B7-H3 and B7-H4 are independent predictors of a poor prognosis in patients with pancreatic cancer

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Abstract. B7-H3 and B7-H4 belong to the peripheral membrane protein B7 family and are hypothesized to regulate immunity. These proteins are expressed in human pancreatic cancer (PC), but their prognostic significance is poorly understood. The present study examined the association between B7-H3 and B7-H4 expression and the overall survival time in patients with PC that underwent surgery at the Second Affiliated Hospital to Zhengzhou University between April 2000 and January 2009. Immunohistochemical analysis demonstrated that B7-H3 and B7-H4 were expressed in 35 (88%) and 30 (75%) tumor tissue samples, respectively, which were obtained from 40 patients with PC. Statistical analysis revealed that B7-H3 expression was associated with an early tumor-node-metastasis stage (stage I and II; P<0.01), and B7-H4 expression was associated with tumors located in the body and tail of the pancreas (P<0.01) and lymph node metastasis (P=0.02). In addition, using Spearman's rank correlation coefficient, the present study demonstrated a positive correlation between B7-H3 expression and B7-H4 expression (r=0.37; P=0.02) in tumor samples. B7-H4 expression (P=0.01), tumors located in the pancreatic body and tail (P<0.01), lymph node metastasis (P=0.02) and combined B7-H3 and B7-H4 expression (P<0.01) were indicators of a poor overall survival time. However, solitary B7-H4 expression (P=0.03) and combined B7-H3 and B7-H4 expression (P=0.04) remained significant prognostic factors following adjustment for other prognostic factors in a multivariate Cox's proportional hazards regression model. Therefore, the present results indicate that solitary B7-H4 expression and a combination of B7-H3 and B7-H4 expression are independent predictors of a poor prognosis in patients with PC.

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Abbreviations: PC, pancreatic cancer; OS, overall survival

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Introduction

Pancreatic cancer (PC) is an aggressive disease and is the fourth leading cause of cancer-associated mortality in the western world, with an overall 5-year survival rate of <6% (1). Surgical resection is the only potentially curative treatment; however, since patients are often diagnosed at advanced stages, only 15-20% of patients are candidates for a pancreatectomy (2). A prognostic evaluation is required for patients with resected pancreatic cancer (3). Such evaluations may distinguish patients with an improved prognosis from those that require additional and more vigorous treatment regimens (3).

The prognosis of patients in various types of cancer is hypothesized to be closely associated with immune evasion by tumor cells (4). Tumor cells may escape from immune surveillance through various mechanisms (5), which are dependent on the balance between co-stimulatory and co-inhibitory signals (6,7). This consists of signals from the peripheral membrane protein B7 family, located on antigen presenting cells (APCs), when they interact with cluster of differentiation (CD)-28, which is located on T cells. The B7 family members (8), including B7-H3 and B7-H4, are hypothesized to play a role in tumor immune evasion, which in turn affects the prognosis of patients.

B7-H3 (9) is an accessory co-stimulatory molecule in the B7/CD28 family, although it is theorized to perform stimulatory and inhibitory functions. The stimulatory properties of B7-H3 consist of promoting T cell proliferation and interferon-γ production (10), while the inhibitory properties consist of impairing type 1 T-helper cell responses and protecting cells from natural killer cell-mediated lysis (11). In certain human malignant tumors, including gastric cancer, the expression of B7-H3 has been revealed to be associated with an improved prognosis of patients (12). By contrast, in non-small cell lung cancer (13), clear-cell renal cell carcinoma (14) and

prostate cancer (15), B7-H3 has been described as an indicator of a poor prognosis. Yamato *et al* (16) reported that B7-H3 was expressed in PC, and revealed that its expression was associated with aggressive clinicopathological characteristics; however, the study did not assess the association between B7-H3 expression and the survival time of patients.

B7-H4 is a negative co-stimulatory molecule that inhibits $CD4^+$ and $CD8^+$ T cell proliferation, cell-cycle progression and interleukin-2 production, and renders tumor cells refractory to apoptosis (17). In breast (18), non-small cell lung (12) and ovarian cancer (19), and renal cell carcinoma (20), aberrant expression of B7-H4 has been demonstrated to be associated with a poor clinical outcome. B7-H4, along with P53, was revealed to be a potential diagnostic marker for PC, and the expression of B7-H4 was associated with adverse pathological features, including an increased tumor grade (21). However, the prognostic value of B7-H4 expression in PC has not been fully elucidated.

The present study evaluated the expression of B7-H3 and B7-H4 in PC and normal tissue samples using immunohistochemical analysis to determine whether B7-H3 and B7-H4 expression is an independent predictor for the overall survival (OS) time in patients with PC.

Materials and methods

Tissue samples. Pancreatic tumor samples and clinical data were obtained from 40 patients with PC (26 men and 14 women; median age at diagnosis, 54 years; range, 34-80 years), who underwent surgery without pre-operative therapy between April 2000 and January 2009 at the Second Affiliated Hospital of Zhengzhou University (Zhengzhou, Henan, China). The present study also obtained 10 normal pancreatic tissue samples, which acted as a control group and were randomly selected during the same time period from benign pancreatic tumor resections. The PC stage was classified according to the 2002 American Joint Committee on Cancer tumor-node-metastasis (TNM) staging system (22). Tumor cell differentiation was determined using the 2000 World Health Organization classification (23). Clinical data consisting of the patient age, patient gender, tumor location, histopathological type, histological grade, tumor stage and lymph node invasion was collected from medical records. The median follow-up time was 58.5 months (range, 12-134 months), and the most recent follow-up occurred on April 30, 2011. The present study was performed subsequent to obtaining informed consent from all patients and approval from the independent Institute Research Ethics Committee of The First Affiliated Hospital of Soochow University (Suzhou, China).

Immunohistochemistry. Immunostaining was performed using an EliVision™ plus kit (Maixin Biotech, Inc., Fuzhou, Fujian, China), according to the manufacturer's protocol. Formalin-fixed paraffin-embedded tissue blocks were sliced in 3-μm sections and mounted on charged glass slides (Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA). Antigen retrieval was performed in citrate buffer (20 mmol/l; pH 6.0; Fuzhou Maixin Biotech Co., Ltd., Fuzhou, China) at 120˚C for 10 min. Endogenous peroxidase activity was blocked with 3.0% hydrogen peroxide (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) for 10 min. Mouse anti-human B7-H3 (dilution, 1:100; clone, 7D7) (24) and mouse anti-human B7-H4 (dilution, 1:100; clone, 3C8) monoclonal antibodies (gifted from Soochow University, Suzhou, China) (25) were used as the primary antibodies. Negative controls were performed using mouse monoclonal immunoglobulin G (NC-1390; Fuzhou Maixin Biotech Co., Ltd.) as the primary antibody. For visualization, the sections were incubated with 3,3'-diaminobenzidine solution (Fuzhou Maixin Biotech Co., Ltd.) and counterstained with hematoxylin (Amresco, LLC, Solon, OH, USA).

Evaluation of immunostaining. B7-H3 and B7-H4 expression was calculated as the percentage of tumor cells exhibiting immunoreactivity in the cytoplasm or on the membrane, which was determined by counting the number of B7-H3 or B7-H4-stained tumor cells out of 1,000 tumor cells in each tissue section. Using an Olympus BH2 microscope (Olympus Corporation, Tokyo, Japan), cell counts were performed at a x400 magnification in ≥5 randomly selected fields in tumor sections. The intensity of the B7-H3 or B7-H4 expressing cells was semi-quantitatively graded as previously described (13), according to the positive cell percentage, as follows: 0, focal expression in <10%; +, focal expression in 10-40%; ++, focal expression in 40-80%; and +++, focal expression in >80%. For the purposes of the present analysis, the samples were classified into 2 groups on the basis of staining intensity, as follows: Negative, <10% expression; and positive, 10-100% expression.

Statistical analysis. χ^2 and Fisher's exact tests were used to examine the association between B7-H3 and B7-H4 expression and various clinicopathological parameters, whilst Spearman's rank correlation coefficient was used to determine correlations. The overall survival times of patients with or without B7-H3 and B7-H4 expression were compared using the Kaplan-Meier method of survival time analysis and the log-rank test. For all patients, the survival time was calculated from the date of pathological diagnosis to the date of the patient succumbing to the disease or the date of the last follow-up. The data from patients that were alive at the last day of follow-up were censored. The median follow-up time was calculated using only censored data. The median survival time was also calculated. Hazard ratios (HR) and 95% confidence intervals (CI) were estimated using univariate and multivariate Cox's proportional-hazard models. All statistical analysis was calculated with SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA), and the P-values were two-tailed. $P<0.05$ was considered to indicate a statistically significant difference.

Results

B7-H3 and B7-H4 expression in PC tissue samples. Staining for B7-H3 and B7-H4 was observed in the cell cytoplasm and membrane in cancerous and non-cancerous cells. B7-H3 expression was increased in PC tissue samples (35 out of 40 patients, 88%; Fig. 1A) compared with normal tissue samples (3 out of 10 patients, 30%; Fig. 1C; $P<0.01$). Similarly,

Table I. Association between clinicopathological parameters and B7-H3 and B7-H4 expression in pancreatic cancer tissue samples.

		B7-H3 expression			B7-H4 expression		
Clinicopathological parameters	n	Present, n	Not present, n	P-value	Present, n	Not present, n	P-value
Total	40	35	5		30	10	
Age at diagnosis, years				0.63			0.47
≤54 years	23	21	2		16	7	
>54 years	17	14	3		14	3	
Gender				0.64			0.25*
Male	26	22	4		18	8	
Female	14	13	1		12	2	
Tumor location in pancreas				0.32			<0.01*
Head	26	24	2		16	10	
Body or tail	14	11	3		14	0	
Histological grade				0.33			1.00
Well	10	10	0		8	2	
Moderate	14	11	3		10	4	
Poor	16	14	2		12	4	
Histopathological type				0.43[a]			0.26
Ductal adenocarcinoma	36	31	5		28	8	
Mucinous cystadenocarcinoma	4	4	0		2	2	
Tumor stage				<0.01			0.10
T1	9	5	4		8	1	
T2	11	11	0		5	6	
T3	12	12	0		10	2	
T4	8	7	1		7	1	
Lymph node metastasis				0.03[a]			0.02
N0	17	17	0		9	8	
N1	20	15	5		18	2	
TNM stage				<0.01			0.73
I	11	11	0		8	3	
II	15	15	0		10	5	
III	8	7	1		7	1	
IV	6	2	4		5	1	

[a]χ^2 test. Lymph node metastasis was not evaluated in 3 patients that underwent a palliative operation. TNM, tumor-node-metastasis.

B7-H4 expression was increased in PC tissue samples (30 out of 40 patients, 75%; Fig. 1B) compared with normal tissue samples (2 out of 10 patients, 20%; Fig. 1D; P<0.01).

Association between B7-H3 and B7-H4 expression and clinicopathological parameters. The association between tumor cell B7-H3 and B7-H4 expression and clinicopathological parameters is revealed in Table I. The present study demonstrated that positive B7-H3 expression in PC tissue samples was associated with an early TNM stage (P<0.01). Positive B7-H4 expression was associated with tumors located in the body and tail of the pancreas, compared with tumors located in the head of the pancreas (P<0.01), and lymph node metastasis (P=0.02). The expression of either protein was not associated with the age of the patient at the time of diagnosis (≤54 vs. >54 years), gender, histological grade, or histopathological type. In addition, the present study identified a positive correlation between B7-H3 expression and B7-H4 expression in PC tissue samples (r=0.37; P=0.02).

Association between B7-H3 and B7-H4 expression and OS time. At the time of the final follow-up, 34 out of 40 patients had succumbed to PC; of the remaining 6 patients, 3 were alive and 3 were lost to follow-up. The patients with tumors that expressed B7-H4 had a poorer OS time compared with the patients with tumors that did not express B7-H4 (P=0.01). Other prognostic factors were also demonstrated to decrease the OS time, including tumors located in the body and tail of the pancreas compared with tumors located in the head of the pancreas (P<0.01) and lymph node metastasis (P=0.02). The

Table II. Multivariate backward stepwise Cox's proportional hazards analyses demonstrating clinicopathological parameters that were associated with a poorer overall survival time in patients with pancreatic cancer.

Clinicopathological parameter	HR	95% CI	P-value
B7-H3 and B7-H4 expression	0.17	0.03-0.94	0.04
B7-H4 expression	0.15	0.03-0.80	0.03
Lymph node metastasis	1.77	0.15-20.77	0.65
Tumor located in body or tail of pancreas	0.73	0.17-3.20	0.68

HR, hazard ratio; CI, confidence interval.

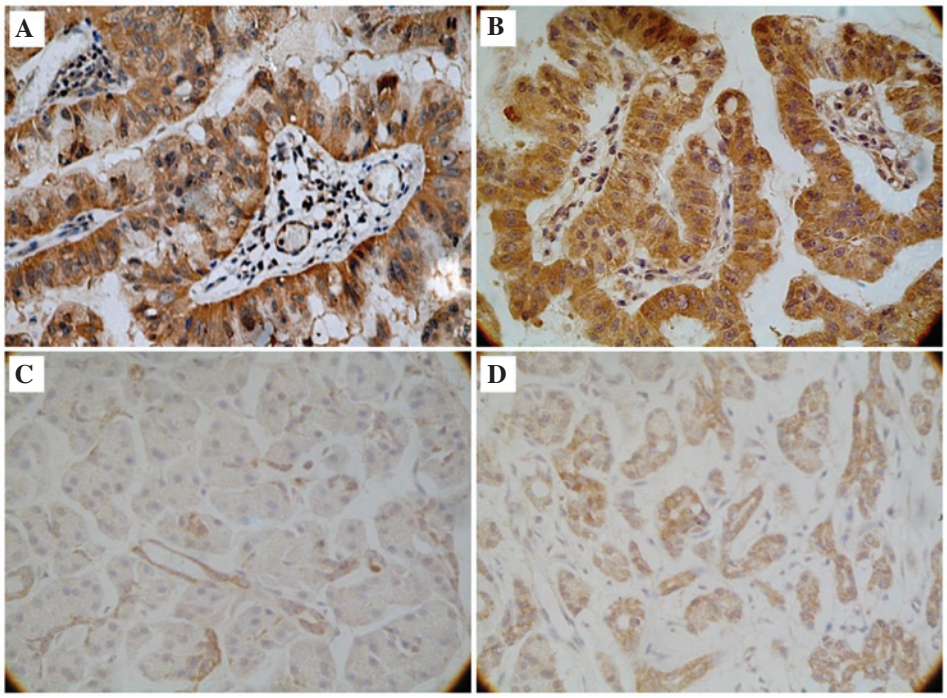

Figure 1. Immunohistochemical staining demonstrating the expression of B7-H3 and B7-H4 in PC and normal pancreatic tissues. (A) Cytoplasmic expression of B7-H3 in PC tissue (magnification, x400). (B) Cytoplasmic expression of B7-H4 in PC tissue (magnification, x400). (C) High expression of B7-H3 in the acini of normal pancreatic tissue (magnification, x100). (D) Low expression of B7-H4 in the intercalated cells of normal pancreatic tissue (magnification, x100). PC, pancreatic cancer.

patients with tumors that did not express B7-H3 and B7-H4 had an improved prognosis compared with the patients with tumors that did express 1 or 2 of the proteins (P<0.01).

Following adjustments for tumor location and lymph node metastasis, the patients with tumors that expressed the 2 proteins B7-H3 and B7-H4 were at an increased risk of mortality compared with the patients with tumors that expressed only 1 protein or did not express either proteins (HR, 0.17; 95% CI, 0.03-0.94; P=0.04; Table II). In addition, patients with tumors that expressed B7-H4 were at an increased risk of mortality compared with the patients with tumors that did not express B7-H4 (HR, 0.15; 95% CI, 0.03-0.80; P=0.03; Table II).

Discussion

B7-H3 and B7-H4 are two novel members of the B7 superfamily of peripheral membrane proteins that are expressed on APCs and have been implicated in tumor immunogenicity and cancer development (13,26,27). The present study demonstrated that B7-H3 and B7-H4 were expressed in the cytoplasm and membrane of cancerous and non-cancerous pancreatic cells, and that B7-H4 expression or the combination of B7-H3 and B7-H4 expression in cancerous cells was associated with a decreased overall survival time, independent of other prognostic factors that are also associated with B7-H3 and B7-H4 expression. Although other studies have demonstrated an association between B7-H3 or B7-H4 expression and clinicopathological parameters, in particular that increased B7-H1 expression in PC tissues is associated with a decreased overall survival time (28), the present study identified that an expression of B7-H4 and a simultaneous combined expression of B7-H3 and B7-H4 are independent predictors of a poor prognosis in patients with PC.

Previous evidence (29) indicates that B7 family molecules are expressed in PC and that their interactions with

corresponding receptors are directly associated with the T cell engagement of an antigen. An upregulation of B7-H3 and B7-H4 expression in tumor tissue was detected in non-small cell lung (13) and prostate cancer (15) in previous studies; however, only a few studies have evaluated B7-H3 or B7-H4 expression levels in PC (30). Yamato *et al* (16) identified that B7-H3 expression in PC was associated with an advanced tumor stage and lymph node metastasis, and Awadallah *et al* (21) reported that B7-H4 was expressed on cytological specimens, which were obtained by endoscopic ultrasound-guided fine needle aspiration and surgical specimens from patients with PC. The overall expression of B7-H4 in benign tissues was relatively decreased compared with that observed in the majority of carcinoma patients. In the survival and correlation analyses of 24 patients (17 surgically resected cases and 7 biopsy cases) that succumbed to pancreatic adenocarcinoma, there was no statistically significant association identified between patient survival rate and the proportion of cells that stained positively for B7-H4 expression (P=0.55) or the intensity of B7-H4 staining (P=0.26) (21). The present study demonstrated that B7-H3 and B7-H4 expression were associated with certain clinicopathological features of patients with PC, including the increased expression of B7-H3 in an early TNM stage (stage I or II; P<0.01), the increased expression of B7-H4 associated with tumors located in the body and tail of the pancreas compared to those located in the head of the pancreas (P<0.01) and lymph node metastasis (P=0.02).

The prognostic role of B7-H3 in cancer is unclear. In gastric cancer (12), an increased B7-H3 expression is revealed to be associated with an improved prognosis compared with a decreased B7-H3 expression; however, in non-small cell lung cancer (13), renal cell carcinoma (14), and prostate cancer (15), B7-H3 served as a marker of poor prognosis or disease metastasis. In the present study, B7-H3 was more likely to be expressed in PC tissue compared with normal pancreatic tissue, which is consistent with a previous study (16). Although the present findings did not demonstrate any survival benefit from the presence of B7-H3 expression, it was observed that B7-H3 expression was increased in late-stage tumors (T2-T4) compared to early-stage tumors (T1) and in tumors that had not metastasized to the lymph nodes compared with tumors that had metastasized (P<0.01 and P=0.03, respectively). These results suggest that B7-H3 may be involved in the development of human PC. However, it is unclear why B7-H3 expression was not a predictor of OS time, considering its positive correlation with B7-H4 expression as well as the decreased OS time observed in patients with tumors that were positive for a combination of B7-H3 and B7-H4 expression. The inconsistent association between B7-H3 expression and prognosis in PC and other cancers may be partly explained by an unknown receptor for B7-H3 (27,31) and the complex tumor microenvironment (32).

The present study revealed that the presence of solitary B7-H4 expression or a combination of B7-H3 and B7-H4 expression in human PC may serve as independent predictors of poor prognosis. Mugler *et al* (33) and Simon *et al* (34) hypothesized that B7-H4 may induce immune evasion through the suppression of T cell activation and cytokine secretion and the development of cytotoxicity. *In vitro* and *in vivo* studies (35) have suggested that B7-H4 overexpression in numerous tumor types may allow tumors to avoid eliciting an antitumor immune response. Although the present results suggest that solitary B7-H4 expression and a combination of B7-H3 and B7-H4 expression may be prognostic factors for PC, the exact mechanisms by which expression of these proteins leads to a decreased survival time remains to be clarified.

A limitation of the present study was the relatively small sample size, which may lead to an overestimation of the magnitude of the association between variables. Considering the potential prognostic value of B7-H3 and B7-H4 in patients with PC, which would aid in the clarification of the most effective treatments to administer to patients, additional retrospective studies of a larger scale are required to confirm the association that the present study observed between the expression of B7-H3 and B7-H4 and the clinical outcomes in PC patients.

In conclusion, the present findings indicate that B7-H3 and B7-H4 are more likely to be expressed in PC tissue compared with normal pancreatic tissue, and that solitary B7-H4 expression and a combination of B7-H3 and B7-H4 expression are associated with a decreased OS time in patients with PC. Therefore, solitary B7-H4 and a combined B7-H3 and B7-H4 expression in PC tissues may be useful independent predictors of prognosis. Large-scale studies are required to validate these findings.

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