Immune Checkpoint Molecules B7-H6 and PD-L1 Co-Pattern The Tumor Inflammatory Microenvironment: Associations and Clinical Significance in Human Breast Cancer

Boutheina Cherif (boutheina.cherif.cbs@gmail.com)
Centre of Biotechnology of Sfax
Hana Triki
Centre of Biotechnology of Sfax
Slim Charfi
Hopital Universitaire Habib Bourguiba
Lobna Bouzidi
Hopital Universitaire Habib Bourguiba
Wala Ben Kridis
Hopital Universitaire Habib Bourguiba
Afef Khanfir
Hopital Universitaire Habib Bourguiba
Kais Chaabane
Hopital Universitaire Hedi Chaker
Tahya Selami-Boudawara
Hopital Universitaire Habib Bourguiba
Ahmed Rebai
Centre of Biotechnology of Sfax

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Abstract

B7-H6 and PD-L1 belong to the B7 family co-stimulatory molecules fine-tuning the immune response. This report exposes for the first time the clinical implication of B7-H6 protein expression in relation with PD-L1 status and Natural Killer Cells infiltration as potential biomarkers in tumor inflammatory microenvironment. Herein, we explore the expression levels of B7-H6 protein by cancer cells and immune infiltrating cells in human breast cancer tissues and evaluate their associations with PD-L1 expression, NK cell status and clinical pathological features as well as the prognosis. Immunohistochemistry labeling method was used to assess B7-H6 and PD-L1 proteins expression by cancer and immune cells. Associations between immune checkpoint, major clinicopathological variables and survival rates were analyzed. B7-H6 protein was revealed in both breast and immune cells. Tumor B7-H6 expression is highly associated with Her2 over expression. B7-H6 + immune cells are highly related to SBR grade and associated with PD-L1 and NK cells status. Survival analysis showed that patients with low expression of B7-H6 by cancer cells had better prognosis. Conversely, B7-H6 + immune cells were significantly associated with longer survival. Our result strongly suggests an interaction between B7 molecules that contributes to a particular design of the inflammatory microenvironment. This may influence the efficiency of therapies based on antibodies blocking the PD-L1/PD1 pathway and can explain why the clinical benefits are seen only in a fraction of patients treated with immune checkpoint inhibitors.

Introduction

Breast cancer is the most diagnosed malignancy in women worldwide and is a highly heterogeneous disease presenting a broad range of molecular and clinical characteristics [1]. Towards this heterogeneity, the establishment of an effective immune response requires the participation of several actors taking into account this variability. Both adaptive and innate immunity contribute to the immune editing of malignancy features [2, 3]. But more attention is devoted to natural immunity particularly to Natural killer (NK) cells which are important components of the innate immune system and play a central role in tumor microenvironment (TME) designing [4] since they participate in adaptive response through their cytokine secretion polarizing T-cell activation [5–7].

Moreover, the modulation of NK cells response can be controlled by the immune checkpoints molecules from the B7 family [8]. The most known immune checkpoint regulators are programmed cell death 1 (PD-1)/PD-1 ligand 1 (PD-L1) and CTL antigen 4 (CTLA-4), which are highlighted in a variety of cancers and their therapeutic advances have encouraged researchers to investigate other targets from the B7/CD28 family. Recently, five new B7 family ligands, B7-H3, B7-H4, B7-H5, B7-H6, and B7-H7, were identified. These later are increasingly investigated in various solid cancers looking for significant association with cancer progression and patient’s prognosis in different clinical retrospective studies [9–12].

Among this group, B7-H6 seems to be a potential target for new immunotherapy strategy. B7-H6 (also known as NCR3LG1) is a ligand for NK-cell–activating receptor NKp30 [13]. Human B7-H6 protein is rarely expressed in normal tissues, but in contrast, is overexpressed in various primary human tumors, including leukemia, lymphoma, and gastrointestinal stromal tumors [14]. However, B7-H6 can be induced at the surface of CD14(+)CD16(+) proinflammatory monocytes and neutrophils upon stimulation by ligands of Toll-like receptors or proinflammatory cytokines such as IL-1β and TNF-α [15]. B7-H6 binds to NKp30 through the complementarity-determining region (CDR)-like loops of its V-like domain in an antibody-like interaction [16]. NK cells eliminate tumor cells expressing B7-H6 directly by cytotoxicity through co-signals balancing between activators and inhibitors ones, or indirectly by cytokines release. Hence the expression of B7-H6 by tumor cells is an important mechanism involved in activation of innate immunity mediated by NK cells. But to divert this visibility to the immune system, the malignant cells release or hide the B7-H6 molecule to prevent NK-mediated recognition [17].

Regarding the clinical significance of B7-H6 expression in human cancers, it has been provided in few studies. In ovarian cancer, positive B7-H6 staining was predominantly observed on the membrane and in the cytoplasm of the ovarian cancer cells. The overall survival rate of the subgroup with lower B7-H6 expression was significantly better compared to those with higher B7-H6 expression [18]. In astrocytoma, B7-H6 positive expression was significantly associated with World Health Organization Grade [19]. Recently, high expression of B7-H6 has been proven to be a predictor of poor prognosis in esophageal squamous cell [20]. In the case of breast cancer disease, B7-H6 expression was revealed to be an unfavorable prognosis biomarker [21].

Despite these established expression in cases of human solid cancers, the association between B7-H6 and PD-L1 expression in human cancer remains unknown. In the current study, we focused on determining the co-expression profiles and clinical significance of B7-H6 and PD-L1 in women breast cancer, we assessed especially the contribution of B7-H6 and PD-L1 expression by tumor cells and immune cells colonizing tumor microenvironment. We also examined and compared their relation with NK cells status in controlling patient’s survival.

Results

B7-H6 and PD-L1 immunodetection in breast cancer

The immunohistochemistry analysis showed positive staining for B7-H6 (Fig. 1a) and PD-L1 (Fig. 1b) both on the membrane and in the cytoplasm of cancer cells, but also in TILs (Fig. 1a and 1b). Representative examples of B7-H6 and PD-L1 immunostaining in breast cancer cells (BCC) and TILs are shown in Fig. 1c and 1d respectively. B7-H6 and PD-L1 expression were evaluated successfully by IHC in 156 BC tissues and positive controls at differential extent and intensity (Fig. 1c). Based on the extent and intensity of cyto-membranous immunostaining in BCC, H-scores were generated as described in methods. In order to investigate the correlation between clinical parameters and the B7-H6 and PD-L1 protein expression levels in BCC, we categorized the 156 patients into two major subgroups according to the intensity of B7-H6 and PD-L1 immunohistochemical staining. Our data show that B7-H6 BCC expression levels were low in 61% (0 ≤ H-score < 100) and high in 39% of cases (H-score ≥ 100). For PD-L1 BCC distribution in our cohort, our results show that PD-L1 BCC expression levels were low in 80% (0 ≤ H-score < 100) and high in 20% of cases (H-score ≥ 100). Regarding TILs densities distributions, TILs-B7-H6 + were predominantly localized in the stroma surrounding BCC clusters and abundant in 14% of cases, conversely, TILs-PD-L1 + were abundant in 60% of cases.

Correlation of B7-H6 and PD-L1 expressions with clinicopathological features
The correlation between patient’s clinical parameters and B7-H6/PD-L1 expression has been shown in Table 1. Statistical analyses show a significant correlation of B7-H6 BCC expression with only Her-2 expression ($p < 0.01$) and molecular subtypes ($p < 0.01$). However, we did not find any correlation with SBR grade, tumor size, lymph node invasion or metastasis. For correlation of TILs-B7-H6 status with clinicopathological parameters, we found significant relation with lymph node status ($p < 0.05$), ER status ($p < 0.01$) and SBR grading ($p < 0.001$). Regarding PD-L1 expression, our results display a significant correlation of PD-L1 BCC expression with histological type ($p < 0.05$) and SBR grade ($p < 0.001$), but no correlation between patient’s clinical parameters and TILs-PD-L1 status was found except of a limited significant correlation with SBR grade ($p = 0.049$).

**Correlations of B7-H6 expressions with PD-L1 and NK-TILs status**

To look for eventual role of B7-H6 in inflammatory tumor microenvironment designing, we investigated the relationship of B7-H6 expression with PD-L1 and NK-TILs status. For more ascertainment, we conducted two types of analyzes. The first way evaluates the relations on the raw results either in H-scores for the BCC expression or in TILs scores shared in 3 classes, as it was described in material and method. Data of these analyzes are shown in Fig. 2. The second way evaluates the relationships between the different parameters after the classification into two classes according to the cut-off mentioned in material and method and based on other previous works [18], [22]. Analyzes based on raw data show significant correlations of PD-L1 BCC expression with the status of NK-TILs ($p < 0.001$), TILs-B7-H6 ($p < 0.001$), and TILs-PD-L1 ($p < 0.001$) (Fig. 2a-c). In contrast, a poor significant correlation of B7-H6 BCC expression with the status of TILs-PD-L1 (Fig. 2d, $p = 0.0318$) and NK-TILs (Fig. 2f, $p = 0.0289$) and insignificant correlation with TILs-B7-H6 (Fig. 2e) was found. Nevertheless, the TILs-B7-H6 status is strongly correlated with the status of TILs-PD-L1 (Fig. 2i, $p < 0.001$) and NK-TILs (Fig. 2h $p < 0.001$) but slightly less TILs-PD-L1 status has a significant relationship with NK-TILs infiltration (Fig. 2g $p < 0.01$). The second way of analysis based on binary classes did not display any significant relationships between B7-H6 BCC expression and all others biomarkers status. However, the status of B7-H6-TILs shows significant associations with the status of NK-TILs ($p = 0.0419$), TILs-PD-L1 ($p = 0.0268$) and PD-L1 BCC expression ($p < 0.01$). In addition, analysis deciphered a limited significant correlation between the status of NK-TILs and TILs-PD-L1 ($p = 0.0284$) and PD-L1 BCC expression ($p = 0.0232$) (Supplementary Table).

**Prognostic significance of B7-H6 expression**

In order to look for the prognostic value of B7-H6 expression in women breast cancer, the log-rank survival analyses were performed according to B7-H6 expression levels in BCC or TILs scores after classification and collection of survival data. As shown in (Fig. 3a), patients with low B7-H6 BCC expression had a markedly longer OS compared to patients with high B7-H6 expression, however DFS curves are inverted but almost confused (Fig. 3b). In regards to TILs-B7-H6 status in survival analysis (Fig. 3c and d), both OS and DFS rates of the subgroup with higher TILs-B7-H6+ are longer than those of the subgroup with lower TILs-B7-H6+, Kaplan-Meier survival curves for OS show significant differences between the two groups ($p = 0.017$) but no significance with DFS curves ($p = 0.128$). We then addressed the prognostic relevance of B7-H6 expression by tumor cells or TILs, we investigated survival data among combined groups; group 1 = TILs-B7-H6Low/B7-H6 BCCLow, group 2 = TILs-B7-H6High /B7-H6 BCCLow/High and group 3 = TILs-B7-H6Low/B7-H6 BCCHigh. As shown in Fig. 3 patients belonging the group 2 had better survival rates compared to patients in the others two groups with significantly longer OS (Fig. 3e, $p = 0.028$) but insignificant longer DFS (Fig. 3f, $p = 0.166$).

**Prognostic significance of the concomitant expression of B7-H6 and the others immune inflammatory biomarkers**

To better understand the impact of B7-H6 expression in relation with PD-L1 and NK cell status on patients survival, different subgroups were created taking into account the classifications according to PD-L1 and NK-cell statutes. Figure 3g-i show survival curves (only for OS) of subgroups formed by combinations between B7-H6 BCC status with PD-L1 BCC or TILs-PD-L1 or NK-TILs status. Figure 3j-l shows survival curves (OS) of subgroups formed by combinations between TILs-B7-H6 status and PD-L1 BCC or TILs-PD-L1 or NK-TILs status. Regardless of the three types of combinations, patients with B7-H6 BCCHigh/PD-L1 BCCLow (Fig. 3g) or B7-H6 BCCHigh/TILs-PD-L1low (Fig. 3h) or B7-H6 BCCHigh/NK-TILsLow (Fig. 3i) status have obviously the shorter OS among the others subgroups. Conversely, OS curves of TILs-B7-H6 combination groups show that a longer survival is always observed in the subgroup with the high profile of TILs-B7-H6 associated with high status of either PD-L1 BCC (Fig. 3j) or TILs-PD-L1 (Fig. 3k) or NK-TILs (Fig. 3l). The differences are important in the case of overall survival to achieve a significant variations in the combinations TILs-B7-H6High/NK-TILsHigh ($p = 0.017$). Nevertheless, TILs-B7-H6High group in combination with PD-L1BCC or TILs-PD-L1 groups always shows a better survival ($p = 0.058$). In regards to multivariate analyzes, cox-regression showed a significant effect of TILs-B7-H6 (hazard ratio [HR] = 0.038, 95% confidence interval [CI] = 0.01–0.730, $p = 0.018$), where all 20 patients with high expression have survived. Furthermore, association of NK-TILs status with OS is also significant in multivariate analyses (Cox-regression) after adjusting for Her2 status (HR = 0.169, CI = 0.039–0.728, $p = 0.017$).

**Prognostic significance of B7-H6 besides others immune inflammatory biomarkers in Her2 positive cancer samples**

In order to better understand the impact of B7-H6 and others immune checkpoint molecules within Her2 positive cancer subtype, we sought correlations between clinical parameters and B7-H6 or PD-L1 expressions by tumor cells and TILs (Table 2), statistical analysis show only significant associations of B7-H6 or PD-L1 BCC expression with SBR grade ($p = 0.025$ and $p = 0.035$ respectively) but a limited significant association between TILs-B7-H6 status and lymph node invasion ($p = 0.055$). Furthermore associations between immunological parameters were performed (Supplementary Fig. 1), results show the same trend across the whole cohort with the exception of the relationship between B7-H6 BCC expression and TILs-B7-H6 status where boxplots show a limited significant association between them (Supplementary Fig. 1e, $p = 0.051$). Similarly but with less intensity in $p$-value, data indicate significant correlations between PD-L1 BCC expression and the status of TILs (Supplementary Fig. 1a-c, $p < 0.05$). Regarding the prognostic value of B7-H6 and PD-L1 through survival analyzes among Her2 positive patients, same observations were recorded with OS curves in whole cohort analyzes showing longer survival for patients with high TILs-B7-H6 status and shorter survival for patients with high B7-H6 BCC expression (Supplementary Fig. 2a-c). In contrast, no difference in
OS curves was observed in high or low TILs-PD-L1 presence but patients with high PD-L1 BCC expression tended to have a better survival (supplementary Fig. 2d-f).

**Discussion**

It remains challenging to build knowledge about the dynamism of the inflammatory tumor microenvironment and to understand the behavior of the main intersecting mediators. A growing number of studies have shown that inflammation plays a critical role in tumorigenesis [23]. The inflammatory tumors microenvironment is characterized by the presence of host leukocytes both in the supporting stroma and in tumor areas [24]. These leukocytes are commonly called Tumor-infiltrating lymphocytes (TILs) which are essential for establishing an immune antitumor response but they may contribute to cancer growth and immunosuppression associated with malignancy [34, 35]. TILs can be adaptive immune cells or innate immune cells such as cytotoxic cells particularly NK cells which we are interested in studying in our cancer research fields. Moreover, it is clear that inflammatory status involves several molecules such as immune checkpoints. These molecules participate mutually but differently in cellular response mediated by NK cells, but the profile of these molecules is not well understood. Members of the B7 family have been shown to be important participants in the TME designing notably B7-H1 (or PD-L1) molecule was widely studied in breast cancer and others solid tumors [27]. Others are less studied such as B7-H6 molecule which appears to be of importance. In this study, we have described the expression of B7-H6 and PD-L1 both on cancer cell and TILs in women breast carcinoma. Our results show for the first time the expression of B7-H6 by TILs suitably within solid tumors. We have analyzed the widest part of the biopsy because we are convinced that TMA’s tissue formats might not provide sufficient representation of the TME notably, immune infiltrating cells. Previous studies have shown only the expression of B7-H6 by cancer cells but no study have reported their expression by immune cell when analyzes are performed on TMA tissues [18, 21], [37]. So, one must be vigilant in certain technical aspects which can limit or even distort results, this point was raised previously by Sobral-Leite and colleagues [29].

Our study is the first to investigate the concomitant profile of B7-H6 and PD-L1 expression in breast carcinoma tissues by exploring their relationship first, and then their implication in innate immunity through their associations with NK cells status, and finally by looking for their combined prognostic value. Our results have shown that the two immune checkpoint molecules of interest participate differently in breast carcinoma physiopathology despite the strong association between them. Originally, our data have shown that the biomarker B7-H6 has a different clinical significance depending on its expression either by tumor cells or by infiltrating immune cells. In fact, statistical analysis demonstrated that high levels of B7-H6 in tumor cells are strongly correlated with Her2 expression, this result is in agreement with those of Sun and colleagues [21]. However, high B7-H6 + TILs are strongly associated with SBR grade, ER expression and lymph node invasion. Secondly, patients with high B7-H6 tumor expression had worse survival than those with low B7-H6 expression, this result is similar to data from other researchers in both ovarian cancer and breast cancer [18, 21]. Nonetheless, patients with high TILs-B7-H6 expression displayed better survival. Regarding PD-L1 biomarker expression, our data showed that its expression by tumor cells or immune stromal cells has the same clinical significance. However, we have noted the strong association of PD-L1 BCC and TILs-PD-L1 with SBR grade. In addition, patient survival curves according to these two biomarkers status display the same trends showing no differences between strong and weak expressions (Supplementary Fig. 3a-d). These data are consistent with the findings of some studies, while others have shown the opposite. This point has been reviewed by numerous experts who have justified the discrepancy in clinical significance of PD-L1 immune checkpoint among published reports [27]. Controversy is argued by the use of different technical supports by different teams and the lack of a complete standardized platform. On the other hand, the great heterogeneity between studied populations may explain also the observed differences.

Further, we seek to explore the relationship between B7-H6 and PD-L1 and their impact on NK cells recruitment. Ours analyzes have shown that there is a strong association between the profiles of PD-L1 in tumor or immune cells with B7-H6-TILs status. In addition, both TILs-B7-H6 and TILs-PD-L1 are highly associated with NK-TILs status. These results consolidate the concept of the mutual crosstalk between tumor cells and immune cells involving different checkpoint molecules which control not only T cell activity, but also NK cells function in a synergistic manner. Knowing that NK cells are associated with better disease outcomes even though they are a minority population in TILs (Supplementary Fig. 4), these cells play an important role in front of the tumor burden. Additionally, survival analyzes were conducted on different combining groups based on the expression of B7-H6, the main biomarker of this study, with the other immunological parameters that we have chosen to associate which are PD-L1 and NK-TILs statuses. Through these analyzes, our data showed that the strong expression of B7-H6 by cancer cells probably leads to a bad progression of the disease among all subgroups. Similarly, but inversely, the high expression of B7-H6 by the immune cells among different subgroups has probably a relation with the awakening process of the immune system involving NK cells and giving the better disease outcomes. This is justified by the data of survival curves according to NK-TILs presence in the tumor or surrounds, we have noted that the best group is for patient having the combination of high NK-TILs and TILs-B7-H6 together. Thus, the immune checkpoint B7-H6 and PD-L1 are linked together and certainly with other molecules in a way which modulates the inflammatory tumor microenvironment, we speculate a double role which can polarize the immune response either towards an effective antitumor response or the opposite by maintaining a chronic inflammatory state leading to immunosuppression. Already, different mechanisms damping the function of NK cells have been shown through B7 molecules action. However, there is a lack of knowledge about the expression of these molecules which act differently, indeed their localization whether expressed on the cellular membrane or sequestered in the cytoplasm or even secreted. These concepts justify the complexity in understanding the mechanisms controlling the tumor inflammatory microenvironment through B7 molecules. Taking into account our finding from the present study, we can suspect a protective role of B7-H6 and PD-L1 molecules when co-expressions are manifested on lymphocytes infiltrating tumors, probably these cells produce interleukins such INF-γ which modulate NK cells function. To better elucidate the immunomodulatory mechanism of B7 molecules, it is important to combine more precise analyzes of their location and to conduct analyzes allowing biomarkers co-revelation. Therefore, immunotherapy targeting checkpoint molecules must be revised. The use of combined immune checkpoint inhibitors may result in reduction of tumor burden and patient benefit in some cases. In others situations probably it cannot be effective because of the double role of these two molecules, targeting other molecules from the B7 family can be an important axis which deserves further investigations to decipher their contribution in innate immunity monitoring, probably, we will need to target several molecules at the same time and use targeted therapy according to the patient characteristics.
Material And Methods

Patients and tissues samples

Formalin-fixed, paraffin-embedded tissue samples were collected from 156 patients with primary invasive breast carcinomas who underwent surgical resection at the Department of Gynecology and Obstetrics of the Hedi Chaker University Hospital (Sfax, Tunisia). All procedures performed in this study were in accordance with the ethical standards of the institutional and the national research committee of Habib Bourguiba and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All specimens were analyzed at the Department of Pathology of the Habib Bourguiba University Hospital (Sfax, Tunisia) and all tumor tissues were confirmed as the serous breast cancer by using hematoxylin and eosin (H&E) staining after surgical resection. Clinical-pathological data of patients were retrieved from the hospital’s electronic records and available paper records. They included age, histological grade, histological type, molecular subtype, tumor size, lymph node status, distant metastasis, and lymphovascular invasion. All tumors were graded according to Elston Ellis modification of the Scarff-Bloom-Richardson (SBR) histological grading system [30]. The clinical stage was determined according to TNM (tumor, lymph node and metastasis) classification adopted by the International Union Against Cancer [31]. Outcomes data were collected from patient follow-ups at the department of medical oncology of the Habib Bourguiba University Hospital (Sfax, Tunisia). Overall survival (OS) was defined as time in days from the beginning of the study observation period until death. If the event, death, did not occur, then the OS was noted as the total study observation period in days. Disease free survival (DFS) was defined from the date of the end of treatment until recurrences or last follow-up.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue samples were cut into 5-µm-thick section. Before immunostaining, haematoxylin and eosin-stained standard slides were reviewed by experimented pathologist (S.C) from each section of breast cancer tissues. A representative tumor region and the corresponding formalin-fixed paraffin-embedded tissue block were selected for use in the tissue array block containing six fragments of 7 mm in diameter.

In order to validate the immunohistochemical staining for special biomarker, striated muscle and placenta tissues were used as positive controls for the revelation of B7-H6 and PD-L1 respectively according to the instructions proposed by the manufacturers. For immunohistochemistry (IHC), each tumor array was cut into 2-µm sections and mounted on Leica Microsystems BOND Plus slides and dried overnight at 60 °C. Immunohistochemistry steps were performed as described in our previous study [32]. Briefly, tissue slides were deparaffinized in xylene followed by subsequent rehydration through graded alcohols then washed in purified water. Heat-induced antigen retrieval was performed at 95 °C for 20 min in a pH = 9 epitope retrieval solution (Leica Novocasta) for 40 min. After heating, slides were allowed to cool down to room temperature and were briefly washed in phosphate-buffered saline (PBS) solution. Immunohistochemical staining was performed using the Novolink Polymer Detection System (RE7150-K, Leica Biosystems). Briefly, endogenous peroxidase activity was neutralized by Peroxidase Block for 5 min. Slides were washed with PBS, followed by application of Protein Block for 30 min. Following another PBS wash, tissue sections were incubated for 60 min at room temperature with primary antibody anti-PD-L1 at 1:100 (Human B7-H1, AF156 R&D systems), or anti-B7-H6 at 1 : 100 (Human B7-H6, NBP1-91144 Novusbio) or with the anti-CDS6 at 1:50 (NCL-CDS6-186, Leica Novocasta). Slides were washed with PBS-Tween, then, only PD-L1 and CD56 antibodies reaction have been submitted to an adequate post primary antibody according to their species for 30 min. All tests received Novolink polymer solution for 30 min. DAB (Novolink DAB substrate) was used as the chromogen and Novolink hematoxylin as the nuclear counterstain. Sections were dehydrated, cleared and mounted.

Tumor immunostaining scoring and molecular subtyping

The B7-H6 immunostaining densities in tumor cells were analyzed according to the H-score method which has been described in a previous publication [33]. Briefly, H-score was assessed on the basis of the percentage of positive tumor cells with clearly brown cytoplasm and/or membrane immunostaining. H-score was calculated as follows = (% tumor cells unstained x0) + (% tumor cells stained weak x1) + (% tumor cells stained moderate x2) + (% tumor cells stained strong x3), and it ranged from 0 (100% negative tumor cells) to 300 (100% strong staining tumor cells). B7-H6 expression was dichotomized into two groups according to the frequency distributions of the H-scores displaying the median as the cut-off value (H-score 0–99 = negative/low expression, and 100–300 = strong expression). B7-H6 expression was dichotomized into two groups according to the frequency distributions of the H-scores displaying the median as the cut-off value (H-score 0–99 = negative/low expression, and 100–300 = strong expression). B7-H6 expression was dichotomized into two groups according to the frequency distributions of the H-scores displaying the median as the cut-off value (H-score 0–99 = negative/low expression, and 100–300 = strong expression). B7-H6 expression was dichotomized into two groups according to the frequency distributions of the H-scores displaying the median as the cut-off value (H-score 0–99 = negative/low expression, and 100–300 = strong expression). B7-H6 expression was dichotomized into two groups according to the frequency distributions of the H-scores displaying the median as the cut-off value (H-score 0–99 = negative/low expression, and 100–300 = strong expression).

Frequency and staining intensity of PD-L1 by tumor cells were analyzed, and PD-L1 expression was quantified using the H-score method which has been described in a previous publication [26, 27].

Breast cancer molecular classification is based on the expression of classical biomarkers including estrogen (ER) and progesterone (PR) receptor, the human epidermal growth factor receptor 2 (Her2) and Ki-67 labeling index as a cell proliferation biomarker. Expression of all biomarkers was carried out using immunohistochemical method. Five molecular subtypes were defined: Luminal A (LA) if ER/PR +, Her2- and Ki-67 < 20%; Luminal B like (LB-Like) if ER/PR +, Her2- and Ki-67 ≥ 20%; Luminal B (LB) if ER/PR + and Her2+; HER2 (H) if ER/PR- and HER2+; Triple Negative Breast Cancer (TNBC) if ER/PR - and Her2- [28–30].

Assessment of tumor-infiltrating lymphocytes (TILs)

TILs PD-L1 were evaluated in at least 3–4 representative high-power field (HPF) areas from each sample, and the mean PD-L1 + TILs count was scored using the following three-tier scoring system: 0–5%/HPF (1), 5–50%/HPF (2), > 50%/HPF (3) [38]. For TILs B7-H6 assessment, we applied the same strategy deployed in PD-L1 enumeration due to the absence of recent publication showing TILs B7-H6 expression in solid tumor, so we have considered the three-tier scoring described above. TILs CD56 + were assessed differently from TILs-PD-L1 and TILs-B7-H6. TILs CD56 + analysis was performed as described in our previous study [24]. Briefly, the number of positive stromal and intratumoral CD56 + lymphocytes was quantified. Scoring of NK-TILs immunostaining was determined as positive or negative by a cut-off value of five cells in ten high power fields (× 40 magnification), yielding an immunoscore (NK-TILs) of 0 (< 5 cells) or 1 (≥ 5 cells).

Statistical analysis
Statistical analyses were performed using the R language and the SPSS 20.0 statistical software for Windows (SPSS Inc., IBM). Bivariate analysis was performed to assess the correlation between biomarkers and clinicopathological characteristics using Chi-square tests for qualitative variables, Pearson-rank correlation for quantitative variables and ANOVA test for quantitative variation among classes of qualitative variables. The cumulative survival (overall survival, OS; recurrence-free survival, RFS) times were calculated using the Kaplan–Meier method and compared with the log-rank test. A cox-regression was also performed in order to evaluate the significance of prognostic factors on survival in a multivariate context (adjusting for confounding variables). All $p$-values less than 0.05 were considered significant.

Abbreviations
Breast Cancer, BC; Breast Cancer Cell, BCC; Tumor Infiltrating Lymphocytes, TILs; NK, Natural Killer; NK-TILs, Tumor Infiltrating Natural Killer Cell; Tumor Microenvironment, TME; Immunohistochemistry, IHC; Overall Survival, OS; Disease Free Survival, DFS; Scarff-Bloom-Richardson, SBR; tumor, lymph node and metastases, TNM; Luminal A, LA; Luminal B like, LB-Like; Her2 positive, H; Triple Negative breast cancer, TNBC; phosphate-buffered saline, PBS.

Declarations

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Author contributions
B.C. takes responsibility for the integrity of the work as a whole. Study design and concepts: B.C. Data acquisition: B.C., H.T., S.C., L.B., W.B.K., A.K. and K. C.; Data analysis and interpretation: B. C., H.T., S.C. and T. B.; Statistical analysis: B. C and A. R.; Manuscript preparation and editing: B. C. and A. R.

Competing interests
The authors declare no competing interests.

Ethical approval
All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and the national research committee of Habib Bourguiba and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Sampling was made only on patient tissues from tissue library of Pathology Department-Habib Bourguiba Hospital and no samples were made specifically for the study.

References
1. A. Jemal, F. Bray, M. M. Center, J. Ferlay, E. Ward, and D. Forman, ‘Global cancer statistics’, CA Cancer J Clin, vol. 61, no. 2, pp. 69–90, Apr. 2011, doi: 10.3322/caac.20107.
2. G. P. Dunn, L. J. Old, and R. D. Schreiber, ‘The immunobiology of cancer immunosurveillance and immunoediting’, Immunity, vol. 21, no. 2, pp. 137–148, Aug. 2004, doi: 10.1016/j.immuni.2004.07.017.
3. D. Mittal, M. M. Gubin, R. D. Schreiber, and M. J. Smyth, ‘New insights into cancer immunoediting and its three component phases—elimination, equilibrium and escape’, Curr. Opin. Immunol., vol. 27, pp. 16–25, Apr. 2014, doi: 10.1016/j.coi.2014.01.004.
4. D. Mittal, M. M. Gubin, R. D. Schreiber, and M. J. Smyth, ‘New insights into cancer immunoediting and its three component phases—elimination, equilibrium and escape’, Curr. Opin. Immunol., vol. 27, pp. 16–25, Apr. 2014, doi: 10.1016/j.coi.2014.01.004.
5. K. D. Moynihan and D. J. Irvine, ‘Roles for innate immunity in combination immunotherapies’, Cancer Res, vol. 77, no. 19, pp. 5215–5221, Oct. 2017, doi: 10.1158/0008-5472.CAN-17-1340.
6. D. Mittal, M. M. Gubin, R. D. Schreiber, and M. J. Smyth, ‘New insights into cancer immunoediting and its three component phases—elimination, equilibrium and escape’, Curr. Opin. Immunol., vol. 27, pp. 16–25, Apr. 2014, doi: 10.1016/j.coi.2014.01.004.
7. M. Vitale et al., ‘NK-dependent DC maturation is mediated by TNFalpha and IFNgamma released upon engagement of the NKP30 triggering receptor’, Blood, vol. 106, no. 2, pp. 566–571, Jul. 2005, doi: 10.1182/blood-2004-10-4035.
8. D. Mittal, M. M. Gubin, R. D. Schreiber, and M. J. Smyth, ‘New insights into cancer immunoediting and its three component phases—elimination, equilibrium and escape’, Curr. Opin. Immunol., vol. 27, pp. 16–25, Apr. 2014, doi: 10.1016/j.coi.2014.01.004.
9. D. Mittal, M. M. Gubin, R. D. Schreiber, and M. J. Smyth, ‘New insights into cancer immunoediting and its three component phases—elimination, equilibrium and escape’, Curr. Opin. Immunol., vol. 27, pp. 16–25, Apr. 2014, doi: 10.1016/j.coi.2014.01.004.
10. G. Deniz et al., ‘Regulatory NK cells suppress antigen-specific T cell responses’, J. Immunol., vol. 180, no. 2, pp. 850–857, Jan. 2008, doi: 10.4049/jimmunol.180.2.850.
11. B. Morandi et al., ‘NK cells provide helper signal for CD8 + T cells by inducing the expression of membrane-bound IL-15 on DCs’, Int. Immunol., vol. 21, no. 5, pp. 599–606, May 2009, doi: 10.1093/intimm/dxp029.
12. M. Vitale et al., ‘NK-dependent DC maturation is mediated by TNFalpha and IFNgamma released upon engagement of the NKP30 triggering receptor’, Blood, vol. 106, no. 2, pp. 566–571, Jul. 2005, doi: 10.1182/blood-2004-10-4035.
13. A. Beldi-Ferchiou and S. Caillat-Zucman, ‘Control of NK Cell Activation by Immune Checkpoint Molecules’, Int J Mol Sci, vol. 18, no. 10, Oct. 2017, doi: 10.3390/ijms18102129.
14. Q. Chen et al., ‘B7-H5/CD28H is a co-stimulatory pathway and correlates with improved prognosis in pancreatic ductal adenocarcinoma’, Cancer Sci., vol. 110, no. 2, pp. 530–539, Feb. 2019, doi: 10.1111/cas.13914.
15. B. Kasten, S. Ferrone, K. R. Zinn, and D. J. Buchsbaum, ‘B7-H3-targeted radioimmunotherapy of human cancer’, Curr. Med. Chem., Feb. 2019, doi: 10.2174/092986732666190228120908.
16. X. Song et al., ‘Prognostic role of high B7-H4 expression in patients with solid tumors: a meta-analysis’, Oncotarget, vol. 7, no. 47, pp. 76523–76533, Nov. 2016, doi: 10.18632/oncotarget.8598.
17. D. Wang, Z. Ran, M. Liu, and Y. Ou, ‘Prognostic Significance of Potential Immune Checkpoint Member HHLA2 in Human Tumors: A Comprehensive Analysis’, Front Immunol, vol. 10, Jul. 2019, doi: 10.3389/fimmu.2019.01573.
13. M. F. Flajnik, T. Tlapakova, M. F. Criscitiello, V. Krylov, and Y. Ohta, ‘Evolution of the B7 family: co-evolution of B7H6 and NKp30, identification of a new B7 family member, B7H7, and of B7’s historical relationship with the MHC’, *Immunogenetics*, vol. 64, no. 8, pp. 571–590, Aug. 2012, doi: 10.1007/s00251-012-0616-2.

14. C. S. Brandt *et al.*, ‘The B7 family member B7-H6 is a tumor cell ligand for the activating natural killer cell receptor NKp30 in humans’, *J. Exp. Med.*, vol. 206, no. 7, pp. 1495–1503, Jul. 2009, doi: 10.1084/jem.20090681.

15. J. Matta *et al.*, ‘Induction of B7-H6, a ligand for the natural killer cell-activating receptor NKp30, in inflammatory conditions’, *Blood*, vol. 122, no. 3, pp. 394–404, Jul. 2013, doi: 10.1182/blood-2013-01-481705.

16. L. Wang *et al.*, ‘VISTA, a novel mouse Ig superfamily ligand that negatively regulates T cell responses’, *J. Exp. Med.*, vol. 208, no. 3, pp. 577–592, Mar. 2011, doi: 10.1084/jem.20100619.

17. E. Schlecker *et al.*, ‘Metalloprotease-mediated tumor cell shedding of B7-H6, the ligand of the natural killer cell-activating receptor NKp30’, *Cancer Res.*, vol. 74, no. 13, pp. 3429–3440, Jul. 2014, doi: 10.1158/0008-5472.CAN-13-3017.

18. Y. Zhou, Y. Xu, L. Chen, B. Xu, C. Wu, and J. Jiang, ‘B7-H6 expression correlates with cancer progression and patient's survival in human ovarian cancer’, *Int J Clin Exp Pathol*, vol. 8, no. 8, pp. 9428–9433, 2015.

19. J.-G. Guo, C.-C. Guo, Z.-Q. He, Z.-G. Liu, Y. Wang, and Y.-G. Mou, ‘Clinical significance of B7-H6 protein expression in astrocytoma’, *Onco Targets Ther*, vol. 9, pp. 3291–3297, 2016, doi: 10.2147/OTT.S103771.

20. H. Zhou *et al.*, ‘The prognostic value of B7-H6 in esophageal squamous cell carcinoma’, *Sci Rep*, vol. 9, Dec. 2019, doi: 10.1038/s41598-019-54731-9.

21. J. Sun *et al.*, ‘Clinical significance of novel costimulatory molecule B7-H6 in human breast cancer’, *Oncol Lett*, vol. 14, no. 2, pp. 2405–2409, Aug. 2017, doi: 10.3892/ol.2017.6147.

22. Z. Li *et al.*, ‘PD-L1 Expression Is Associated with Tumor FOXP3(+) Regulatory T-Cell Infiltration of Breast Cancer and Poor Prognosis of Patient’, *J Cancer*, vol. 7, no. 7, pp. 784–793, 2016, doi: 10.7150/jca.14549.

23. M. Karin, ‘Nuclear factor-kappaB in cancer development and progression’, *Nature*, vol. 441, no. 7092, pp. 431–436, May 2006, doi: 10.1038/nature04870.

24. R. P. Negus, G. W. Stamp, J. Hadley, and F. R. Balkwill, ‘Quantitative assessment of the leukocyte infiltrate in ovarian cancer and its relationship to the expression of C-C chemokines’, *Am. J. Pathol.*, vol. 150, no. 5, pp. 1723–1734, May 1997.

25. W.-W. Lin and M. Karin, ‘A cytokine-mediated link between innate immunity, inflammation, and cancer’, *J. Clin. Invest.*, vol. 117, no. 5, pp. 1175–1183, May 2007, doi: 10.1172/JCI31537.

26. M. J. Smyth, G. P. Dunn, and R. D. Schreiber, ‘Cancer immunosurveillance and immunoeediting: the roles of immunity in suppressing tumor development and shaping tumor immunogenicity’, *Adv. Immunol.*, vol. 90, pp. 1–50, 2006, doi: 10.1016/S0065-2776(06)90001-7.

27. A. Matikas *et al.*, ‘Prognostic Implications of PD-L1 Expression in Breast Cancer: Systematic Review and Meta-analysis of Immunohistochemistry and Pooled Analysis of Transcriptomic Data’, *Clin. Cancer Res.*, vol. 25, no. 18, pp. 5717–5726, Sep. 2019, doi: 10.1158/1078-0432.CCR-19-1131.

28. T. Jiang *et al.*, ‘High expression of B7-H6 in human glioma tissues promotes tumor progression’, *Oncotarget*, vol. 8, no. 23, pp. 37435–37447, Mar. 2017, doi: 10.18632/oncotarget.16391.

29. M. Sobral-Leite *et al.*, ‘Assessment of PD-L1 expression across breast cancer molecular subtypes, in relation to mutation rate, BRCA1-like status, tumor-infiltrating immune cells and survival’, *Oncotarget*, vol. 7, no. 12, p. e1509820, 2018, doi: 10.1080/2162402X.2018.1509820.

30. C. W. Elston and I. O. Ellis, ‘Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. C. W. Elston & I. O. Ellis. Histopathology 1991; 19: 403–410’, *Histopathology*, vol. 41, no. 3A, pp. 151–152, discussion 152–153, Sep. 2002.

31. B. O'Sullivan *et al.*, ‘The TNM classification of malignant tumours-towards common understanding and reasonable expectations’, *Lancet Oncol.*, vol. 18, no. 7, pp. 849–851, 2017, doi: 10.1016/S1470-2045(17)30438-2.

32. H. Triki *et al.*, ‘CD155 expression in human breast cancer: Clinical significance and relevance to natural killer cell infiltration’, *Life Sci.*, vol. 231, p. 116543, Aug. 2019, doi: 10.1016/j.lfs.2019.116543.

33. S. Pesce *et al.*, ‘B7-H6-mediated downregulation of NKp30 in NK cells contributes to ovarian carcinoma immune escape’, *Oncoimmunology*, vol. 4, no. 4, p. e1001224, Apr. 2015, doi: 10.1080/2162402X.2014.1001224.

34. S. Muenst *et al.*, ‘Expression of programmed death ligand 1 (PD-L1) is associated with poor prognosis in human breast cancer’, *Breast Cancer Res. Treat.*, vol. 146, no. 1, pp. 15–24, Jul. 2014, doi: 10.1007/s10549-014-2988-5.

35. M. E. H. Hammond, D. F. Hayes, A. C. Wolff, P. B. Mangu, and S. Temin, ‘American society of clinical oncology/college of american pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer’, *J Oncol Pract*, vol. 6, no. 4, pp. 195–197, Jul. 2010, doi: 10.1200/JOP.2010.003931.

36. R. Tashima *et al.*, ‘Evaluation of an Optimal Cut-Off Point for the Ki-67 Index as a Prognostic Factor in Primary Breast Cancer: A Retrospective Study’, *PLoS ONE*, vol. 10, no. 7, p. e0119565, 2015, doi: 10.1371/journal.pone.0119565.

37. A. C. Wolff *et al.*, ‘Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer: American Society of Clinical Oncology/College of American Pathologists Clinical Practice Guideline Focused Update’, *J. Clin. Oncol.*, vol. 36, no. 20, pp. 2105–2122, 10 2018, doi: 10.1200/JCO.2018.77.8738.

38. R. Salgado *et al.*, ‘The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014’, *Ann. Oncol.*, vol. 26, no. 2, pp. 259–271, Feb. 2015, doi: 10.1093/annonc/mdu450.

**Tables**
Table 1: Associations of B7-H6 and PD-L1 expression with clinical parameters in primary breast cancer patients
| Clinicopathological Parameter | All patients n = 156 | B7-H6 BCC | P-value | TILs-B7-H6 | P-value | PD-L1 BCC | P-value | TILs-PD-L1 |
|------------------------------|----------------------|-----------|---------|-----------|---------|-----------|---------|-----------|
|                             |                      | <median n=96 | ≥ median n=60 |         | Low n = 126 | High n = 20 | Unk n = 10 |         | Low n = 58 | Hi n = 31 |
| Age in years                | 1                    | 0.224      | 0.62    | 0.70      | 0.426    | 0.035*    | 0.24    | 0.002**   | 0.23    |
| <median =48                 | 70                   | 43         | 27      | 54        | 13       | 3         | 54      | 16        | 23      | 44      |
| ≥ median =48                | 86                   | 53         | 33      | 72        | 7        | 7         | 71      | 15        | 35      | 44      |
| Histological type           |                      |            |         |           |          |           |         |           |         |
| CCI                         | 129                  | 78         | 51      | 102       | 18       | 9         | 99      | 30        | 45      | 75      |
| Others                      | 27                   | 18         | 9       | 24        | 2        | 1         | 26      | 1         | 13      | 13      |
| ER                          | 0.24                 |            |         |           |          |           |         |           |         |
| Negative                    | 57                   | 39         | 18      | 39        | 14       | 4         | 42      | 15        | 16      | 37      |
| Positive                    | 99                   | 57         | 42      | 87        | 6        | 6         | 83      | 16        | 42      | 51      |
| PR                          | 0.98                 | 0.199      | 0.37    |           |          |           |         |           |         |
| Negative                    | 69                   | 43         | 26      | 52        | 12       | 5         | 53      | 16        | 24      | 40      |
| Positive                    | 87                   | 53         | 34      | 74        | 8        | 5         | 72      | 15        | 34      | 48      |
| HER2                        | 0.002**              | 0.787      | 1       |           |          |           |         |           |         |
| Negative                    | 106                  | 74         | 32      | 87        | 12       | 7         | 84      | 22        | 43      | 56      |
| Positive                    | 50                   | 22         | 28      | 39        | 8        | 3         | 41      | 9         | 15      | 32      |
| MolecularSubtypes           | 0.003***             | 0.141      | 0.30    |           |          |           |         |           |         |
| H                           | 22                   | 9          | 13      | 16        | 5        | 1         | 18      | 4         | 6       | 15      |
| LA                          | 31                   | 23         | 8       | 27        | 1        | 3         | 28      | 3         | 16      | 12      |
| LB                          | 28                   | 13         | 15      | 23        | 3        | 2         | 23      | 5         | 10      | 16      |
| LB-LIKE                     | 47                   | 27         | 20      | 41        | 5        | 1         | 37      | 10        | 18      | 28      |
| TNBC                        | 28                   | 24         | 4       | 19        | 6        | 3         | 19      | 9         | 8       | 17      |
| SBR grading                 |                      |            |         |           |          |           |         |           |         |
| I                           | 23                   | 18         | 5       | 21        | 0        | 2         | 23      | 0         | 13      | 8       |
| II                          | 66                   | 41         | 25      | 57        | 4        | 5         | 59      | 7         | 27      | 34      |
| III                         | 67                   | 37         | 30      | 48        | 16       | 3         | 43      | 24        | 18      | 46      |
| Tumor size (cm)             |                      |            |         |           |          |           |         |           |         |
| T1 ≤ 2                      | 29                   | 16         | 13      | 22        | 4        | 3         | 26      | 3         | 8       | 18      |
| 2< T2 ≤5                    | 87                   | 55         | 32      | 72        | 10       | 5         | 65      | 22        | 34      | 48      |
| T3 > 5                      | 21                   | 14         | 7       | 14        | 5        | 2         | 18      | 3         | 7       | 12      |
| T4                          | 18                   | 11         | 7       | 17        | 1        | 0         | 15      | 3         | 8       | 10      |
| Unknown                     | 1                    | 0          | 1       | 1         | 0        | 0         | 1       | 0         | 1       | 0       |
| Lymph Node status           |                      |            |         |           |          |           |         |           |         |
| N0                          | 56                   | 39         | 17      | 43        | 10       | 3         | 42      | 14        | 20      | 33      |
| N1                          | 53                   | 30         | 23      | 47        | 2        | 4         | 44      | 9         | 21      | 28      |
| N2                          | 26                   | 16         | 10      | 17        | 7        | 2         | 22      | 4         | 8       | 16      |
| N3                          | 19                   | 10         | 9       | 17        | 1        | 1         | 15      | 4         | 7       | 11      |
| Unknown                     | 2                    | 1          | 1       | 2         | 0        | 0         | 2       | 0         | 2       | 0       |
| Metastasis                  |                      |            |         |           |          |           |         |           |         |
| M0                          | 108                  | 69         | 39      | 83        | 18       | 7         | 86      | 22        | 39      | 62      |
| M+                          | 13                   | 7          | 6       | 13        | 0        | 0         | 23      | 0         | 7       | 6       |
Variable between groups presented in frequency tables evaluated by Chi-Square test. Two sided p-values are considered statistically significant if <0.05 and are indicated in bold. (Unk;Unknown)

Table 2: Associations of B7-H6 and PD-L1 expression with clinical parameters in Her2 positive primary breast cancer patients

| Clinicopathological Parameter | All patients < median n=22 | ≥ median n=28 | P value | TIL≤B7-H6 | ≥ median n=39 | High n=8 | UnK n=3 | PD-L1 < median n=41 | ≥ median n=9 | UnK n=32 | TIL≤PD-L1 |
|-------------------------------|-----------------------------|---------------|---------|-----------|--------------|--------|---------|---------------------|-------------|----------|-----------|
| Age in years                  | 0.77                        | 0.75          | 0.11    |           |               |        |         |                     |             |          |           |
| <median =48                   | 27                          | 11            | 16      | 22        | 5            | 0      | 20      | 7                   | 10          | 17       | 0         |
| ≥ median =48                  | 23                          | 11            | 12      | 17        | 3            | 3      | 21      | 2                   | 5           | 15       | 3         |
| Histological type             | 1                           |               | 0.34    | 0.26      |               |        |         |                     |             |          |           |
| CCI                           | 45                          | 20            | 25      | 35        | 8            | 2      | 36      | 9                   | 14          | 29       | 2         |
| Others                        | 5                           | 2             | 3       | 4         | 0            | 1      | 5       | 0                   | 1           | 3        | 1         |
| ER                            | 0.61                        |               | 0.11    | 0.26      |               |        |         |                     |             |          |           |
| Negative                      | 25                          | 11            | 14      | 18        | 6            | 1      | 19      | 6                   | 7           | 17       | 1         |
| Positive                      | 25                          | 11            | 14      | 21        | 2            | 2      | 22      | 3                   | 8           | 15       | 2         |
| PR                            | 0.77                        |               | 0.85    | 0.76      |               |        |         |                     |             |          |           |
| Negative                      | 30                          | 14            | 16      | 23        | 5            | 2      | 25      | 5                   | 9           | 19       | 2         |
| Positive                      | 20                          | 8             | 12      | 16        | 3            | 1      | 16      | 4                   | 6           | 13       | 1         |
| Molecular Subtypes            | 0.94                        |               | 0.32    | 0.91      |               |        |         |                     |             |          |           |
| H                             | 22                          | 10            | 12      | 17        | 5            | 0      | 18      | 4                   | 6           | 16       | 0         |
| LB                            | 28                          | 12            | 16      | 22        | 3            | 3      | 23      | 5                   | 9           | 16       | 3         |
| SBR grading                   | 0.94                        |               | 0.32    | 0.91      |               |        |         |                     |             |          |           |
| I                             | 3                           | 3             | 0       | 2         | 0            | 1      | 3       | 0                   | 1           | 1        | 1         |
| II                            | 22                          | 12            | 10      | 19        | 1            | 2      | 21      | 1                   | 7           | 13       | 2         |
| III                           | 25                          | 7             | 18      | 18        | 7            | 0      | 17      | 8                   | 7           | 18       | 0         |
| Tumor size (cm)               | 0.92                        |               | 0.80    | 0.43      |               |        |         |                     |             |          |           |
| T1 ≤ 2                        | 8                           | 3             | 5       | 5         | 2            | 1      | 7       | 1                   | 0           | 7        | 1         |
| 2< T2 ≤5                      | 24                          | 11            | 13      | 19        | 4            | 1      | 18      | 6                   | 9           | 14       | 1         |
| T3 > 5                        | 8                           | 3             | 5       | 7         | 1            | 0      | 8       | 0                   | 2           | 6        | 0         |
| T4                            | 10                          | 5             | 5       | 8         | 1            | 1      | 8       | 2                   | 4           | 5        | 1         |
| Lymph Node status             | 0.89                        |               | 0.055   | 0.53      |               |        |         |                     |             |          |           |
| N0                            | 9                           | 5             | 4       | 6         | 3            | 0      | 6       | 3                   | 1           | 8        | 0         |
| N1                            | 19                          | 8             | 11      | 16        | 0            | 3      | 17      | 2                   | 8           | 8        | 3         |
| N2                            | 12                          | 5             | 7       | 8         | 4            | 0      | 10      | 2                   | 3           | 9        | 0         |
| N3                            | 10                          | 4             | 6       | 9         | 1            | 0      | 8       | 2                   | 3           | 7        | 0         |
| Metastasis                    | 0.84                        |               | 0.11    | 0.51      |               |        |         |                     |             |          |           |
| M0                            | 29                          | 14            | 15      | 21        | 7            | 1      | 23      | 6                   | 9           | 19       | 1         |
| M+                            | 9                           | 4             | 5       | 8         | 0            | 1      | 8       | 1                   | 4           | 4        | 1         |
| Unknown                       | 12                          | 4             | 8       | 10        | 1            | 1      | 10      | 2                   | 2           | 9        | 1         |
Variable between groups presented in frequency tables evaluated by Chi-Square test. Two sided p-values are considered statistically significant if <0.05 and are indicated in bold.

**Figures**

**Figure 1**

Representative images of immune checkpoint molecules B7-H6 and PD-L1 localizations and abundance in breast cancer tissues using immunohistochemical staining. B7-H6 IHC analysis showed three localizations types (a): membranous tumor cells (a, green gated area), cytoplasmic tumor cells (a, blue gated area) and in the stroma at the level of immune infiltrated cells (TILs) (a, red gated area). Identically, PD-L1 IHC analysis showed three localizations types (b): membranous tumor cells (b, green gated area), cytoplasmic tumor cells (b, blue gated area) and in the stroma at the level of TILs (b, red gated area). (c) B7-H6 and PD-L1 expression analysis by immunohistochemical staining at the level of breast cancer cells (IHC BCC). B7-H6 and PD-L1 IHC showed positive stained tumor cells at three different intensities; strong, moderate and weak. Positive controls for IHC staining of B7-H6 (Striated muscles tissue) and PD-L1 (Placenta tissue) respectively. (d) B7-H6 and PD-L1 IHC showed positive stained immune cells in three different attendances; low, medium and high B7-H6+TILs or PD-L1+TILs distribution in the stroma area are presented. (Original magnification x 400)
Figure 2

B7-H6 and PD-L1 H expressions features associations with TILs-PD-L1, TILs-B7-H6 and NK-TILs statutes. Boxplot representations of H-score PD-L1 distribution according to TILs-PD-L1 status (a, p < 0.001), TILs-B7-H6 status (b, p < 0.001) and NK-TILs status (c, p < 0.001). Boxplot representations of H-score B7-H6 distribution according to TILs-PD-L1 status (d, p = 0.05), TILs-B7-H6 status (e, p = 0.768) and NK-TILs status (f, p < 0.05). Barplot representations showing NK-TILs distribution according to TILs-PD-L1 status (g, p < 0.01) and TILs-B7-H6 status (h, p < 0.001). Barplot representation of TILs-B7-H6 distribution among TILs-PD-L1 status (i, p < 0.001).
Figure 3

Survival analysis according to B7-H6 expression and subgroups of combination between B7-H6 BCC or TILs-B7-H6 expression and immune parameters in breast cancer tissues. (a-b) Kaplan-Meier curves stratified on B7-H6 expression by cancer cells (B7-H6 BCC) for overall survival (OS) and disease free survival (DFS). (c-d) Kaplan-Meier curves stratified on B7-H6 expression by immune infiltrating cells (TILs-B7-H6) for OS and DFS. OS (e) and DFS (f) curves according to B7-H6 expression by tumor and immune cells together leading to three subgroups: subgroup 1: TILs-B7-H6Low/ B7-H6 BCCLow, subgroup 2: TILs-B7-H6High/ B7-H6 BCCLow/High and subgroup 3: TILs-B7-H6Low/ B7-H6 BCCHigh. (g) OS curves according to B7-H6 BCC expression combined with PD-L1 BCC expression leading to three subgroups as follows: subgroup 1: B7-H6 BCCLow/ PD-L1 BCCHigh/Low, subgroup 2: B7-H6 BCCHigh/ PD-L1 BCCLow, subgroup 3: B7-H6 BCCHigh/ PD-L1 BCCHigh. (h) OS curves according to TILs-B7-H6 status combined with PD-L1 BCC expression leading to three subgroups as follows: subgroup 1: TILs-B7-H6 BCCLow/ PD-L1 BCCHigh/Low, subgroup 2: TILs-B7-H6High/ PD-L1 BCCLow, subgroup 3: TILs-B7-H6High/ PD-L1 BCCHigh. (i) OS curves according to B7-H6 BCC expression combined with TILs-PD-L1 status leading to three subgroups as follows: subgroup 1: TILs-B7-H6 BCCLow/ TILs-PD-L1 Low, subgroup 2: TILs-B7-H6 BCCHigh/ TILs-PD-L1 High, subgroup 3: TILs-B7-H6 BCCHigh/ TILs-PD-L1 High. (j) OS curves according to TILs-B7-H6 status combined with TILs-PD-L1 status leading to three subgroups as follows: subgroup 1: TILs-B7-H6 BCCLow/ TILs-PD-L1 Low, subgroup 2: TILs-B7-H6 BCCHigh/ TILs-PD-L1 Low, subgroup 3: TILs-B7-H6 BCCHigh/ TILs-PD-L1 High. (k) OS curves according to B7-H6 BCC expression combined with NK-TILs status leading to three subgroups as follows: subgroup 1: B7-H6 BCCLow NK-TILs High/Low, subgroup 2: B7-H6 BCCHigh/ NK-TILs Low, subgroup 3: B7-H6 BCCHigh/ NK-TILs High. (l) OS curves according to TILs-B7-H6 status combined with NK-TILs status leading to three subgroups as follows: subgroup 1: B7-H6 BCCLow NK-TILs Low, subgroup 2: B7-H6 BCCLow/ NK-TILs High, subgroup 3: B7-H6 BCCHigh/ NK-TILs High/Low.

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