, which remains unknown [107, 108]. Hence, the role of B7-H3 in BC may vary depending on the pathophysiological context. Therefore, further studies with more extensive series are necessary to better define its role among BC subtypes.

Our data on *JAK2* agree with those of Liu et al. [68], who described its underexpression in BC compared to that of healthy tissue and other neoplasms. This study also reported a correlation between high mRNA levels and age<50, smaller tumor size, early clinical stage, and PR expression. Immunohistochemistry studies have confirmed its association with a higher density of TILs, mainly T and B lymphocytes, macrophages, neutrophils, and dendritic cells [109, 110]. The published data are

also consistently associated with luminal B/HER2-negative tumors [68, 111]. These results are congruent, given the association of JAK2 signaling through the prolactin receptor (PLR) with an increased risk of developing luminal tumors [112–114]. Indeed, the PLR/JAK2/STAT5 pathway plays a significant role in the pathogenesis of some neoplasms in murine models [115].

Furthermore, JAK2 has been involved in the mechanisms of resistance to tamoxifen, a selective ER modulator used in treating luminal phenotypes [50]. Collectively, these data suggest that JAK2 plays a crucial role in the development of luminal tumors and the acquisition of resistance to hormone treatment. However, since our findings have only been partially corroborated and there is limited published data, further studies are necessary.

Finally, *FOXO1* expression in BC exhibits variability [69], with inconsistent association across different BC subtypes. While some studies, including ours, show a significant correlation with smaller tumor size, negative lymph nodes, HR-positive status, and HER2-negative expression [56, 116], other studies show associations only with negative PR, HER2 expression, and elevated Ki-67 [69]. Furthermore, FOXO1 may promote or repress ER and PR functions in BC, depending on the cellular context [117–120]. Recent findings suggest that *FOXO1* expression is modulated by the transcription factor E2F1 [121], associated with tamoxifen resistance [122]. Therefore, these data highlight the complex role of *FOXO1* in the pathogenesis of luminal phenotypes and emphasize the need for further research to define its role in this molecular subtype.

Regarding patients' outcomes, none of the genes of interest showed significant association with OS in our study. However, we observed a trend suggesting that patients with tumors overexpressing *FOXO1* had longer OS, consistent with its correlation with favorable clinicopathological features. Nonetheless, while several studies indicated that *FOXO1* expression may modulate progression and metastasis in BC [123, 124], this has not been confirmed by Lallemand et al. [69].

Concerning DFS, we observed fewer recurrences when tumors overexpressed *CTLA-4*, which was shown as an independent prognostic factor in BC patients. Our results agree with a recent molecular study [67], although opposite results have been published [73, 125, 126]. These discrepancies may be partially attributed to methodological differences (mRNA vs. protein expression) or data interpretation. For instance, Yu et al. [125] evaluated CTLA-4 protein expression in tumor cells and TILs. They found that high CTLA-4 expression in TILs correlated with a better prognosis, whereas its positivity in tumor cells was associated with a worse outcome. Therefore, our results might be influenced by CTLA-4-positive TILs in TME.

The analysis of OS and DFS based on *CTLA-4* expression levels, stratified by phenotype, revealed an association with favorable prognosis in patients with HER2-enriched tumors. Similarly, it has also been associated with improved survival, particularly in TN/BL tumors [67, 75]. On the contrary, CTLA-4 overexpression, at both mRNA and protein levels, has been linked to poorer OS and DFS [125, 127]. These contrasting results suggest a significant role for CTLA-4+TILs in this tumor subtype. Nonetheless, further independent studies are necessary to confirm these findings.

Studies in animal models have demonstrated that anti-CTLA-4 treatment promotes T-lymphocyte activation and neoplastic cell eradication [128, 129]. Consequently, investigation into two humanized monoclonal antibodies targeting CTLA-4 (ipilimumab and tremelimumab) have been undertaken in different clinical trials. In a phase I trial, tremelimumab was evaluated with exemestane in 26 patients with advanced luminal BC, where 42% (11/26) exhibited stable disease for at least 12 weeks [130]. Moreover, increased levels of CD4+and CD8+T-lymphocytes and reduced Treg were observed peripherally [130]. Similar results were noted in a pilot study combining ipilimumab with cryoablation in 19 patients with early BC [131].

In contrast, the phase II clinical trial NCT02536794 (registration date 2015-Sept-01) assessed the efficacy of the combined durvalumab (anti-PD-L1) and tremelimumab treatment in 18 patients with luminal or TN/BL metastatic BC [132]. Preliminary results indicated clinical benefit in 71% of patients with TN/BL tumors, although the study was discontinued because the objective response rate (ORR) did not meet the required criteria. Currently, several clinical trials are ongoing in patients with TN/BL tumors, exploring the combination of nivolumab (anti-PD-1) and ipilimumab with cryoablation, radiotherapy, or oncolytic viruses, as well as durvalumab and tremelimumab alone or in combination with nab-paclitaxel and neoantigen vaccines. However, their preliminary results are awaited.

Conclusions

In conclusion, our findings suggest that *CTLA-4*, *PDCD1* (PD-1), *CD274* (PD-L1), and *PDCD1LG2* (PD-L2) expression correlates with unfavorable clinicopathological characteristics, while *CD276* (B7-H7), *JAK2* and *FOXO1* expression may not directly contribute to BC aggressiveness. Paradoxically, our data indicate that *CTLA-4* expression is a significant independent favorable prognostic factor for DFS in BC patients, particularly in the HER2-enriched subtype. These results support the hypothesis that the expression of immunoregulatory genes depends on tumor phenotype and may hold clinical implications in BC patients.

Abbreviations

BC	Breast cancer
CI	Confidence Interval
CTLA-4	Cytotoxic T lymphocyte antigen 4
DBUGH	Dr. Balmis University General Hospital
DFS	Disease-free survival
EFS	Event-free survival
EMT	Epithelial-to-mesenchymal transition
ER	Estrogen receptor
FDA	Food and Drug Administration
FFPE	Formalin-fixed paraffin-embedded
HER2	Human epidermal growth factor receptor 2
HR	Hormone receptor
IC	Immune checkpoints
ICI	Immune checkpoint inhibitors
OR	Odds ratio
OS	Overall survival
PD-1	Programmed cell death protein 1
PFS	Progression-free survival
PR	Progesterone receptor
qPCR	Real-time quantitative polymerase chain reaction
RQ	Relative quantification
STATs	Signal transducer and activator of transcription proteins
sTIL	Stromal tumor-infiltrating lymphocytes
TILs	Tumor-infiltrating lymphocytes
TME	Tumor microenvironment
TN/BL	Triple-negative/basal-like

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12935-024-03554-4.

Supplementary Material 1

Supplementary Material 2

Acknowledgements

We want to particularly acknowledge patients and both DBUGH and HUB-ICO-IDIBELL Biobanks (PT20/00171) for their collaboration, being the latter integrated into the ISCIII Biobanks and Biomodels Platform and Xarxa Banc de Tumors de Catalunya (XBTC).

Author contributions

Conceptualization, Y.G.M.-P., and G.P.; methodology, Y.G.M.-P., J.J.P., S.D.-G., T.A.M., H.B., E.C.-M., A. R.-M., I. L.-C., J.M.S.-O., and G.P.; validation, Y.G.M.-P.; formal analysis, Y.G.M.-P.; investigation, Y.G.M.-P., and G.P.; resources, J.J.P., S.D.-G., T.A.M., H.B., E.C.-M. A. R.-M., I. L.-C., and G.P.; data curation, Y.G.M.-P., and G.P.; writing—original draft preparation, Y.G.M.-P.; writing—review and editing, Y.G.M.-P. and G.P.; visualization, Y.G.M.-P. and G.P.; supervision, G.P.; project administration, G.P.; funding acquisition, J.M.S.-O. and G.P. All authors have read and agreed to the published version of the manuscript.

Funding

The Alicante Institute for Health and Biomedical Research (ISABIAL) funded this research (Grants: NR-180202; NR-19-293; 2020 – 0325; 2022 – 0280).

Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

The study was conducted following the Declaration of Helsinki and approved by the Institutional Ethics Committee of Dr. Balmis University General Hospital (protocol code PI2018/048, approved on 30 May 2018; protocol code PI2019-058, approved on 29 January 2020; protocol code PI2020-242, approved on 15 December 2020 and protocol code PI2022-092, approved on 29 June 2022). Written informed consent was obtained from individual participants.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Received: 14 June 2024 / Accepted: 2 November 2024
Published online: 10 November 2024

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