The coexpression and clinical significance of costimulatory molecules B7-H1, B7-H3, and B7-H4 in human pancreatic cancer

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Aim: We investigated the expression of the inhibitory costimulatory molecules B7-H1, B7-H3, and B7-H4 in human pancreatic cancer to define their clinical significance and mechanism in a tumor microenvironment.

Patients and methods: Sixty-three pancreatic cancer tissues and 12 normal pancreatic tissues were examined in our research. Patients were enrolled in the study between December 2000 and August 2010. Expression levels of the B7 family of molecules and densities of tumor-infiltrating lymphocytes in the tissues were characterized with immunohistochemical assays.

Results: More than 50% of the patients expressed B7-H1 and B7-H4, and nearly 100% of the patients expressed B7-H3. B7-H1 expression was correlated with tumor size, B7-H3 expression was correlated with lymph-node metastasis and differentiation grade, and B7-H4 expression was correlated with tumor size, lymph-node metastasis, and invasion depth. High B7-H4 expression was also correlated with poor survival in pancreatic cancer. We determined the value of these three B7 family molecules in the postoperative survival prognosis for patients with pancreatic cancer, and pancreatic cancer patients with less coexpression of the B7 family of molecules had a significantly higher survival rate. B7-H1 expression was found to be negatively related to the intensity of both CD3+ T cells and CD8+ T cells, and B7-H4 expression was negatively related to CD3+ T-cell infiltration intensity, but not to CD8+ T cells.

Conclusion: B7-H1, B7-H3, and B7-H4 are involved in pancreatic cancer progression, and their coexpression could be a valuable prognostic indicator. Negative regulation of T-cell infiltration might be the main mechanism of action of the B7 family of molecules in pancreatic cancer.

Keywords: pancreatic cancer, B7-H1, B7-H3, B7-H4, tumor-infiltrated T cell

Introduction
Pancreatic cancer is one of the most devastating human malignancies, with a 5-year survival rate of less than 5%. Because of its extremely high malignant potential, it is usually diagnosed in its advanced stages, and is often not suitable for present curative surgery. New approaches are needed for a complete cure of pancreatic cancer, especially targets for suppression of tumor immune escape.

Tumor cells have the ability to build a microenvironment by changing their immunogenic phenotypes, particularly some costimulatory molecules that are not expressed in normal tissues, such as the B7 family of molecules. In the past decade, the value of negative B7 family molecules in tumor surveillance has been confirmed by many research groups, and clinical experiments are being conducted that target these molecules. B7-H1, B7-H3, and B7-H4 are the most significant molecules of the B7 family in human tumor immune surveillance, and they display similar characteristics in...
the regulation of T-cell activation, although the precise function of each molecule in the tumor immune response remains unclear. B7-H1 is abundantly and constitutively expressed by many cells and in various tissues. The interaction of B7-H1 and its receptor, PD-1, controls the induction and maintenance of peripheral immune tolerance, and is responsible for the functional impairment of antigen-specific CD8+ T-cell responses during malignant transformation.10–12 B7-H3 messenger ribonucleic acid (mRNA) and protein expression have been found in many lymphoid and nonlymphoid cells and peripheral organs.13,14 Although B7-H4 mRNA transcription occurs widely in peripheral tissues and in most stromal and hematopoietic cells, protein expression is absent in most somatic tissues and only detected in the epithelial cells of the kidney, lung, and pancreas.15 Because a receptor for B7-H3 and B7-H4 has not yet been confirmed, functional analyses are currently difficult to perform, and the role of B7-H3 and B7-H4 in T-cell regulation has yet to be defined.16

The expression and clinical significance of B7-H1, B7-H3, and B7-H4 have been investigated in many human malignancies, including pancreatic cancer, but the results have been ambiguous, and the significance of coexpression of these three molecules remains unclear. In the present study, we investigated B7-H1, B7-H3, and B7-H4 expression and their relations to the T-cell-based tumor immune response in 63 pancreatic tissues, and analyzed the clinical significance of the coexpression to future applications for clinical treatment of human pancreatic cancer.

Patients and methods
Sixty-three cases of pancreatic cancer tissue were examined in our research. Formalin-fixed, paraffin-embedded tumor-tissue blocks of pancreatic cancer were collected from the First Affiliated Hospital, Suzhou University. All of the 63 pancreatic cancer patients underwent surgical resection between December 2000 and August 2010. None of the patients received chemotherapy or radiotherapy before surgery. Pathology reports were reviewed, and tumor–node–metastasis (TNM) stages were assigned according to the American Joint Committee on Cancer staging system.17 Follow-up was until death or until August 2013. In addition, 12 normal pancreatic tissues were obtained from surgical specimens other than pancreatic cancer. All of the research was reviewed and approved by the ethics committee of the hospital.

Immunohistochemistry
Formalin-fixed, paraffin-embedded tissues were cut into 3 µm-thick consecutive sections, dewaxed in xylene, and rehydrated by graded washes in ethanol solutions. Antigens were retrieved by enzyme digestion or by heating the tissue sections at 100°C for 30 minutes in citrate (10 mmol/L, pH 6.0) or ethylenediaminetetraacetic acid (1 mmol/L, pH 9.0) solution when needed. Next, the sections were immersed in a 0.3% hydrogen peroxide solution for 30 minutes to block endogenous peroxidase activity, rinsed in phosphate-buffered saline for 5 minutes, and incubated with primary antibodies against B7-H1 (mouse antihuman monoclonal antibody, clone 2H11; final concentration in use, 10 µg/mL),18 B7-H3 (mouse antihuman monoclonal antibody, clone 21D4; final concentration in use, 10 µg/mL),19 B7-H4 (mouse antihuman monoclonal antibody, clone 3C8; final concentration in use, 4 µg/mL),20 CD3 (mouse antihuman monoclonal antibody, ready to use; Maixin Biotechnology, Fuzhou, People’s Republic of China), CD8 (mouse antihuman monoclonal antibody, ready to use; Maixin Biotechnology), and CD68 (mouse antihuman monoclonal antibody, ready to use; Maixin Biotechnology). A negative control was performed by omitting the primary antibodies. The sections were then incubated with horseradish peroxidase-labeled goat antimouse/antirabbit secondary antibody (ready to use; Maixin Biotechnology). Diaminobenzene was used as the chromogen and hematoxylin as the nuclear counterstain. Sections were dehydrated, cleared, and mounted.

Immunostaining evaluation
B7-H1, B7-H3, and B7-H4 immunostaining densities were assessed according to a method described previously.18–20 The expression evaluation was determined according to the percentage of tumor cells showing brown in the cytoplasm and/or the membrane, and the percentage was calculated by counting the number of stained tumor cells among 1,000 tumor cells in each section. Cases with >10% cells clearly stained were considered positive expression. The scale to determine the staining intensity is defined as follows: grade 0, negative, <10% cells stained; grade 1, weakly positive, 10%–30% cells stained; grade 2, moderately positive, 30%–60% cells stained; and grade 3, strongly positive; >60% cells stained. For analysis, immunostaining intensities were classified as follows: sections that contained grade 0 and grade 1 were defined as the low-expression group, and other sections containing grade 2 and grade 3 were defined as the high-expression group. The intensity of tumor-infiltrating lymphocytes (TILs) in tumor tissues was determined according to the immunohistochemical staining of CD3, CD8, and CD68. TILs in the tumor tissues were counted as follows: five areas of tumor tissue with the most intense infiltrating lymphocytes were selected at low magnification (40×), and then the TILs were counted and recorded.
in a high-power field (200× magnification). The averages of the counts in these five areas were considered the intensity of TILs and used in the statistical analysis. All data were obtained independently by two pathologists who were not informed of the patients’ clinical data.

Statistical analysis
The \( \chi^2 \) test was used to analyze the correlations between TILs and patient clinical parameters, and correlations between TILs and B7-H1, B7-H3, and B7-H4 expression were also analyzed by \( \chi^2 \) test. Each patient’s postoperative prognosis related to B7-H1, B7-H3, and B7-H4 expression and TILs was examined by log-rank survival analysis. All statistical analyses were performed with the GraphPad Prism 4.0 software package (GraphPad Software, La Jolla, CA, USA). All statistical tests were two-tailed.

Results
B7-H1, B7-H3, and B7-H4 expression in human pancreatic cancer tissues
B7-H1, B7-H3, and B7-H4 immunohistochemical staining was observed on the pancreatic tumor-cell membrane and in tumor-cell cytoplasm (Figure 1A), whereas no expression of B7-H1 or B7-H4 and very weak expression of B7-H3 was found in normal pancreatic tissues (Figure 1B). The expression intensities of B7-H1, B7-H3, and B7-H4 in pancreatic tissues were remarkably higher than in normal pancreatic tissues (Figure 1C). Using staining intensity, we categorized 63 patients into two subgroups according to the staining of B7-H1, B7-H3, and B7-H4, as follows: the lower B7-H1-expression group (39 cases) and the higher B7-H1-expression group (24 cases); the lower B7-H3-expression group (20 cases) and the higher B7-H1-expression group (43 cases); and the lower B7-H4-expression group (32 cases) and the higher B7-H4-expression group (31 cases). In sum, 57.1% of patients (36 of 63) showed positive B7-H1 expression (including grade 1, weakly positive; grade 2, moderately positive; and grade 3, strongly positive); 93.7% of patients (59 of 63) showed positive B7-H3 expression; and 61.9% of patients (39 of 63) showed positive B7-H4 expression. Only one patient showed no expression of any of the three molecules; other patients showed various expression patterns of the three B7-family molecules.

**Figure 1** B7-H1, B7-H3, and B7-H4 expression was detected by immunohistochemical (IHC) assay in pancreatic cancer tissue (A) and normal pancreatic tissue (B). (C) The expression levels of B7-H1, B7-H3, and B7-H4 molecules in cancer tissues and normal tissues were compared.

**Note:** Scale bar = 100 \( \mu \)m.
Correlations between B7-H1, B7-H3, and B7-H4 expression and pathological parameters

Costimulatory molecules have been implicated as a possible regulator of antitumor immunity in several human malignancies. Therefore, we investigated the correlations between the expression of the B7 family of molecules in pancreatic tumor tissues and patients’ pathological parameters (Table 1). The statistical results indicated a significant difference of the B7 family of molecules in the pathological changes in pancreatic cancer. Patients with high B7-H1 expression had larger tumors ($P=0.03$), patients with high B7-H3 expression had more lymph-node metastasis ($P=0.01$) and a lower differentiation grade ($P=0.05$), patients with high B7-H4 expression had larger tumors ($P<0.01$) and more lymph-node metastasis ($P=0.02$), and B7-H4 expression was correlated with the invasion depth of the tumor ($P<0.01$). No correlations were observed between the three B7-family molecules and patients’ sex, age, tumor location, or distant metastasis.

Expression of B7 family of molecules correlates with poor survival in pancreatic cancer patients

The B7 family of molecules has been suggested to be a valuable marker of the prognosis of various human malignancies. The correlations between B7-H1, B7-H3, and B7-H4 and survival time for patients with pancreatic cancer were studied in our research. Statistical analysis showed that compared with patients’ individual survival times, B7-H1- and B7-H3-expression intensities were not correlated with patients’ overall survival time ($P=0.089$, $P=0.159$), whereas high B7-H4 expression was correlated with poor survival in pancreatic cancer ($P<0.001$) (Figure 2A). To determine the value of these three B7-family molecules in the postoperative survival prognosis of pancreatic cancer patients, we divided patients into one of four subgroups according to the coexpression of the three molecules as follows: high expression of all three molecules ($3^{high}$), high expression of two molecules and low expression of one molecule ($2^{high}1^{low}$), low expression of two

Table 1 Correlations between B7-H1, B7-H3, and B7-H4 expressions and patient pathological parameters

<table>
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<tr>
<th>Pathological parameters</th>
<th>Cases</th>
<th>B7-H1 expression</th>
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<th>B7-H3 expression</th>
<th>P-value</th>
<th>B7-H4 expression</th>
<th>P-value</th>
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<td></td>
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<td></td>
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<td>7</td>
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<td>10</td>
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<td>14</td>
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<td>Distant metastasis (M)c</td>
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<td>23</td>
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<td>0.21</td>
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<td></td>
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<td>M1</td>
<td>30</td>
<td>16</td>
<td>14</td>
<td></td>
<td>11</td>
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Notes: aThe depth of tumor invasion is classified as follows: pT1, invasion of lamina propria or submucosa; pT2, invasion of muscularis propria; pT3, invasion of adventitia; and pT4, invasion of adjacent structures; blymph-node metastasis is classified as follows: N0, no regional lymph-node metastasis; N1, regional lymph-node metastasis; cDistant metastasis is classified as follows: M0, no distant metastasis; M1, metastasis to cervical nodes, celiac nodes, and other distant metastases. $P<0.05$ was considered significant.
molecules and high expression of one molecule (2low1high), and low expression of all three molecules (3low). The results showed that pancreatic cancer patients with less B7-family molecule expression had a significantly higher survival rate ($P=0.0051$) (Figure 2B).

**B7-H1 and B7-H4 expression regulates the infiltration of T cells in pancreatic cancer**

CD3$^+$/CD8$^+$-effective T cell subsets were found infiltrated in pancreatic cancer tissues (Figure 3A). T-cell-mediated immunity was the dominant antitumor immune response. Our results also showed that CD3-stained total T-cell infiltration in pancreatic cancer tissue was associated with patient survival: high infiltration led to better survival. Although CD8$^+$ T cells were thought to be the effective antitumor T-cell subset, we did not find any correlation between CD8$^+$ T-cells and survival rate in these patients with pancreatic cancer (Figure 3B). We further investigated the relations between B7-family molecule expression and T-cell infiltration densities in pancreatic cancer. As shown in Table 2, B7-H1 expression was found to be negatively related to the intensity of both CD3$^+$ T cells and CD8$^+$ T cells, in contrast to B7-H3 expression, which was not correlated with T-cell infiltration intensities. B7-H4 expression was negatively related to CD3$^+$ T-cell infiltration intensity, but was not related to CD8$^+$ T-cell intensity. Therefore, the present data further support an underlying role of the B7 family of molecules in suppressing T-cell-mediated cellular immune surveillance of human pancreatic cancer.

**Discussion**

Pancreatic cancer is one of the most aggressive human cancers and a leading cause of cancer-related deaths in the world today. Despite advancements in surgical treatment and new chemotherapeutic agents, a complete cure is rarely available, and the mortality rate is only weakly depressed. Immunotherapy might be the most potentially curative therapy for pancreatic cancer. The B7 family of molecules has attracted great attention in the development of effective immunotherapy in human malignancies. To elicit a sufficient tumor-specific T-cell response, inhibition of the negative T-cell pathway might represent a breakthrough in the regulation of tumorous T cells. An intervention targeting B7/CTLA-4 or B7/4-1BB has been used in cancer immunotherapy, but the use of blocking antibodies is likely to be limited in current clinical trials. Therefore, attempts to identify new negative regulators are needed. B7-H1, B7-H3, and B7-H4 are the newest members of the B7 family to be identified in the past decade, and have been proven to play important roles in T-cell regulation. The abnormal expression of these three molecules in various human malignancies implies their potential effect on the tumor-specific immune response, although the exact significance of the three molecules in pancreatic cancer remains poorly known and ambiguous.
In this study, we first examined the expression of B7-H1, B7-H3, and B7-H4, and found that more than 50% of patients expressed B7-H1 and B7-H4, whereas nearly 100% of patients expressed B7-H3. After the coexpression analysis, we found that only one patient did not express any of these three molecules, whereas the other patients showed different levels of expression of each of the three molecules. Because these three molecules are not expressed in normal pancreatic tissues, their abnormal expression in pancreatic cancer confirms their potential role in the progression of pancreatic cancer. Further study revealed that the abnormal expression of these three B7-family molecules was correlated with tumor size, lymph-node metastasis, differentiation grade, and invasion depth, which implies that these three molecules might be involved in the different pathways of tumor development. Our results are consistent with several previous studies in other malignancies, including lung cancer and prostate cancer, as well as pancreatic cancer. Survival analysis confirmed the negative effect of these three B7-family molecules in pancreatic cancer. B7-H4 expression was found to be negatively correlated with survival rate, and more notably the coexpression of these three molecules led to the worst survival rate, whereas lesser expression of the B7 family of molecules resulted in better survival. Our research, for the first time, reveals the coexpression of B7-family molecules in pancreatic cancer and strongly supports the prognostic value of the B7 family of molecules in pancreatic cancer, suggesting that the coexpression analysis of these three molecules could be an indicator of pancreatic cancer prognosis.

Table 2 Correlations between B7 family molecules and T-cell subset infiltration in pancreatic cancer

<table>
<thead>
<tr>
<th>T-cell infiltration density</th>
<th>Cases</th>
<th>B7-H1 expression P-value</th>
<th>B7-H3 expression P-value</th>
<th>B7-H4 expression P-value</th>
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<tr>
<td></td>
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<td>Low</td>
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<tr>
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<td>CD8</td>
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<tr>
<td>Low</td>
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<tr>
<td>Total</td>
<td>63</td>
<td>39</td>
<td>24</td>
<td>&lt;0.001</td>
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Figure 3 (A and B) T-cell subset infiltration and survival analyses. (A) CD3+ T cells and CD8+ T cells were found in pancreatic cancer tissues; (B) survival analyses of T-cell subsets.
In pancreatic cancer, some conclusions have been obtained about the mechanism of B7-family molecules. The latest reports showed that B7-H4 expression in pancreatic cancer cells could boost cell proliferation and migration. Moreover, loss of B7-H4 could induce the apoptosis and inhibition of the Erk1/2 signaling pathway of pancreatic cancer cells. B7-H3 knockdown decreased cell migration and transwell invasion in vitro, and in vivo essay showed that B7-H3 expression reduced pancreatic cancer metastasis in vivo. Further study indicated that silencing of B7-H3 could increase drug-induced apoptosis of pancreatic cancer cells. The overexpression of B7-H1 in pancreatic cancer cells promoted cell proliferation. Conversely, the small hairpin RNA knockdown of B7-H1 inhibited pancreatic cancer cell proliferation.

Our previous studies showed that higher expression of B7-family inhibitory molecules by tumor cells was significantly correlated with the densities of TILs in human malignancies. All the studies mentioned indicated that the B7 family of molecules might be involved in cancer progression via two possible mechanisms, one of which serves as a negative regulator of T-cell-mediated antitumor immunity, and the other renders tumor cells refractory to apoptosis and ability of proliferation. In the present study, we investigated T-cell infiltration in pancreatic cancer tissues as well. Previously, we investigated the prognostic value of T-cell subsets in pancreatic cancer, and found that CD3-stained total T-cell infiltration was critical to patient survival, but CD8+-effective T-cell infiltration was irrelevant to survival. This result is consistent with research that has shown that the majority of tumor-infiltrated CD8+ T cells are ultimately proven to be exhausted T cells that could not exert an antitumor effect. Furthermore, we analyzed the relationships between the B7 family of molecules and T-cell densities in pancreatic tumor tissues. The results revealed that B7-H1 and B7-H4 expression were related to T-cell subset infiltration, which suggests that B7-H1 and B7-H4 might promote pancreatic cancer through negative regulation of the T-cell-based immune response. B7-H3 was not related to T-cell infiltration, and this might be because of its unknown receptor, which was not considered constantly expressed on T cells.

In conclusion, our findings indicate that the inhibitory costimulatory molecules B7-H1, B7-H3, and B7-H4 are involved in pancreatic cancer progression, and their coexpression could be a valuable prognostic indicator. Negative regulation of T-cell infiltration might be the main mechanism of action of the B7 family of molecules in pancreatic cancer. Our data suggest that efforts to develop immunotherapeutic approaches that target the B7 family of molecules for the treatment of pancreatic cancer are needed.

**Acknowledgment**

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**Disclosure**

The authors report no conflicts of interest in this work.

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