Prognostic Values of B7-H3, B7-H4, and HHLA2 Expression in Human Pancreatic Cancer Tissues Based on mIHC and Spatial Distribution Analysis

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Research Article

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Abstract

Background: Pancreatic cancer (PC) is one of the most malignant cancers and its 5-year survival rate remains poor. Although immunotherapy has achieved certain therapeutic efficacy in some clinical trials, such treatment still shows low responsiveness and overall remission rate. Therefore, it is urgently necessary to dissect the tumor microenvironment and optimize the immunotherapeutic strategies against this malignancy.

Methods: Using the multi-color immunohistochemistry, we investigated the expressions of B7-H3, B7-H4, HHLA2, CD8, and CD68 in 63 cases of PC tissues with the tissue microarray. Moreover, we analyzed immunolocalization features, prognostic values of this immune contexture, and clinical associations.

Results: The expressions of B7-H3, B7-H4, and HHLA2 could be detected in cytokeratin $(CK)^+$ tumor cells, $CD68^+$macrophages, and even stromal cells. Higher expression of B7-H3 in tumor cells could predict a better survival of the PC patients. A positive correlation was found between the expressions of B7-H3 and HHLA2 in tumor cells, while there was a negative correlation between the expressions of B7-H4 and HHLA2 in tumor cells. A positive correlation was found between the expressions of B7-H3 and B7-H4 or HHLA2 in tumor-associated macrophages (TAMs), but not B7-H4 and HHLA2. Tumor-infiltrating $CD8^+$T cells in combination with $CD68^+$TAMs could serve as an important predictor for the postoperative prognosis of PC patients. Higher expression of B7-H3, or HHLA2 in $CD68^+$TAMs could serve as an important predictor for poorer prognosis of PC patients. Patients with B7-$H3^{low}$B7-$H4^{low}$, B7-$H3^{low}$$HHLA2^{low}$, or B7-$H4^{low}$$HHLA2^{low}$ on $CD68^+$TAMs could have a better postoperative prognosis compared with the other sub-populations in the combinational analysis.

Conclusions: Taken together, our study indicated variable expressions and prognostic values of B7-H3, B7-H4, and HHLA2, in human PC tissues, and distinctively demonstrated that these immunosuppressive co-stimulators expressed by $CD68^+$TAMs could be used as important bio-markers for the prognostic prediction of PC patients. Moreover, these results supported that the evaluation of these markers could be used as essential candidate targets for immunotherapy against PC.

Background

Pancreatic cancer (PC) is one of the most malignant cancers, and it is the leading cause of the cancer-related death [1]. Approximately 80% of the PC patients are unresectable at diagnosis, and the 5-year survival rate is less than 6%. Although 15%-20% of the PC patients may have the opportunity to receive surgical resection, the 5-year survival rate of those PC patients remains less than 20% [2]. Moreover, due to the insensitivity of PC cells to traditional radiotherapy or chemotherapy, PC patients usually cannot benefit a lot from adjuvant therapies [3]. Therefore, it is urgently necessary to identify novel biomarkers and develop therapeutic strategies for this malignancy. Recently, the immunotherapeutic strategies, such as immune checkpoint inhibitors, adoptive cell therapy, cancer vaccine, or even immunotherapy in combination with traditional therapies, have achieved certain therapeutic efficacy in some clinical trials

[4–10]. However, because of the tumor heterogeneity and the immune-suppressive tumor microenvironment (TME), the immunotherapy still shows low responsiveness and overall remission rate against this malignancy [11].

B7-family members, such as PD-L1, B7-H3, and B7-H4, have been considered as important suppressors in the T-cell-mediated anti-tumor immunity, and the increased expressions of these molecules in human PC cancer cells significantly associated with cancer progression and poor prognosis of the patients [12–16]. Moreover, HHLA2 (also known as B7-H7), another important homolog molecule, still remains unknown in human PC. HHLA2 is a new member of the B7 family, and it is not expressed in mice but higher primates [16]. The *in vitro* studies have demonstrated that HHLA2 can inhibit proliferation and function of T cells [17]. Similar to other co-inhibitory molecules, HHLA2 is also expressed in a majority of cancer tissues. Our previous study has shown that over-expression of HHLA2 in renal cancer tissues is significantly associated with cancer progression and patient's poor prognosis [18]. And this conclusion is also confirmed by another study showing that co-expression of HHLA2 and PD-L1 can predict poor prognosis in patients with renal cancer [19]. As we know, the detailed expression patterns and the spatial distribution features of these co-inhibitory molecules in cancer tissues, as well as their correlations with infiltrating immune cells play a critical role in TME and optimization of cancer immunotherapy [20].

In our present study, we used multiplex staining to investigate the expression of B7-H3, B7-H4, HHLA2, CD8, and CD68 in a human PC tissue microarray (TMA) to further examine their immunolocalization features and prognostic values of this immune contexture. As we know, besides its expression in epithelial cancer cells, the expression of B7-H3 can also be induced in immune cells such as T cells, natural killer (NK) cells, antigen-presenting cells (APCs) and macrophages [16]. Moreover, B7-H4 has been also considered to be upregulated in TAMs, leading to the suppression of $CD8^+$T cell-mediated anti-tumor immunity [21, 22]. In present study, we systematically examined the expression of B7-H3, B7-H4, andHHLA2 in human PC tissues, including tumor cells, $CD68^+$TAMs, and stromal cells. Furthermore, the clinical associations and prognostic values were also analyzed. In addition, we also assessed the prognostic values of the intensities of tumor-infiltrating $CD8^+$T cells and $CD68^+$TAMs in human PC tissues.

Materials And Methods

Patients and tissue samples

The PC TMA (catalog: HPanA120Su02) was purchased from Shanghai Outdo Biotech Co., Ltd., Shanghai, China. A total of 66 patients (37 males and 29 females, aged from 38 to 85 years) who underwent surgery from January 2009 to August 2013 were enrolled in this study. Incomplete tissue samples and several missing tissue samples were excluded during the heat-induced antigen retrieval, and finally, 63 cases were involved in the present work and statistical analysis. The detailed clinical parameters of these patients were shown in Table 1.

Table 1
Patient's detailed clinical parameters

Parameters Cases	
Median age at surgery (range, years)	66(38–85)
Median survival (range, months)	10(1–65)
Median tumor diameter (range, cm)	4(2–9)
Gender	
M	35
F	28
T stage	
I	3
II	14
III	46
TNM stage	
I	13
II	46
IV	4
Pathological grade	
II	36
III	27
Distance metastasis	
Yes	4
No	59
Total	63

Multi-color Immunohistochemistry (Mihc)

The mIHC was carried out by using the Opal 7-color fluorescent IHC kit (catalog No. NEL811001KT, PerkinElmer, USA) in combination with automated quantitative analysis (PerkinElmer, USA) based on the manufacture's instructions to characterize the expressions and localization of B7-H3, B7-H4, HHLA2, CD8, and CD68 in tumor tissues. Cytokeratin (CK) was used to identify the epithelial cancer cells, and 4',6-diamidino-2-phenylindole (DAPI) was used to stain the nucleus. Briefly, the concentration of the six

antibodies above-listed against the markers were optimized, and the spectral library was built based on the single-stained slides. The PC TMA slide was dewaxed and rehydrated through a series of xylene-to-alcohol washes before incubated in distilled water. Subsequently, heat-induced antigen retrieval was carried out in citric acid solution (PH = 6.0), followed by the mIHC staining using the primary antibodies as follows, anti-B7-H3 (1:3,000 dilution, Catalog No. ab226256, Abcam, Cambridge), anti-B7-H4 (1:50 dilution, catalog No. ab209242, Abcam, Cambridge), anti-HHLA2 (1:500 dilution, catalog No. ab214327, Abcam, Cambridge), anti-CD8 (, catalog No. PA067, BioDot, USA), anti-CD68 (1:500 dilution, catalog No. M0876, DAKO, Denmark), and anti-CK (1:2 dilution, catalog No. PA125, BioDot, USA). The PC TMA slide was then incubated with HRP-conjugated secondary-antibodies (PerkinElmer, USA) in Opal working solution (PerkinElmer, USA). The slide was mounted with ProLong Diamond Antifade Reagent with DAPI (Thermofisher, USA).

Imaging Analysis

First, the TissueFAXS system (TissueGnostics Asia Pacific Limited, Austria) was used to conduct panoramic multispectral scanning of the slide, and then acquired images were processed using StrataQuest analysis software (Version No. 7.0.1.165, TissueGnostics Asia Pacific Limited, Austria), in which each fluorophore was spectrally unmixed into individual channels and saved as a separate file. DAPI was used to generate a binary mask of all viable cells in the image. Similarly, the expression of B7-H3, B7-H4, and HHLA2 were used in combination with DAPI to create binary masks of all cells expressing these biomarkers of interest. Additionally, the binary mask of CK was used to limit the analysis of tumor cells, and the binary mask of CD8 or CD68 was used to determine the intensity of infiltrating lymphocytes. (detailed descriptions were shown in Supplementary Table 1).

Statistical Analyses

Statistical analyses were performed using Prism 7 software (GraphPad) and RStudio 6.3. Chi-square test was used to compare the disease-related factors in patients with low and high expression of B7-H3, B7-H4, and HHLA2 in different cell populations. Log-rank survival analysis was used to predict the postoperative overall survival (OS) of patients. The Cox model was used to evaluate the prognostic values of different parameters involving B7-H3, B7-H4, and HHLA2 expressions in different cell populations. $P < 0.05$ was considered statistically significant.

Results

Expressions and localization of B7-H3, B7-H4, and HHLA2 in PC tissues

In the present study, we examined the localization of B7-H3, B7-H4, HHLA2, CD8, and CD68 in 63 cases of PC tissues by using mIHC. Figure 1 shows that the expressions of B7-H3, B7-H4, and HHLA2 could be

predominantly detected on the membrane and in the cytoplasm of cancer cells, stromal cells, and TAMs. Supplementary Fig. 1–2 reveal that the expression of B7-H3 in stromal cells in cancer tissues, was significantly higher compared with the normal tissues. However, there were no significant differences between cancer tissues and normal tissues regarding the expressions of B7-H3, B7-H4, and HHLA2 in tumor cells or $CD68^+$macrophage, and neither B7-H4 or HHLA2 was expressed in stromal cells. Moreover, the expression of B7-H3 and the infiltration intensity of $CD68^+$macrophages in cancer tissues were significantly higher compared with the normal tissues. Supplementary Table 1–4 show that there was a significant correlation between the expression of HHLA2 in tumor cells and gender and a significant correlation between the expression of B7-H3 in $CD68^+$macrophages and tumor size. No other significant correlations were observed between the expressions of B7-H3, B7-H4, and HHLA2 in tumor cells, stromal cells, $CD68^+$macrophages, and PC tissue cores and clinical parameters.

Prognostic values of the expressions of B7-H3, B7-H4, and HHLA2 in tumor cells and stromal cells

Figure 2 shows that patients with low expression of B7-H3 in tumor cells had a significantly worse OS than those with high expression (cut-off = 80; HR = 2.36, 95% CI: 1.19–4.70, $P = 0.05$, Fig. 2a), patients with high expression of B7-H4 in tumor cells tended to have a worse OS than those with low expression (cut-off = 90.5; HR = 1.70, 95% CI: 0.77–3.77, $P = 0.11$, Fig. 2b), patients with low expression of HHLA2 in tumor cells tended to have a worse OS than those with high expression (cut-off = 26.7; HR = 1.81, 95% CI: 1.77–4.24, $P = 0.08$, Fig. 2c). With regards to the expressions of B7-H3, B7-H4, and HHLA2 in stromal cells, patients in the high-expression sub-groups had no significantly different OS compared with the low-expression sub-groups. (Fig. 2d-f).

Prognostic values of the expressions of B7-H3, B7-H4, and HHLA2 in CD68 $^+$ TAMs

Figure 3 shows that patients with high expression of B7-H3 in $CD68^+$macrophages had a significantly worse OS than those with low expression (cut-off = 29.2, HR = 2.79, 95% CI: 1.33–5.86, $P < 0.001$, Fig. 3a). Patients with high expression of HHLA2 in $CD68^+$macrophages had a significantly worse OS than those with low expression (cut-off = 59.6, HR = 4.26, 95% CI: 1.46–12.4, $P = 0.01$, Fig. 3c), while patients with high expression of B7-H4 sub-group had no significant different OS compared with low-expression sub-group (cut-off = 33.0, Fig. 3b). Furthermore, the comparison of different combinations between the expressions of B7-H3 and B7-H4 in $CD68^+$macrophages showed that B7-$H3^{low}$B7-$H4^{low}$ patients had a significantly better OS than B7-$H3^{high}$B7-$H4^{low}$ patients (HR = 0.45, 95% CI: 0.18–1.13, $P < 0.05$, Fig. 3d) or B7-$H3^{high}$B7-$H4^{high}$ patients (HR = 0.28, 95% CI: 0.08–0.98, $P < 0.001$, Fig. 3d).The comparison of different combinations between the expression of B7-H3 and HHLA2 in $CD68^+$macrophages revealed that B7-$H3^{low}$$HHLA2^{low}$ patients had a significantly better OS than B7-$H3^{high}$$HHLA2^{low}$ patients (HR = 0.35, 95% CI: 0.14–0.92, $P < 0.01$, Fig. 3e) or B7-$H3^{high}$$HHLA2^{high}$ patients (HR = 0.30, 95% CI: 0.08–1.11, $P < 0.01$, Fig. 3e),. We also found that B7-$H4^{low}$$HHLA2^{low}$ patients had a significantly better OS than B7-$H4^{low}$$HHLA2^{high}$ patients (HR = 0.39, 95% CI: 0.13–1.16, $P < 0.01$, Fig. 3f).

Prognostic values of tumor-infiltrating CD8 $^{+}$ T cells and CD68 $^{+}$ TAMs in PC tissues

In our present study, we also analyzed the prognostic values of tumor-infiltrating $CD8^{+}$T cells and $CD68^{+}$macrophages in PC tissues. Figure 4 shows that patients with high density of infiltrating $CD8^{+}$T cells tended to have a better OS than those with low infiltration (cut-off = 0.34, HR = 0.52, 95% CI: 0.19–1.47, $P = 0.09$, Fig. 4a), and patients with low density of $CD68^{+}$TAMs tended to have a better OS than those with high infiltration (cut-off = 10.5, HR = 0.61, 95% CI: 0.33–1.15, $P = 0.09$, Fig. 4b). $CD8^{low}CD68^{high}$ Patients had a significantly worse OS than $CD8^{high}CD68^{low}$ patients (HR = 0.20, 95% CI: 0.01–4.16, $P < 0.05$, Fig. 4c) or $CD8^{high}CD68^{high}$ patients (HR = 0.25, 95% CI: 0.02–3.71, $P < 0.05$, Fig. 4c).

Cox model analyses based on the expressions of B7-H3, B7-H4, and HHLA2 in tumor cells, stromal cells, and CD68 $^{+}$ TAMs

Table 2 reveals that patients with T stage of III + IV had a significantly reduced death risk (HR = 0.32, 95% CI: 0.14–0.75, $P = 0.01$) compared with patients with T stage of I + II after adjustment of gender, age, tumor size, TNM stage, pathological stage, infiltration of $CD8^{+}$T cells, infiltration of $CD68^{+}$macrophages, expression of B7-H3 and *etc*. Patients with pathological stage of III + IV had a significantly increased death risk (HR = 2.98, 95% CI: 1.27–7.01, $P = 0.01$) compared with patients with pathological stage of I + II. Patients with low infiltration of $CD8^{+}$T cells had a significantly increased death risk (HR = 13.4, 95% CI: 2.95–60.6, $P < 0.001$) compared with patients with high infiltration of $CD8^{+}$T cells). Patients with high infiltration of $CD68^{+}$macrophages had a significantly increased death risk (HR = 11.4, 95% CI: 3.69–35.3, $P < 0.001$) compared with patients with low $CD68^{+}$macrophages infiltration. Patients with high expression of B7-H3 had a significantly increased death risk (HR = 4.12, 95% CI: 1.06-16.0, $P = 0.04$) compared with patients with low expression of B7-H3. Patients with low expression of B7-H3 in tumor cells had a significantly increased death risk (HR = 3.98, 95% CI: 1.19–13.3, $P = 0.02$) compared with patients with high expression of B7-H3 in tumor cells. Patients with low expression of HHLA2 in tumor cells had a significantly increased death risk (HR = 2.28, 95% CI: 0.71–7.26, $P = 0.05$) compared with patients with high expression of HHLA2 in tumor cells. Patients with high expression of HHLA2 in stromal cells had a significantly increased death risk (HR = 6.94, 95% CI: 1.67–28.8, $P = 0.01$) compared with patients with low expression of HHLA2 in stromal cells.

Table 2

Univariate analyses and Cox model analyses based on the clinicopathological features, the expression of B7-H3, B7-H4, and HHLA2 in tumor cells, stromal cells, and $CD68^+$TAMs

Variables	Unfavorable/favorable	Univariate analysis		Multivariate Cox regression analysis	
		HR (95% CI)	*P* value	HR (95% CI)	*P* value
Gender	Male/Female	1.21(0.69–2.15)	0.51	1.70(0.68–4.23)	0.26
Age	≥ 66/<66	1.42(0.80–2.50)	0.23	1.85(0.85–4.02)	0.12
Tumor size	≥ 4/<4	0.85(0.47–1.55)	0.59	1.60(0.71–3.57)	0.26
TNM stage	IV/I + II	3.67(1.26–10.7)	**0.02**	1.70(0.68–4.23)	0.26
T stage	III/I + II	0.88(0.48–1.62)	0.68	0.32(0.14–0.75)	**0.01**
Pathological stage	III/II	2.01 (1.13–3.56)	**0.02**	2.98(1.27–7.01)	**0.01**
$CD8^+$T cells infiltration	Low/high	1.92(0.68–5.38)	0.09	13.4(2.95–60.6)	< **0.001**
$CD68^+$ macrophages infiltration	High/low	1.63(0.87–3.04)	0.09	11.4(3.69–35.3)	< **0.001**
B7-H3 expression	High/low	1.07(0.42–2.71)	0.88	4.12(1.06-16.0)	**0.04**
B7-H4 expression	High/low	2.41(1.07–5.42)	**0.03**	0.62(0.10–3.78)	0.6
HHLA2 expression	Low/high	1.29(0.70–2.35)	0.41	3.47(0.83–14.5)	0.09
B7-H3 expression in tumor cells	Low/high	2.36(1.19–4.70)	**0.05**	3.98(1.19–13.3)	**0.02**
B7-H4 expression in tumor cells	High/low	1.70(0.77–3.77)	0.11	2.12(0.67–6.70)	0.20
HHLA2 expression in tumor cells	Low/high	1.81(1.77–4.24)	0.08	2.28(0.71–7.26)	**0.05**

Bold signifies $P < 0.05$.

Variables	Unfavorable/favorable	Univariate analysis		Multivariate Cox regression analysis	
		HR (95% CI)	*P* value	HR (95% CI)	*P* value
B7-H3 expression in stroma cells	High/low	1.35(0.76–2.38)	0.30	1.00(0.42–2.42)	1.00
B7-H4 expression in stroma cells	High/low	0.75(0.42–1.32)	0.31	1.23(0.42–3.61)	0.71
HHLA2 expression in stroma cells	High/low	1.59(0.77–3.30)	0.27	6.94(1.67–28.8)	**0.01**
B7-H3 expression in $CD68^+$TAMs	High/low	2.79(1.33–5.86)	< **0.001**	3.06(0.96–9.72)	0.16
B7-H4 expression in $CD68^+$TAMs	High/low	1.44 (0.63–3.26)	0.32	0.51(0.13-2.00)	0.80
HHLA2 expression in $CD68^+$TAMs	High/low	4.26(1.46–12.4)	< **0.01**	1.22(0.39–3.81)	0.57
Bold signifies $P < 0.05$.					

Correlations among the expression levels of B7-H3, B7-H4, and HHLA2 in tumor cells, stromal cells and CD68 $^+$ macrophages

Figure 5a shows that there was no correlation between the expressions of B7-H3 and B7-H4 in tumor cells. There was a significant positive correlation between the expressions of B7-H3 and HHLA2 in tumor cells ($P < 0.0001$, Fig. 5b), while there was a significant negative correlation between the expressions of B7-H4 and HHLA2 in tumor cells ($P < 0.01$, Fig. 5c). There were significant positive correlations between the expressions of B7-H3 and B7-H4 ($P = 0.01$, Fig. 5d) or HHLA2 ($P < 0.0001$, Fig. 5e) in stromal cells, respectively. However, there was no correlation between the expressions of HHLA2 and B7-H4 in stromal cells (Fig. 5f). With regards to the correlations among the expressions of B7-H3, B7-H4, and HHLA2 in $CD68^+$macrophages, we found that there were significant positive correlations between the expressions of B7-H3 and B7-H4 ($P < 0.01$, Fig. 5g) or HHLA2 ($P < 0.0001$, Fig. 5i) respectively. However, there was no significant correlation between the expressions of HHLA2 and B7-H4 in $CD68^+$macrophages (Fig. 5h).

Correlations among the expression levels of B7-H3, B7-H4, and HHLA2 in tumor cells and tumor-infiltrating immune cells

Figure 6 shows that there was no correlation between the infiltration intensity of $CD8^+$T cell and B7-H3 expression in tumor cells, and neither B7-H4 nor HHLA2 (Fig. 6a-c). There were significant positive correlations between the infiltration intensity of $CD68^+$macrophages and B7-H3 expression ($P = 0.03$, Fig. 6d) or HHLA2 expression in tumor cells ($P < 0.0001$, Fig. 6f). However, there was a significant negative correlation between the infiltration intensity of $CD68^+$macrophage and B7-H4 expression in tumor cells ($P = 0.01$, Fig. 6e). Figure 7 indicates that there was a significant negative correlation between the infiltration intensity of $CD8^+$T cell and B7-H3 expression in stromal cells ($P = 0.01$, Fig. 7a), but not B7-H4 or HHLA2 expression in stromal cells (Fig. 7b-c). With regards to the correlations between B7-H3, B7-H4 or HHLA2 expression in stromal cells and the infiltration intensity of $CD68^+$macrophages, we found that there was a significant negative correlation between the infiltration intensity of $CD68^+$macrophage and B7-H4 expression in stromal cells ($P = 0.01$, Fig. 7e), while there was a significant positive correlation between the infiltration intensity of $CD68^+$macrophage and HHLA2 expression in stromal cells ($P < 0.0001$, Fig. 7f). However, we did not find any significant correlation between the infiltration intensity of $CD68^+$macrophage and B7-H3 expression in stromal cells (Fig. 7d). Moreover, there were no significant correlations between B7-H3, B7-H4, and HHLA2 expression in $CD68^+$macrophages and the infiltration intensity of $CD8^+$T cells (Supplementary Fig. 3a-c). Moreover, there was no significant correlation in the infiltrating density between $CD68^+$macrophages and $CD8^+$T cells (Supplementary Fig. 4).

Discussion

It has been suggested that some co-inhibitory molecules, including B7-H3, B7-H4, and HHLA2, from the B7 family, are important immunotherapeutic targets in many human cancers [23–25]. For example, targeting B7-H3 via chimeric antigen receptor T (CAR-T) cells can significantly inhibit the growth of PC, ovarian cancer and neuroblastoma [5]. B7-H4-CAR T cells also show anti-tumor reactivity in the treatment of B7-H4-positive ovarian cancer, although the delayed toxicity can be found unexpectedly [26]. Therefore, detailed descriptions of the expression patterns and immunolocalization of these molecules in the TME will help optimize the schematic design of targeted immunotherapeutic strategies.

A previous study has shown that the constitutive or inducible expressions of these three molecules can be detected in a broad spectrum of cell types [27]. For example, B7-H3 can not only be expressed by APCs, T cells, and epithelial cancer cells, but also by stromal cells and its expression is associated with immune suppression [23, 28]. Similar to B7-H3, higher expression of B7-H4 both in tumor cells and tumor stromal cells are significantly associated with lower intensities of tumor-infiltrating lymphocytes in breast cancer [29]. Moreover, different expression patterns of B7-H4 have also been found in human cancers. Increased circulating $B7\text{-}H4^+CD68^+$macrophages are significantly correlated with the clinical stage of lung carcinomas [30]. In human ovarian cancer tissues, the intracellular expression of B7-H4 is found in tumor cells, while the B7-H4 expression on cell surface is found in TAMs, and the macrophages expressing B7-H4 but not tumor cells, play an important role in the suppression of antigen-specific T cell immunity [31]. B7-H4 expressed by tumor-infiltrating myeloid cells can also significantly lead to the dysfunction of

$CD8^+$T cell-mediated anti-tumor response [32]. As a novel member from B7-family, HHLA2 can also be expressed by monocytes and B cells [17].

In our present study, we primarily aimed to study the immunolocalization and the expression pattern of B7-H3, B7-H4, and HHLA2 in human PC tissues by using the TMA, and the correlations with the intensities of $CD8^+$ T cells and $CD68^+$macrophages were also investigated. Based on the mIHC and computer scanning analysis, all these three molecules could be found in CK^+ tumor cells, $CD68^+$ macrophages, and even stromal cells. Among these three molecules, we only found the expression of B7-H3 in stromal cells in cancer tissues, was significantly higher compared with the normal tissues. However, there were no significant differences between cancer tissues and normal tissues regarding the expressions of these three molecules in tumor cells, or $CD68^+$macrophages, and neither the expressions of B7-H4 and HHLA2 in stromal cells. Moreover, the infiltration intensity of $CD68^+$macrophages in cancer tissues was significantly higher compare with the normal tissues, and the infiltration intensity of $CD8^+$T cells tended to be higher in cancer tissues.

Second, we also evaluated the prognostic values of B7-H3, B7-H4, and HHLA2 expression in human PC, as well as the infiltration intensities of $CD8^+$T cells and $CD68^+$macrophages. Interestingly, the survival analysis revealed that higher expression of B7-H3 in tumor cells was significantly correlated with better survival of the patients, while there were no significant correlations between the expression of B7-H4 or HHLA2 expression in tumor cells and patients' prognoses. The prognostic value of B7-H3 in human cancers remains controversial. Some reports have shown that higher expression of B7-H3 in human cancers is correlated with poorer survival of the patients [33–36]. However, in consistent with some studies, our present results showed that higher expression of B7-H3 in tumor cells could predict a better survival of PC patients [37]. Herein, we also didn't find any significant correlations between the expression of B7-H3, B7-H4, or HHLA2 expression in stromal cells and the post-operative prognoses of PC patients. Moreover, our present study showed a positive correlation between correlation B7-H3 and HHLA2 in tumor cells, while there was a negative correlation between the expressions of B7-H4 and HHLA2 in tumor cells. We also presented a positive correlation between the expressions of B7-H3 and B7-H4 or HHLA2 in TAMs, but not between B7-H4 and HHLA2.

With regards to the tumor-infiltrating $CD8^+$T cells and $CD68^+$macrophages, we also found that the $CD8^+T^{high}$ patients, or $CD68^+macrophage^{low}$ patients tended to have a better prognosis compared with $CD8^+T^{low}$ patients or $CD68^+macrophage^{low}$ patients. Moreover, based on the combinational analysis, $CD8^+T^{high}CD68^+macrophage^{low}$ patients favored a better survival compared with the subpopulations with $CD8^+T^{low}CD68^+macrophage^{high}$, or $CD8^+T^{high}CD68^+macrophage^{high}$, and also tended to have a better prognosis compared with the $CD8^+T^{low}CD68^+macrophage^{low}$ patients. Our results further pointed out that tumor-infiltrating $CD8^+$T cells in combination with $CD68^+$TAMs, could serve as an important predictor for postoperative prognoses of cancer patients, such as gastric cancer, hepatocellular carcinoma, and ovarian cancer.[38–40]. Moreover, our present study also showed a negative correlation between the expression of HHLA2 in tumor cell and the infiltration intensity of $CD8^+$T cells, a positive correlation

between the expression of HHLA2 in tumor cell and the infiltration intensity of $CD68^+$ macrophages, a negative correlation between the expression of B7-H4 in tumor cell expressed and the infiltration intensity of $CD68^+$macrophages, a negative correlation between the expression of B7-H3 in stromal cell and the infiltration intensity of $CD8^+$T cells, a negative correlation between the expression of HHLA2 in stromal cell and the infiltration intensity of $CD8^+$T cells, a positive correlation between the expression of HHLA2 in stromal cell and the infiltration intensity of $CD68^+$macrophages, a negative correlation between the expression of B7-H4 in stromal cell and the infiltration intensity of $CD68^+$macrophages. All these results suggested that, among these molecules and their expression patterns, the expression of HHLA2 in tumor cells or stromal cells played an important role in regulating immune suppression in the TME of human PC.

Third, we also evaluated the prognostic values of these three co-inhibitory molecules expressed by $CD68^+$TAMs. We found that higher expression of B7-H3 in $CD68^+$macrophages, and higher expression of HHLA2 in $CD68^+$macrophages, could serve as an important predictor for poorer prognosis of PC patients. Moreover, we further carried out the combinational analysis on the expressions of these molecules in $CD68^+$macrophages, and the results demonstrated that the patients with B7-$H3^{low}$B7-$H4^{low}$, B7-$H3^{low}$$HHLA2^{low}$, or B7-$H4^{low}$$HHLA2^{low}$, in $CD68^+$macrophages could favor a better post-operative prognosis compared with the other sub-populations. Our present results also supported the notion that the immunosuppressive co-stimulators, such as B7-H3 and B7-H4 expressed by tumor-infiltrating immune cells were significantly correlated with the poor survival of the patients [41, 42].

Taken together, our study indicated variable expressions and prognostic values of B7-family ligands, namely B7-H3, B7-H4, and HHLA, in human PC tissues, and distinctively demonstrated that these immunosuppressive co-stimulators expressed by infiltrating immune cells could be used as important bio-markers for prognostic prediction of PC patients. Collectively, these results supported that these immunosuppressive co-stimulators could be used as essential candidate targets for immunotherapy against PC.

Abbreviations

DAPI
4',6-diamidino-2-phenylindole;
IHC
Immunohistochemistry;
PC
Pancreatic cancer;
TMA
Tissue microarray;
NT
Normal tissues;
CA

Cancer tissues;
B7-H3
B7-Homolog 3;
B7-H4
B7-Homolog 4;
HHLA2
Human endogenous retrovirus subfamily H long terminal repeat associating protein 2;
ICB
Immune checkpoint blockade

Declarations

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Not applicable.

Authors' contributions

Study conception and design: L. Chen.

Image analysis: L. Chen, Y. Zhu, J. Chen, T. Jiang, Y. Li.

Statistical analyses: Y. Zhu, Y. Liu. J. Feng, X. Zheng.

Drafting of manuscript: Y. Zhu, L. Chen.

Critical revision of manuscript: All authors.

Supervision & securing of study resources: J. Zhang, L. Chen.

All authors read and approved the final manuscript.

Ethics approval and consent to participate

All patients were given informed consent for participation, and the protocol for the present study was approved by the ethics committee of the Third Affiliated Hospital of Soochow University.

Consent for publication

The authors declare no competing interests.

Availability of data and materials

De-identified datasets used and/or analyzed during the current study are available from the corresponding authors upon reasonable request.

Competing interests

The authors declare no competing interests.

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Figures

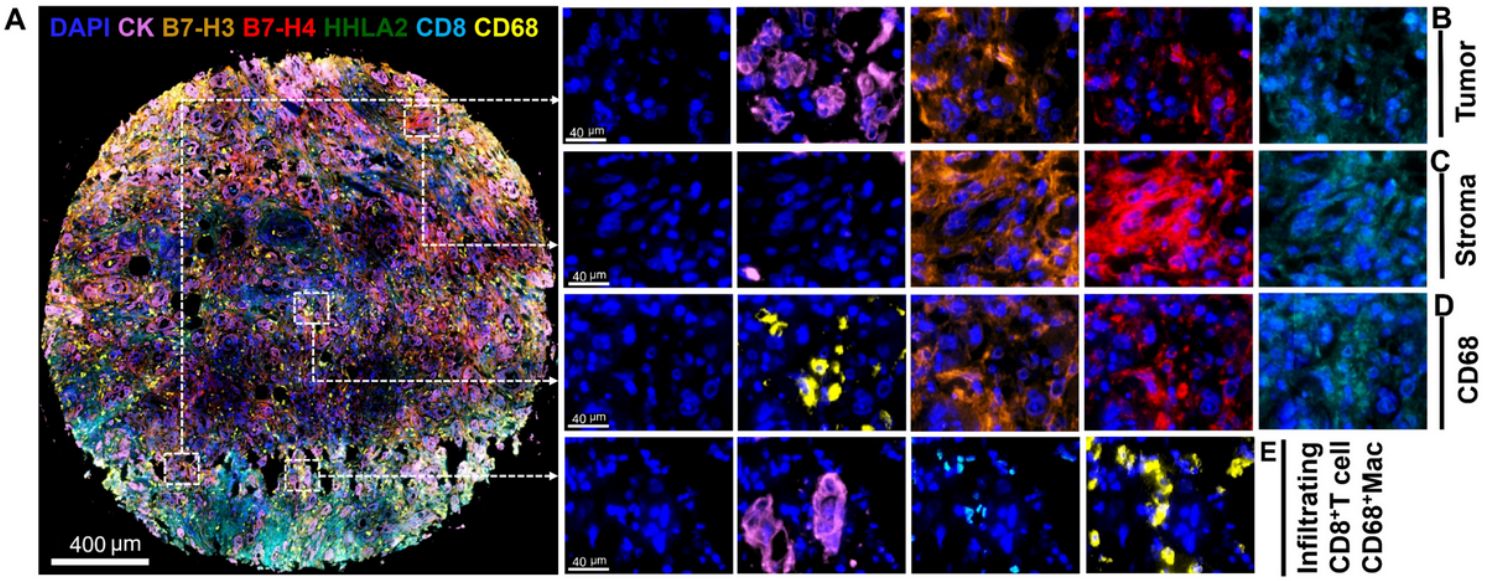

Figure 1

Characterization of the expressions and localization of B7-H3, B7-H4, HHLA2, CD8, and CD68 in human PC tissues by using Opal seven-color multiplex analysis. A. Representative composite image displaying the TMA core after spectral unmixing. B-E. An enlarged subsection of the core highlighted in A, showing each of the individual markers in the composite image after spectral unmixing, together with the DAPI nuclear marker (pseudo-colored blue) and the autofluorescence signal (pseudo-colored black). B. The expression of B7-H3 (membrane and cytoplasm, pseudo-colored brown), B7-H4 (membrane and cytoplasm, pseudo-colored red) and HHLA2 (membrane and cytoplasm, pseudo-colored green) in tumor cells identified by CK (membrane and cytoplasm, pseudo-colored magenta), respectively. C. Expressions of B7-H3, B7-H4, and HHLA2 in stromal cells. D. Expressions of B7-H3, B7-H4, and HHLA2 in CD68+ (membrane and cytoplasm, pseudo-colored yellow) TAMs. E. CD8 (membrane and cytoplasm, pseudo-colored cyan) and CD68 infiltration in PC tissues.

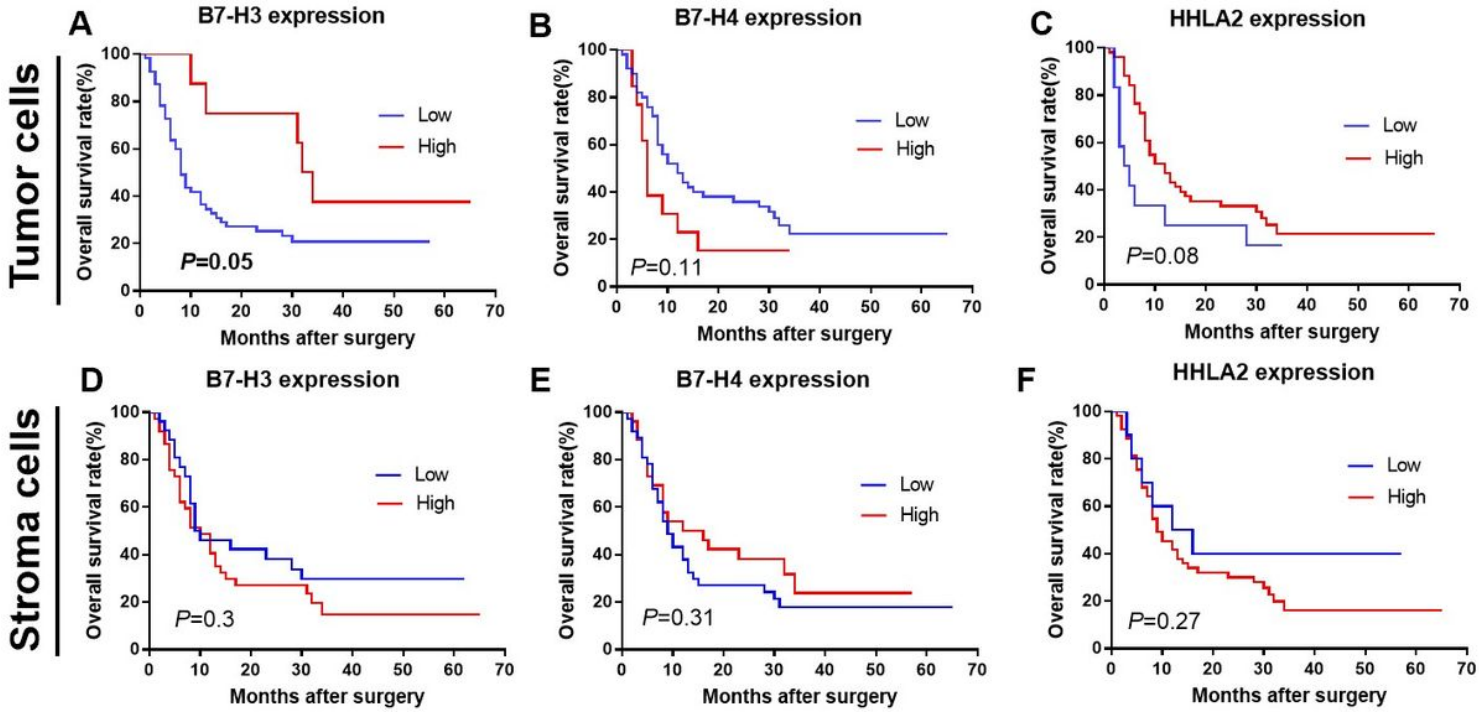

Figure 2

Survival analysis of the expressions of B7-H3, B7-H4 and HHLA2 in tumor cells and stromal cells in PC tissues. A. Patients with high expression of B7-H3 in tumor cells had a significantly better OS than those with low expression (P=0.05). B. Patients with low expression of B7-H4 in tumor cells tended to have a better OS than those with high expression. C. Patients with high expression of HHLA2 in tumor cells tended to have a better OS than those with low expression. D-F. There was no significantly difference between patients with high and low expressions of B7-H3 in stroma cells, and neither B7-H4 nor HHLA2 expression in stromal cells.

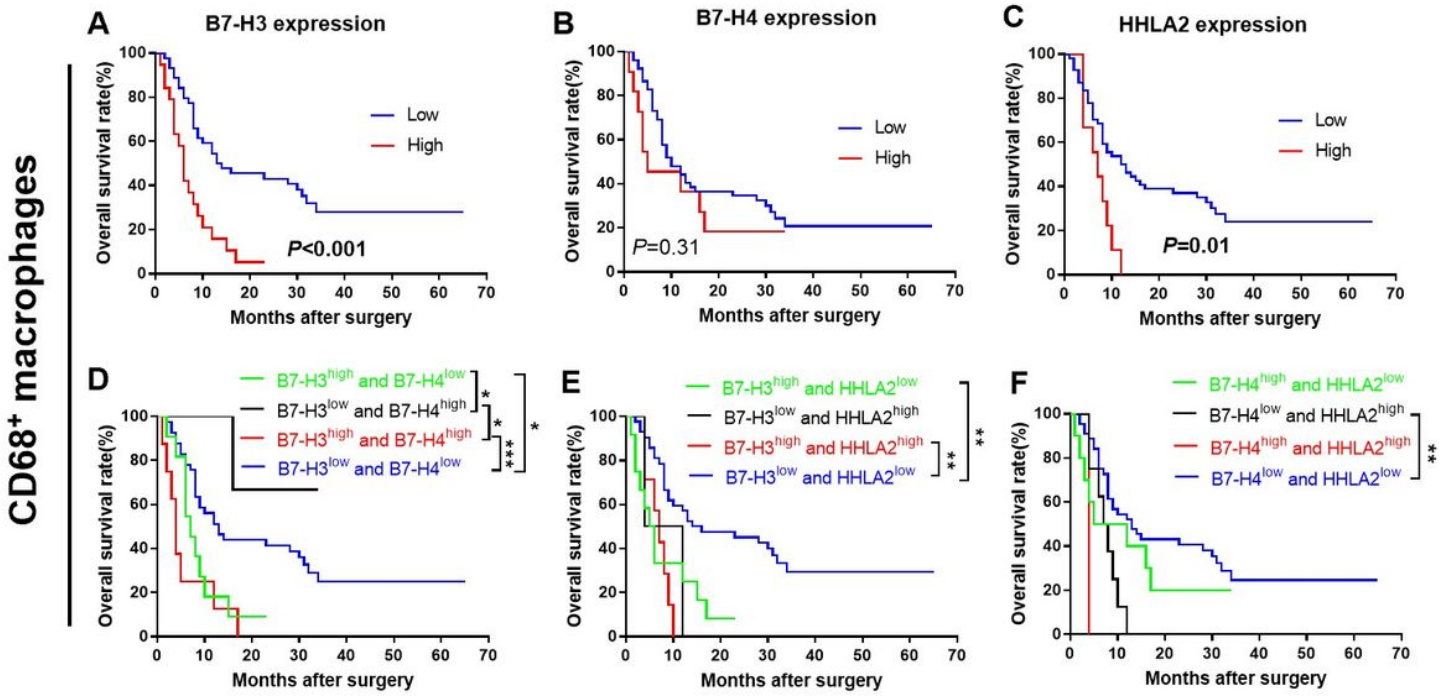

Figure 3

Survival analysis of the expressions of B7-H3, B7-H4, and HHLLA2 in CD68+TAMs in PC tissues. A. Patients with low expression of B7-H3 in CD68+TAMs had a significantly better OS than those with high expression (P<0.001). B-C. Patients with low expression of HHLA2 in CD68+TAMs had a significantly better OS than those with high expression (P=0.01), but there was no significant difference between high and low B7-H4 expressions in CD68+macrophages. D. B7-H3lowB7-H4low patients had a significantly better OS than B7-H3highB7-H4low patients (P<0.05) or B7-H3highB7-H4high patients (P<0.001). E. B7-H3lowHHLA2low patients had a significantly better OS than B7-H3highHHLA2low patients (P<0.01) or B7-H3highHHLA2high patients (P<0.01). F. B7-H4lowHHLA2low patients had a significantly better OS than B7-H4lowHHLA2high patients (P<0.01).

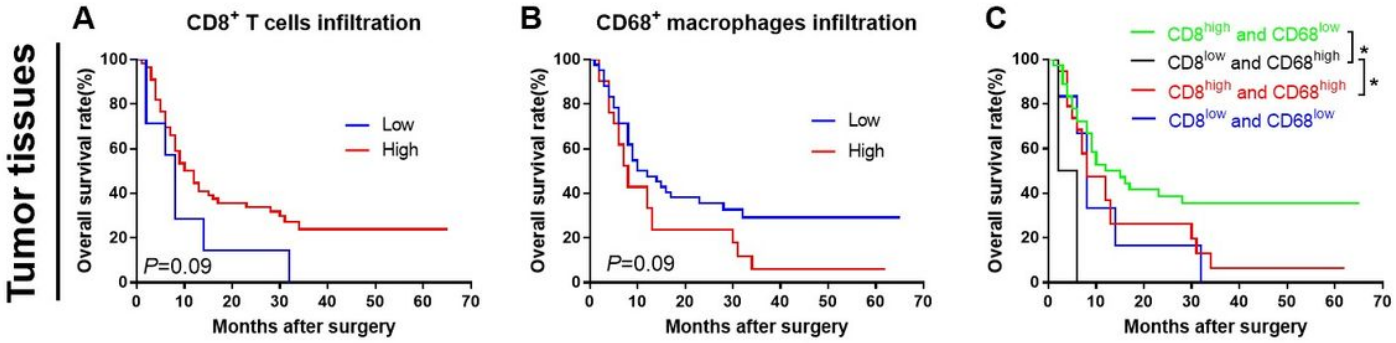

Figure 4

Survival analysis of the infiltration densities of CD8+T cells and CD68+TAMs in PC tissues. A. Patients with high infiltration of CD8+T cells tended to have a better OS than those with low infiltration (P=0.09). B. Patients with low infiltration of CD68+TAMs tended to have a better OS than those with high infiltration (P=0.09). C. CD8highCD68low patients had the best OS. CD8lowCD68high patients had a significantly worse OS than CD8highCD68low patients (P<0.05) or CD8highCD68high patients (P<0.05).

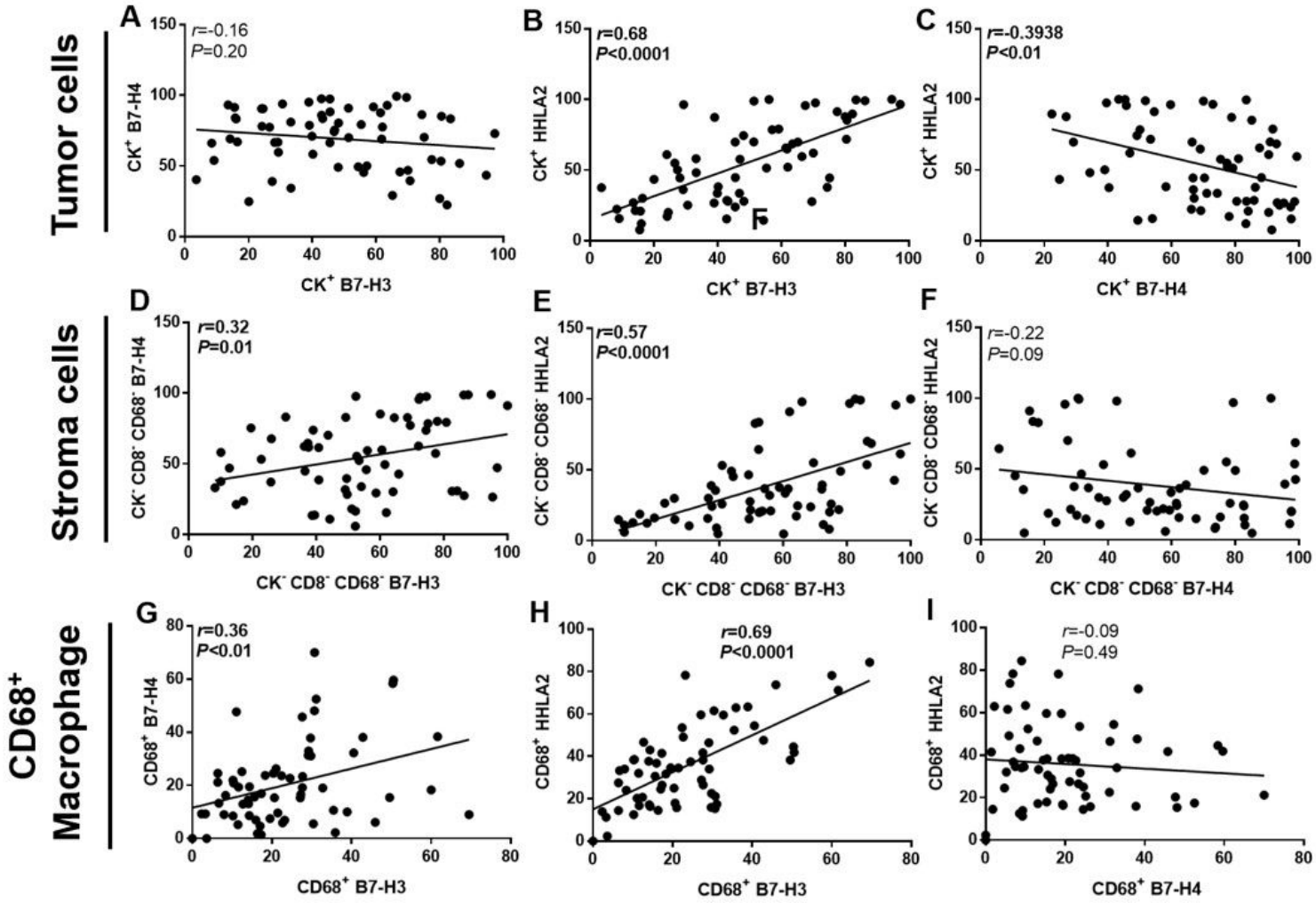

Figure 5

Correlations of expressions of B7-H3, B7-H4, and HHLA2 in tumor cells, stromal cells, and CD68+TAMs. A. There was no correlation between the expressions of B7-H3 and B7-H4 in tumor cells. B-C. There was a significant positive correlation between the expressions of B7-H3 and HHLA2 in tumor cells(P<0.0001), while there was a significant negative correlation between the expressions of B7-H4 and HHLA2 in tumor cells(P<0.01). D. There was a significant positive correlation between the expressions of B7-H3 and B7-H4 in stromal cells expressed (P=0.01). E. There was a significant positive correlation between the expressions of B7-H3 and HHLA2 in stromal cells(P<0.0001). F. There was no correlation between the expressions of HHLA2 and B7-H4 in stromal cells. G. There was a significant positive correlation between the expressions of B7-H3 and B7-H4 in CD68+TAMs (P<0.01). H. There was a significant positive correlation between the expressions of B7-H3 and HHLA2 in CD68+TAMs (P<0.0001). I. There was no correlation between the expressions of HHLA2 and B7-H4 in CD68+TAMs.

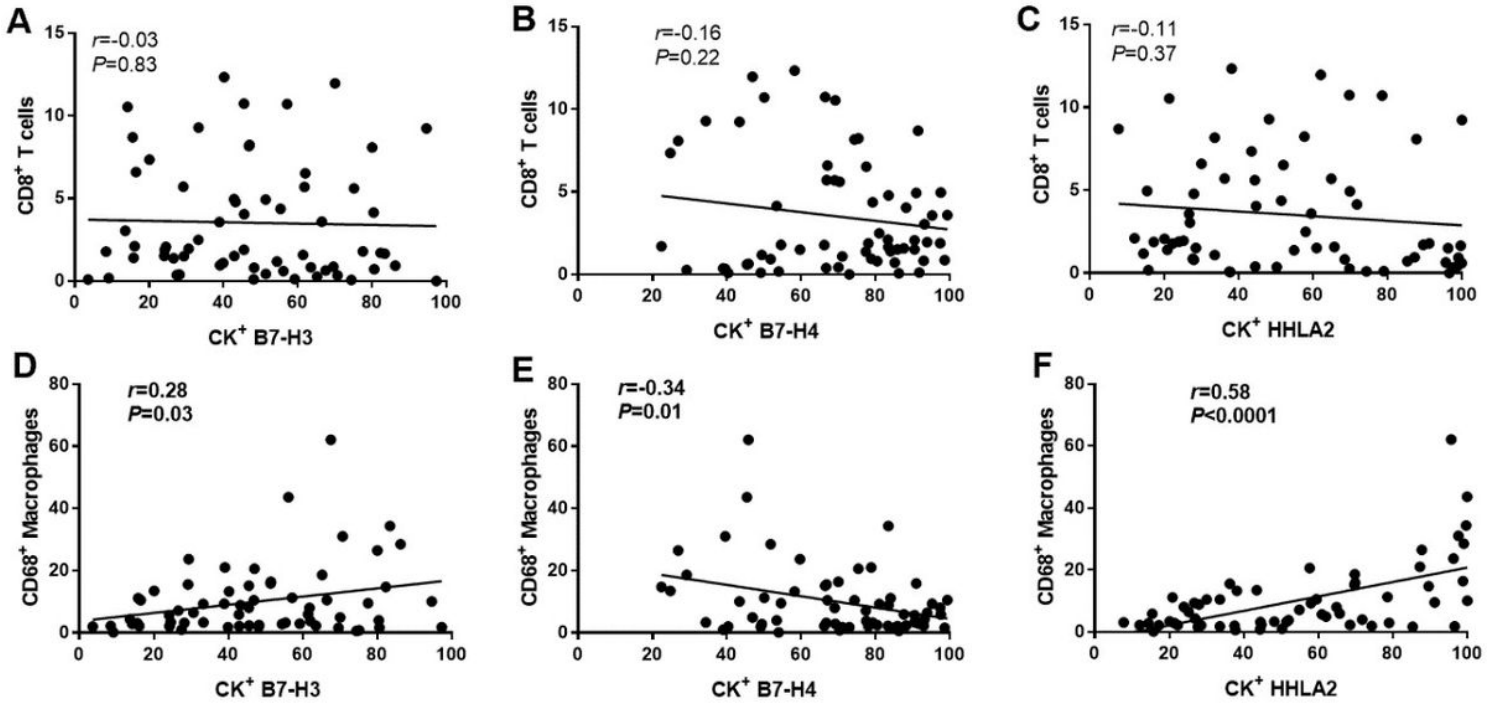

Figure 6

Correlations between the expressions of B7-H3, B7-H4, and HHLA2 in tumor cells and the infiltration intensities of CD8+T cells and CD68+TAMs. A. There was no significant correlation between the B7-H3 expression in tumor cells and the infiltration intensity of CD8+T cells. B. There was no significant correlation between the B7-H4 expression in tumor cells and the infiltration intensity of CD8+T cells. C. There was no significant correlation between the HHLA2 expression in tumor cells and the infiltration intensity of CD8+T cells. D. There was a significant positive correlation between the B7-H3 expression in tumor cells and the infiltration intensity of CD68+TAMs (P=0.03). E. There was a significant negative correlation between the B7-H4 expression in tumor cells and the infiltration intensity of CD68+TAMs (P=0.01). F. There was a significant positive correlation between the HHLA2 expression in tumor cells and the infiltration intensity of CD68+TAMs (P<0.0001).

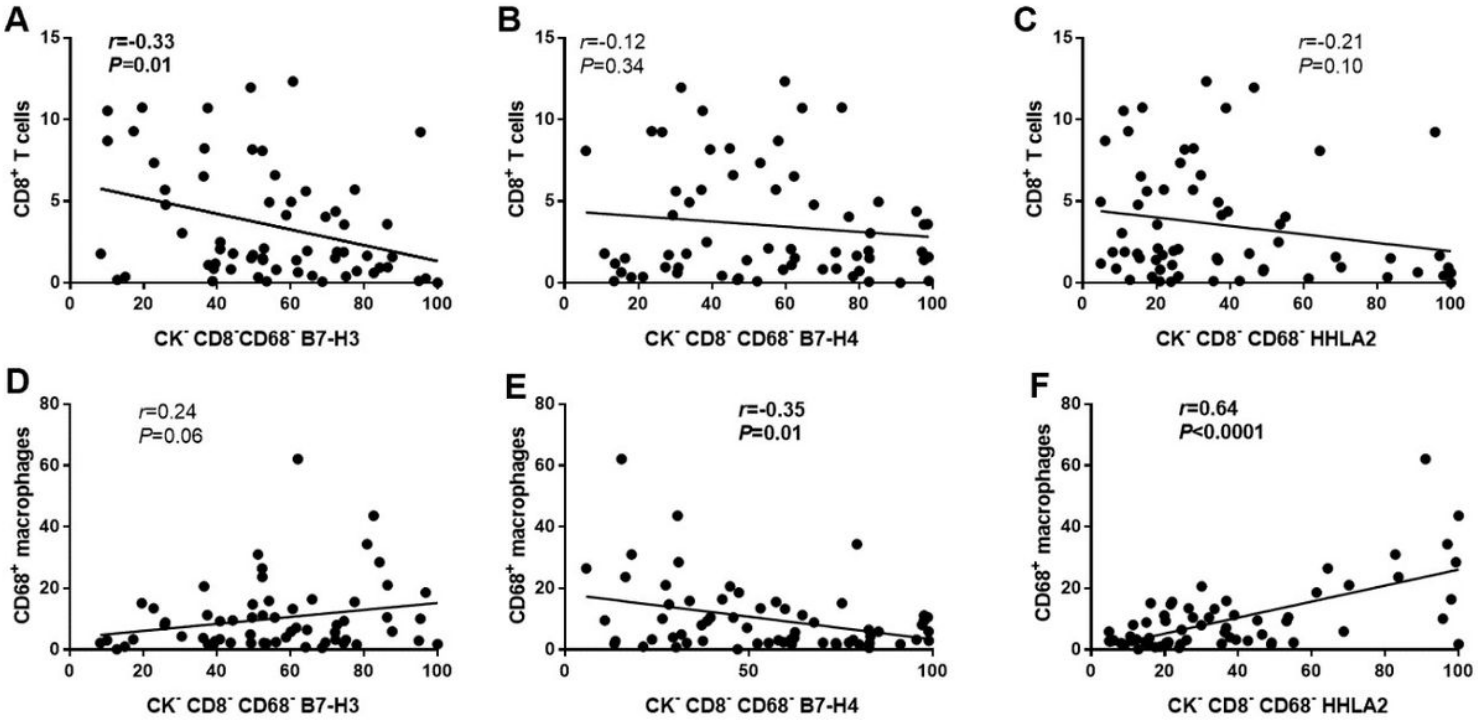

Figure 7

Correlations between the expression of B7-H3, B7-H4, and HHLA2 in stromal cells and the infiltration intensities of CD8+T cell and CD68+TAMs. A. There was a significant negative correlation between the B7-H3 expression in stromal cells and the infiltration intensity of CD8+T cells (P=0.01). B. There was no significant correlation between the B7-H4 expression in stromal cells and the infiltration intensity of CD8+T cells. C. There was no significant correlation between the HHLA2 expression in stromal cells and the infiltration intensity of CD8+T cells. D. There was no significant correlation between the B7-H3 expression in stromal cells and the infiltration intensity of CD68+TAMs. E. There was a significant negative correlation between the B7-H4 expression in stromal cells and the infiltration intensity of CD68+TAMs (P=0.01). F. There was a significant positive correlation between the HHLA2 expression in stromal cells expressed and the infiltration intensity of CD68+TAMs (P<0.0001).

Supplementary Files

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