Aberrant expression of B7-H4 correlates with poor prognosis and suppresses tumor-infiltration of CD8+ T lymphocytes in human cholangiocarcinoma

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Received December 31, 2015; Accepted February 14, 2016

DOI: 10.3892/or.2016.4807

Abstract. B7-H4, as a member of the costimulatory B7 family, serves a critical role in the negative regulation of T cell-mediated antitumor immune responses. Cholangiocarcinoma (CCA) has a poor prognosis due its invasiveness and associated metastasis. The present study investigated the expression of B7-H4 in patients with CCA and its association with patient prognosis. The correlation between B7-H4 expression and $CD4^+$ and $CD8^+$ tumor-infiltrating lymphocytes was also investigated. The results demonstrated that high B7-H4 expression was detected in cancer tissues (54/110; 49.1%) compared with that noted in chronic inflammatory bile duct tissues (4/19; 21.1%). Furthermore, all 8 biliary adenoma samples showed negative staining. The expression of B7-H4 was significantly associated with adverse clinical and pathological features including histologic grade (P<0.001), tumor status (P=0.025), lymph node metastasis (P=0.035) and 6th Union for International Cancer Control stage (P=0.019). Kaplan-Meier survival analysis and Cox regression analysis indicated that aberrant B7-H4 expression was a significant independent predictor of poor overall survival and early recurrence. In addition, the present study demonstrated that B7-H4 expression in tumor cells was negatively correlated with the density of $CD8^+$ T cells in the tumor stroma. Co-culture assays indicated that knockdown of B7-H4 increased $CD8^+$ T cell-mediated cytotoxicity *in vitro*, suggesting that the expression of B7-H4 may serve a role in shielding tumors from immune surveillance by suppression of tumor-infiltrating $CD8^+$ T lymphocytes in CCA. In conclusion, the present study showed that aberrant expression of B7-H4 was correlated with poorer prognosis and suppressed $CD8^+$ tumor-infiltrating lymphocytes in CCA.

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Key words: cholangiocarcinoma, B7-H4, tumor immune escape, tumor-infiltrating lymphocytes, prognosis

Introduction

Cholangiocarcinoma (CCA) is a severe tumor originating from epithelial cells in the intrahepatic and extrahepatic bile ducts and is associated with a poor prognosis (1,2). Surgical resection is the predominant treatment, however, the majority of patients are diagnosed too late to resect or present with metastatic disease (3). Additionally, the lack of biomarkers and effective non-surgical therapeutic modalities limit current treatment options. To date, no second-line therapy has definitely demonstrated improved long-term survival (4). Therefore, improving the understanding of the molecular mechanisms of carcinogenesis is required to develop novel therapies for the treatment of CCA.

The immunological response to cancer is an important protective mechanism against cancer. However, the escape of tumors from immune surveillance has been attributed to immune system dysfunction, leading to tumor progression, metastasis and recurrence. There are a number of strategies that enable tumor cells to escape immune surveillance, including dysfunctional major histocompatibility complex class I molecule, immunosuppressive factors and aberrant expression of costimulatory molecules. While the regulatory mechanisms by which costimulatory molecules regulate the immune system have received research focus, the B7 family has not been investigated in detail.

The costimulatory B7 family are cell-surface protein ligands, providing stimulatory and inhibitory signals to regulate the T cell response. The B7 family comprises seven members, B7.1 (CD80), B7.2 (CD86), B7-DC (CD273, PD-L2), B7-H1 (CD274, PD-L1), B7-H2 (ICOS-L), B7-H3 (CD276) and B7-H4 (B7x, B7S1) (5). B7-H4 is a new member of the B7 family, and is a type I-transmembrane protein and functions via a glycosyl phosphate-dylinositol linkage, binding to a currently unidentified receptor. B7-H4 is expressed at low levels in various peripheral tissues, including lung, colon, liver, kidney, pancreas, small bowel, breast and uterus (6,7). Aberrant expression of B7-H4 has been observed in several

tumor types, including those of the breast, skin, lungs, colon, kidney, brain and ovaries (8). B7-H4 has been demonstrated to effect the negative regulation of T cell-mediated immune responses by inhibiting T cell activation, proliferation, cytokine production and cytotoxic activity (6). Furthermore, previous studies have reported that the expression levels of B7-H4 are correlated with clinicopathological parameters, and B7-H4 is currently considered to be a prognostic marker in various tumors. However, the expression levels of B7-H4 have not been investigated in different tumor types, and its correlation with clinical outcomes remains controversial (8). In particular, the expression of B7-H4 in CCA and its clinical significance have not been analyzed in detail. Further investigation of the association between B7-H4 and CCA is required, and may provide potential molecular targets for improved methods of detection and treatment.

In the present study, the expression of B7-H4, and its correlation with clinicopathological parameters in CAA were investigated. The prognostic value of B7-H4 was evaluated using the Kaplan-Meier estimator and Cox regression analysis. The present study aimed to examine the tumor microenvironment by investigating the association between B7-H4 protein and the density of various T lymphocytes. Additionally, to understand the functional role of B7-H4 in antitumor T cell responses, we carried out co-culture with $CD8^+$ T cytotoxic lymphocytes (CTLs) to identify its impact on the suppression of CTL activity. Together, the results suggest that B7-H4 may represent a novel prognostic predictor, in addition to being a potential target for antitumor immunotherapy for patients with CCA.

Materials and methods

CCA patients and clinical samples. Tissues were obtained from 137 patients who underwent surgery at the Southwest Hospital (Chongqing, China) between 2005 and 2011. Patients who underwent pre-operative treatment, such as radiotherapy and/or chemotherapy, were excluded. A total of 110 cancerous and 28 lymph node metastatic samples from the patients were collected. A total of 19 chronic inflammatory bile duct samples from patients with hepatolithiasis and 8 biliary adenoma samples were also collected from Southwest Hospital. All samples were obtained with informed consent from all patients, according to the protocols approved by the Institutional Review Board of the Southwest Hospital, Third Military Medical University. The pathological reports were reviewed and the clinical features of the 110 patients with CCAs are presented in Table I. Tumor-node-metastasis stages were assigned according to the 6th Union for International Cancer Control. Overall survival was calculated from the date of surgery to the date of mortality or last contact. Recurrence-free survival was computed from the date of surgery to the date of recurrence.

Immunohistochemistry (IHC). Formalin-fixed, paraffin-embedded resected tissue blocks were cut into 4-mm sections and mounted on charged glass slides, deparaffinized and rehydrated in a graded series of ethanol. Endogenous peroxidase activity was blocked with a solution of 3% H_2O_2 in methanol for 30 min. Following washing in phosphate-buffered saline (PBS), antigen retrieval was performed in a citrate buffer (pH 6.0) at 120˚C for 15 min. After cooling and washing 3 times with PBS (pH 7.4) for 5 min each, sections were incubated with the primary antibodies against B7-H4 (LLC.250473; dilution 1:200; Abbiotec, San Diego, CA, USA) (9), CD4 (TA802240S; dilution 1:150), CD8 (TA802079S; dilution 1:200) (both from OriGene Technologies, Inc., Rockville, MD, USA) in a humid chamber at 4˚C overnight. The sections were then washed with PBS and incubated with a secondary polymeric peroxidase-labeled rabbit anti-mouse antibody (Dako, Glostrup, Denmark) for 1 h at 37˚C. Subsequently, the nuclei were counterstained with hematoxylin and visualized with 3,3-diaminobenzidine tetrahydrochloride (Dako). A negative control was performed using PBS instead of the primary antibodies under the same conditions. The sections were dehydrated, cleared and mounted.

Evaluation of IHC staining. Two independent investigators (G.F. and J.P.), who were blinded to the patient clinicopathological data, analyzed the IHC images. Expression of B7-H4 was analyzed in 10 different high-power fields (HPFs). IHC for B7-H4 showed cytoplasmic and membrane staining. The intensity (I) of staining was scored as negative (0), weak (1), moderate (2), or strong (3). The proportion (P) of B7-H4-positive cells was defined as: 0, no staining; 1, <10% tumor cells with staining; 2, 10-50% tumor cells with staining; 3, 50-80% tumor cells with staining; 4, >80% tumor cells with staining. Samples with IHC scores (P x I) ≤3 were considered as negative and with scores >3 were defined as positive.

Additionally, the expression levels of CD4/CD8-positive T lymphocytes in the tumor nest and tumor stroma were separately determined according to IHC staining. The evaluation was based on a review of 10 different HPFs that showed the highest level of lymphocytic infiltrates for each case (low <10%, high ≥10%).

CCA cell lines and transfection. Two human CCA cell lines (QBC939 and RBE), were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) (10,11), and cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS; GE Healthcare Life Sciences, Chalfont, UK). All cell lines were incubated at 37˚C in a humidified atmosphere containing 5% CO_2.

Cells were transfected with lentiviral vectors encoding short hairpin RNA (Shanghai GenePharma Co., Ltd., Shanghai, China) targeting human B7-H4 for B7-H4 knockdown or a scrambled shRNA-NC (Shanghai GenePharma Co., Ltd.) as the control. The QBC939 and RBE cell lines were co-transfected with lentiviral vectors. The recombinant vector was named GFP&Puro-B7-H4-shRNA, according to the manufacturer's recommendations. For the QBC939 and RBE cell lines, a multiplicity of infection of 10 was used to achieve >90% transfection. The cells were cultured for 72 h following transfection. Stably transfected QBC939 and RBE cells were selected using puromycin. The efficiency of knockdown was detected using western blotting.

Western blot analysis. Seventy-two hours after co-transfection with the lentiviral vectors, cell extracts were prepared on ice using RIPA lysis buffer plus a complete protease inhibitor

Table I. Correlation between B7-H4 expression and the CCA patient clinical features.

Clinical parameters	Cases	B7-H4 expression		χ	P-value[b]
		Positive (%)	Negative (%)		
Gender				1.163	0.184
Men	68	30 (44.1)	38 (55.9)		
Women	42	24 (57.1)	18 (42.9)		
Age (years)				0.546	0.460
≤60	69	32 (46.4)	37 (53.6)		
>60	41	22 (53.7)	19 (46.3)		
Tumor location				0.896	0.334
Intrahepatic	18	7 (38.9)	11 (61.1)		
Extrahepatic	92	47 (51.1)	45 (48.9)		
Tumor size (cm)				0.115	0.734
≤5	91	44 (48.4)	47 (51.6)		
>5	19	10 (52.6)	9 (47.4)		
Tumor (T) status[a]				9.385	0.025[c]
T1	23	6 (26.1)	17 (73.9)		
T2	41	20 (48.8)	21 (51.2)		
T3	30	16 (53.3)	14 (46.7)		
T4	16	12 (75.0)	4 (25.0)		
Lymph node (N) metastasis				4.464	0.035[c]
With	42	26 (61.9)	16 (38.1)		
Without	68	28 (41.2)	40 (58.8)		
Distant metastasis (M)				3.434	0.064
With	19	13 (68.4)	6 (31.6)		
Without	91	41 (45.1)	50 (54.9)		
UICC stage[a]				9.895	0.019[c]
I	47	15 (31.9)	32 (68.1)		
II	31	20 (64.5)	11 (35.5)		
III	8	5 (62.5)	3 (37.5)		
IV	24	14 (58.3)	10 (41.7)		

[a]According to the T classification of the 6th UICC-TNM staging. [b]P-value is for t-test (continuous variables) or Chi-square or Fisher exact test (categorical variables). [c]P<0.05, statistical significance. UICC, International Union Against Cancer; TNM, tumor-node-metastasis.

cocktail (both from Beyotime, China). Protein quantitation was measured by a BCA protein assay kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. Ten micrograms of protein/lane were separated on 8% acrylamide gels by sodium dodecyl sulfate (SDS) gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes. After blockage of non-specific binding sites using 5% skim milk with Tris-buffered saline with Tween-20 (TBST) at 4˚C overnight, the membranes were incubated for 2 h at 37˚C with goat polyclonal affinity purified anti-human B7-H4 antibody (ab130151; dilution 1:1,000; Abcam, Cambridge, MA, USA). A GAPDH antibody (10494-1-AP; dilution 1:1,000; Proteintech, China) was used as an internal control. Following primary antibody incubation, the membranes were washed in TBST and incubated with horseradish peroxidase (HRP)-conjugated appropriate secondary antibodies (Dako) for 1 h at 37˚C. The membranes were washed with TBST followed by visualization using enhanced chemiluminescence (Millipore, Billerica, MA, USA) according to the manufacturer's protocol.

Generation of CCA-specific CTLs. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Histopaque-1077 (Sigma-Aldrich, Munich, Germany) from 11 healthy donors. PBMCs were seeded into 6-well culture plates containing 2 ml RPMI-1640 medium and 10% FBS at a final concentration of 5-10x10^6 cells/well. Following 2 h of incubation, non-adherent cells were removed by gentle washing with warm medium. The non-adherent cells (effector lymphocytes) were cryopreserved in FBS supplemented with 10% dimethyl sulfoxide. The resultant adherent cells containing dendritic cells (DCs) were cultured in medium supplemented with 500 U/ml recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF)

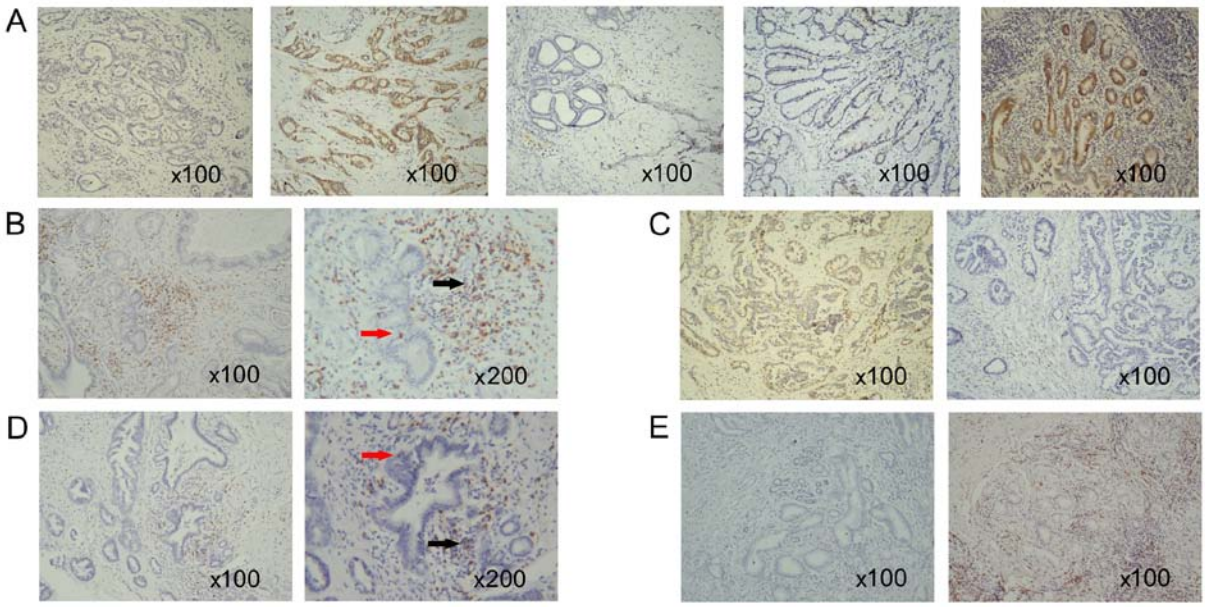

Figure 1. (A) B7-H4 expression in tissue samples. From left to right: negative staining of B7-H4 in cancerous tissue; positive staining in cancerous tissue; negative staining in an inflammatory bile duct sample (epithelial cells); negative staining in biliary adenoma samples; and positive cytoplasmic staining in lymph node metastatic tissue samples. (B) Positive cytoplasmic staining of $CD4^+$ T cells in the tumor stroma (black arrow) and tumor nest (red arrow). (C) Positive staining of B7-H4 and low-level staining of $CD8^+$ cells in cancerous tissue. (D) Positive cytoplasmic staining of $CD8^+$ T cells in the tumor stroma (black arrow) and tumor nest (red arrow). (E) Negative staining of B7-H4 and high-level staining of $CD8^+$ cells in cancerous tissue.

and 1,000 U/ml recombinant human interleukin 4 (IL-4) (both from PreproTech, Inc., Rocky Hill, NJ, USA) at 37˚C in 5% CO_2 (12). Every 2 days, one-half of the medium was replaced with fresh medium containing a double concentration of GM-CSF and IL-4 as indicated above. Following 5 days in culture, 10 ng/ml of recombinant human tumor necrosis factor-α (TNF-α; PreproTech, Inc.) was added to the medium to induce phenotypic and functional maturation of DCs (12). CCA cells were induced to apoptotic tumor cells (ATCs) with 100 μg/ml mitomycin for 24 h (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), and were presented by DCs to induce specific CTLs *in vitro*. Subsequently, the isolated non-adherent effector lymphocytes were co-cultured with the ATC-pulsed autologous DCs in a 6-well plate in the presence of 10 ng/ml recombinant human interleukin-7 (IL-7; PreproTech, Inc.). Half the medium was replaced with complete medium supplemented with 30 IU/ml recombinant human interleukin 2 (IL-2; PreproTech, Inc.) every 3 days. Following 7 days in culture, the lymphocytes were re-stimulated with the ATC-pulsed autologous DCs in medium containing 10 ng/ml IL-7 and 20 U/ml IL-2. On day 10, following the fourth round of re-stimulation, the cells were harvested and tested using CCA-specific CTL assay (13). $CD8^+$ T-mediated CTLs were purified by negative depletion using a $CD8^+$ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany).

CTL cytotoxicity assay. CTL activity was evaluated using the CytoTox 96® non-radioactive cytotoxicity assay (Promega, Madison, WI, USA) based on lactate dehydrogenase (LDH) release. After washing, the target cells were counted and seeded into 96-well V-bottomed culture plates. Varying numbers of CTLs were added to a final volume of 100 μl at the effector to target (E/T) ratios of 2.5:1, 5:1, 10:1 and 20:1 and incubated for 4 h at 37˚C. The supernatants were harvested and the assay plates were incubated for 30 min at room temperature, protected from light. The absorbance at 490 nm was recorded within 1 h after adding the stop solution. The corrected values were used in the following formula to compute percent cytotoxicity: Cytotoxicity = [(Experimental - Effector Spontaneous - Target Spontaneous)/(Target Maximum - Target Spontaneous)] x 100%.

Statistical analysis. Data were analyzed using SPSS software, version 19.0 (IBM SPSS, Armonk, NY, USA). Group comparisons of continuous data were made using t-test on independent means. For categorical data, Chi-square analysis or the Fisher's exact test was used. The Kaplan-Meier estimator and Cox analysis were used for overall survival and recurrence-free survival. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of B7-H4 as detected by IHC analysis in CCA. Representative IHC images of B7-H4 are presented in Fig. 1. The expression of B7-H4 protein was detected in 54/110 (49.1%) cancerous tissues, 15/28 (53.6%) lymph node metastatic tissues and 4/19 (21.1%) chronic inflammatory bile duct tissue samples (Table II). Notably, in the inflammatory bile duct tissues, B7-H4 was predominantly expressed in the infiltrating mononuclear cells rather than the epithelial cells of the bile duct, which is in line with its role during inflammatory reactions. In addition, 8 biliary adenoma samples (Fig. 1) stained negative for B7-H4. As shown in Table II, positive staining of B7-H4 was detected in cancerous and lymph node metastatic samples, which was significantly greater compared with the non-tumorous tissues. These data indicated that the high expression of B7-H4 was specific to the CCA tissues.

Table II. Differences in B7-H4 expression in CCA and chronic inflammatory bile duct samples.

Sample	Cases	B7-H4 expression		P-value
		Positive (%)	Negative (%)	
Cancerous	110	54 (49.1)	56 (50.9)	Control
Lymph node metastatic	28	15 (53.6)	13 (46.4)	0.672
Chronic inflammatory bile duct	19	4 (21.1)	15 (78.9)	0.023[a]

[a]P<0.05, statistical significance, compared with the cancerous sample group. CCA, cholangiocarcinoma.

Table III. Univariate analysis of various clinicopathological parameters in relation to the survival of patients with CCA.

Clinical parameters	N (%)	Overall survival		Disease-free survival	
		Median (months)	Log-rank (P-value)	Median (months)	Log-rank (P-value)
Gender					
Male	57 (62.0)	13.4	0.914	15.3	0.855
Female	35 (38.0)	14.0		16.0	
Age (years)					
≤60	58 (63.0)	16.9	0.160	16.0	0.192
>60	34 (37.0)	12.6		11.5	
Tumor size (cm)					
≤5	81 (88.0)	17.1	<0.001[b]	17.2	<0.001[b]
>5	11 (12.0)	6.2		5.8	
Tumor (T) status[a]					
T1	11 (12.0)		<0.001[b]		<0.001[b]
T2	39 (42.4)	16.3		15.2	
T3	27 (29.3)	8.5		6.1	
T4	15 (16.3)	8.9		9.6	
Lymph node (N) metastasis					
With	37 (40.2)	8.4	<0.001[b]	6.9	0.03[b]
Without	55 (59.8)	21.9		16.2	
Distant metastasis (M)					
With	17 (18.5)	8.2	<0.001[b]	6.1	<0.001[b]
Without	55 (81.5)	18.2		16.0	
UICC stage[a]					
I	39 (42.4)	23.2	<0.001[b]	31.9	<0.001[b]
II	30 (32.6)	8.4		10.6	
III	6 (6.5)	17.2		15.7	
IV	17 (18.5)	8.5		6.4	
B7-H4 expression					
Positive	47 (51.1)	12.6	0.015[b]	10.9	0.046[b]
Negative	45 (48.9)	19.5		16.7	

[a]According to the T classification of the 6th UICC-TNM staging. [b]P<0.05, statistical significance. CCA, cholangiocarcinoma; UICC, International Union Against Cancer; TNM, tumor-node-metastasis.

Expression of B7-H4 is significantly associated with clinicopathological features including tumor status, lymph node metastasis and International Union Against Cancer (UICC) stage in CCA. As B7-H4 was highly expressed in the cancer tissues of patients with CCA, its expression was investigated as to whether it correlates with clinicopathological parameters in patients with CCA. The clinicopathological features of the 110 cases of CCA were grouped by positive or negative

Table IV. Correlation between B7-H4 expression and the densities of TILs in the CCA tissue sections.

B7-H4 expression	Cases	CD4+ T cells						CD8+ T cells					
		In tumor nest			In tumor stroma			In tumor nest			In tumor stroma		
		Low	High	P-value	Low	High	P-value	Low	High	P-value	Low	High	P-value
Positive	54	42	12	0.567	48	6	0.822	38	16	0.776	35	19	0.004[a]
Negative	56	46	10		49	7		38	18		21	35	

[a]P<0.05, statistical significance. TILs, tumor-infiltrating lymphocytes; CCA, cholangiocarcinoma.

Table V. Differences in Cox analysis for overall survival and recurrence-free survival of CCAs after surgical resection (n=110).

Factors	Overall survival		Recurrence-free survival	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Expression of B7-H4 (+/-)	1.786 (1.110-2.872)	0.017[a]	2.062 (1.160-3.665)	0.014[a]
Gender (male/female)	1.049 (0.647-1.701)	0.603	0.816 (0.480-1.387)	0.452
Age, years (≤60, >60)	0.728 (0.443-1.195)	0.112	0.796 (0.472-1.343)	0.392
Location (hilar/distal)	1.451 (0.700-3.008)	0.241	2.610 (1.025-6.650)	0.044[a]
Histologic grade (G1-2/G3)	1.435 (0.841-2.447)	0.204	1.598 (0.890-2.867)	0.116

[a]P<0.05, statistical significance. CCAs, cholangiocarcinomas; HR, hazard ratio; CI, confidence interval.

B7-H4 expression. As shown in Table I, B7-H4 expression in CCA tissues was significantly associated with tumor status (P=0.025), lymph node metastasis (P=0.035) and UICC stage (P=0.019), however, not with gender, age, tumor location and size. The data indicate that the cases of CCA positive for B7-H4 exhibited more extensive metastatic behavior.

Expression of B7-H4 indicates poorer prognosis in patients with CCA. The results indicated that the expression of B7-H4 correlates with adverse pathological features, which are associated with patient prognosis. First, the correlation of B7-H4 expression with disease prognosis was analyzed. The results indicated that the expression of B7-H4 is associated with a poorer outcome following surgery. The median overall survival time of patients negative for B7-H4 was 19.5 months, which is longer than the 12.6 months observed in patients positive for the expression of B7-H4 (P=0.015; Table III). In addition, the median disease-free survival time of patients negative for B7-H4 expression was longer than that of patients with positive B7-H4 expression (16.7 vs. 10.9 months; P=0.046; Table III). Kaplan-Meier analysis indicated that the expression of B7-H4 is associated with reduced survival (log-rank P=0.015; Fig. 2A) and time to recurrence (log-rank P=0.046; Fig. 2B) in the 110 cases. To further validate these observations, multivariate Cox analysis was used, which showed that B7-H4 expression was an independent indicator of poorer overall survival and early recurrence [hazard ratio (HR)=1.786; 95% confidence interval (CI), 1.110-2.872; P=0.017; and HR=2.062; 95% CI, 1.160-3.665; P=0.014; Table V]. Taken together, the data suggest that aberrant expression of B7-H4 may be a risk factor for poorer prognosis in patients with CCA following surgical resection.

Expression of B7-H4 in tumor cells is inversely associated with the density of CD8+ T cells in the tumor stroma. Previous studies have shown that B7-H4 is able to directly or indirectly modulate immune infiltrate cells, which are comprised predominantly of CD4+ helper T cells and CD8+ cytotoxic T cells (8,14-16). The presence of tumor-infiltrating lymphocytes (TILs) within the tumor stroma or nest is considered an indicator of the host immune response to the tumor (17). Therefore, whether the expression of B7-H4 correlates with CD4+ and CD8+ TILs was investigated in the 110 CCA tissues. As shown in Table IV, levels of B7-H4 expression in the tumor cells was inversely correlated with the density of CD8+ T cells in the tumor stroma (P=0.0004), however, was not correlated with the density of CD8+ T cells in the tumor nest (P=0.776). In addition, there was no significant association between B7-H4 expression and the CD4+ T cells in the tumor stroma or nest (P=0.567 and P=0.822, respectively). Therefore, these data provide further evidence of the potential role of B7-H4 in the suppression of cellular immune surveillance in patients with CAA, and in particular tumor infiltrating CD8+ T cells.

Knockdown of B7-H4 increases CD8+ T-mediated cytotoxicity (CTL) in CCA cell lines. Considering the above observations, which indicated a negative correlation between B7-H4 expression and the density of CD8+ T cells in the tumor stroma, the impact of B7-H4 on CD8+ T cells was further investi-

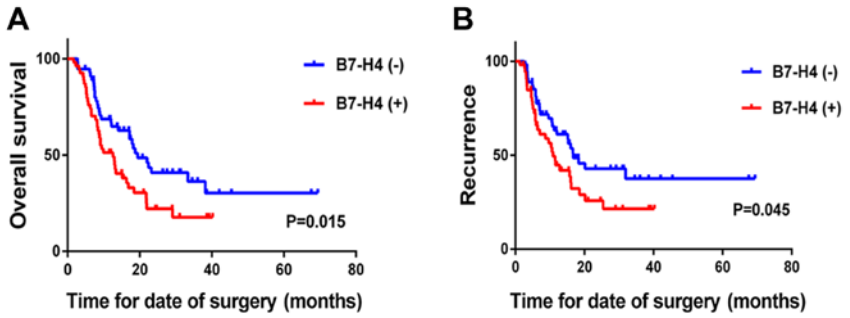

Figure 2. Association of tumor B7-H4 expression with cancer-specific survival in 110 patients with CCA. (A) Overall survival of patients in association with B7-H3 protein expression. The median survival time of patients was 19.5 months (B7-H4-negative) compared with 12.6 months in B7-H4-positive patients. (B) Cancer recurrence in patients by B7-H4 protein expression level. The median time to recurrence was 16.7 months in B7-H4-negative patients compared with 10.9 months in B7-H4-positive patients.

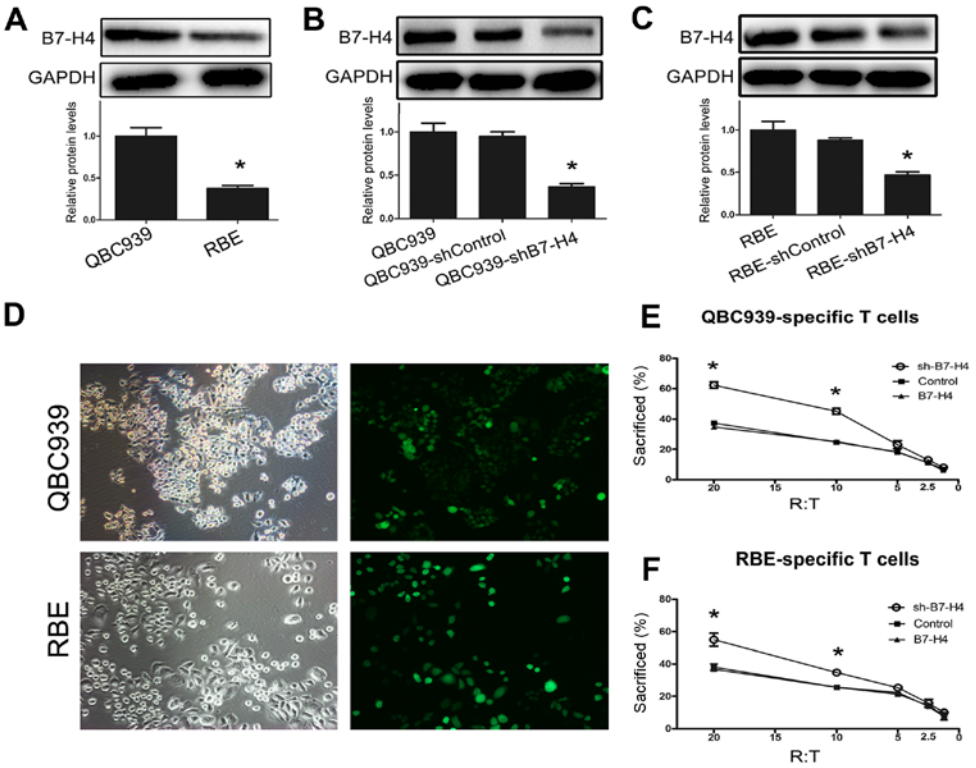

Figure 3. (A) B7-H4 protein levels in QBC939 and RBE cells measured by western blot analysis. (B and C) Validation of B7-H4 protein levels following shRNA transfection by western blotting. (D) The B7-H4 shRNA transfected cells were examined by immunofluorescence (green, shRNA with fluorescent protein). (E) A CytoTox 96 Non-Radioactive Cytotoxicity Assay was used to measure cell death in the QBC939 peptide-loaded B7-H4-shRNA transfected cells compared with the sh-control transfected and untransfected cells in co-culture with different ratios of QBC939-specific T cells. (F) A CytoTox 96 Non-Radioactive Cytotoxicity Assay measured cell death in the QBCRBE peptide-loaded B7-H4-shRNA-transfected cells compared with sh-control-transfected and untransfected cells in co-culture with different ratios ofRBE-specific T cells. *P<0.05.

gated *in vitro*. Western blot analysis showed that B7-H4 was expressed in QBC939 and RBE cells (Fig. 3A). Notably, the protein expression levels of B7-H4 were significantly greater in QBC939 cells compared with RBE cells (Fig. 3A). Knockdown of B7-H4 was performed in QBC939 and RBE cells using B7-H4-shRNA lentiviral transfection (Fig. 3B and C). By culturing with $CD8^+$ cytotoxic T cells, the cytotoxicity of $CD8^+$ T cells was markedly improved by the knockdown of B7-H4 in QBC939 and RBE cells (Fig. 3E and F). These data indicate that knockdown of B7-H4 in tumors increases $CD8^+$ T cell-mediated cytotoxicity *in vitro*.

Discussion

Epidemiological studies have demonstrated that the incidence of CCA has been increasing in recent years (18). However, patients with CCA have a poor prognosis, with a median survival of <24 months, due to late diagnosis and the limited efficacy of non-surgical therapies (19). B7-H4, as a negative regulator of T cell responses, has been observed to be expressed in a variety of human tumors. Numerous studies that focus on the clinical significance of B7-H4 have been reported (20-23). However, there are limited studies regarding

the expression of B7-H4 in CCA, and its functional relevance has not been reported in detail. The present study, to the best of our knowledge, is the first demonstration that the expression of B7-H4 is low in noncancerous and high in CCA tissues and lymph node metastases. These results are in accordance with previous studies in which the expression of B7-H4 has been observed in gastric and lung cancer tissues, with a positive rate of 44.9 and 40.7% (24,25). Furthermore, ovarian, breast and esophageal squamous carcinoma have demonstrated higher expression of B7-H4 in 93.5, 94.8 and 95.5% of cases, respectively (2,20,26). In addition, the present study observed that positive expression of B7-H4 is associated with tumor status, lymph node metastasis and tumor stage in CCA. These data are in accordance with the association between the expression of B7-H4 and clinicopathological factors associated with the prognosis of tumor patients reported in previous studies (20-23). In contrast to the present study, Tringler *et al* observed no significant association between B7-H4 expression and grade, stage or other clinicopathological features in breast cancer (2). This discrepancy may be explained by the tumor heterogeneity between CCA and breast cancer. Therefore, the present study demonstrates that B7-H4 expression is associated with advanced CCA, and indicates a more aggressive biological potential.

Due to the aforementioned results, the present study further analyzed the association between B7-H4 and the prognosis of patients with tumors. The results suggest that B7-H4 is an independent factor in the prognosis of patients with CCA.

The presence of T-lymphocytes within the tumor microenvironment is considered an important component of the antitumor immune response and reflects the process of 'cancer immunoediting' in solid tumors (27). In the present study, the immune responses against cancer cells were investigated using IHC of TILs in CCA. TILs in the tumor microenvironment are predominantly $CD4^+$ and $CD8^+$ T cells, which are considered to be the effector cells in the Th2 and Th1 antitumor immune responses, respectively. A subset of $CD4^+$ TILs was selected to examine the T-helper population, and a subset of $CD8^+$ TILs was selected to specifically examine the cytotoxic T cell population (17,28). The $CD8^+$ TILs serve a vital role in the killing of tumor cells. Previous studies have shown that increased B7-H4 expression is involved in shaping the tumor microenvironment by modulating the infiltration of $CD3^+$ and $CD8^+$ TILs in breast cancer (14), however, the subtypes of the T lymphocytes were not further analyzed. The association between B7-H4 and $CD4^+$, $CD8^+$ TILs has not been reported in CCA. The present study demonstrated that the expression of B7-H4 is inversely correlated with the density of $CD8^+$, not with $CD4^+$ TILs in the tumor stroma. Notably, $CD4^+$ and $CD8^+$ TILs exhibit low expression in the tumor nest regardless of the expression levels of B7-H4. This phenomenon may be due to the immunosuppression of the local tumor nest. These data suggest that B7-H4 may reduce the total number of infiltrating lymphocytes in tumor stroma, in particular, $CD8^+$ TILs rather than $CD4^+$ TILs, by inhibiting their recruitment or survival in the tumor microenvironment.

The present study indicated that B7-H4 acts as a negative regulator of T cells, by inhibiting the infiltration of the $CD8^+$ TILs. However, the functionality of the immune cells is of greater importance compared to the frequencies of immune infiltrates (28,30). Therefore, the impact of cancer-associated B7-H4 on CCA-specific $CD8^+$ T cytotoxicity was further explored *in vitro*. Sica *et al* (6) reported that B7-H4 inhibits T cell proliferation and cytotoxicity against allogeneic antigens *in vitro*. Additionally, it has been demonstrated in lung cancer that blockade of B7-H4 using neutralizing monoclonal antibodies promotes the apoptosis of T cells, and inhibits CTL-mediated cytotoxicity (29). In contrast to inhibiting the function of B7-H4 using B7-H4 Ig, the present study knocked down the expression of B7-H4 using lentiviral vectors encoding shRNA. This enables the direct observation of the reduction of B7-H4 in tumor cells, and the screening for stable tumor cell lines which express low levels of B7-H4. Following co-culture with CCA cells transfected with lentiviral vectors, $CD8^+$ T cell-mediated cytotoxicity was measured. Following the reduction in the expression of B7-H4, the $CD8^+$ T cell-mediated cytotoxicity was increased. Therefore, the present study suggests that it may be possible to increase $CD8^+$ T cell-mediated cytotoxicity using treatments to reduce or block B7-H4.

B7-H4 may contribute to the inhibition of $CD8^+$ T cell-mediated cytotoxicity, however, the mechanism has not been investigated in detail. Previous studies have suggested various possible explanations for the suppression of cytotoxicity. B7-H4 has been reported to interfere with T cell activation, at least in part, through signaling pathways downstream of CD28, including protein kinase B, extracellular signal-regulated kinase and c-Jun N-terminal kinase (30). As a cell-surface protein, B7-H4 is extensively N-glycosylated, which appears to regulate surrounding T cell function (31). Alternatively, B7-H4 may partially contribute to the production of cytokines such as TGF-β and IL-6 (16,32). These cytokines are able to induce $CD8^+$ CTLs to differentiate into non-cytotoxic IL-17-producing cells (33). The results of the present study provide evidence that B7-H4 may serve an important role in shielding tumors from immune surveillance by reducing the number and cytotoxic ability of $CD8^+$ TILs.

In conclusion, the present study showed that B7-H4 is overexpressed in CCA and is associated with multiple aggressive tumor features and poor prognosis. The aberrant expression of B7-H4 observed in CCA cells may significantly suppress the number and cytotoxicity of $CD8^+$ T cells in the tumor microenvironment. However, the precise role of B7-H4 in T cell regulation and the underlying mechanisms remain to be fully elucidated. Further studies are required to explore the specific role of B7-H4 in CCA. The present study indicates that B7-H4 may be a promising new target for the diagnosis and treatment of patients with CCA.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (nos. 81071729 and 8127237). We thank Spandidos Publications for providing English Language editing Service.

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