

# Aberrant expression of B7-H4 may contribute to the development of hepatocellular carcinoma

LINGLING YUAN<sup>1\*</sup>, LIJIE DONG<sup>1\*</sup>, GUOHUA YU<sup>2\*</sup>, WANFENG FAN<sup>1</sup>,  
LIN ZHANG<sup>1</sup>, PEIYUAN WANG<sup>1</sup>, XUEMEI HU<sup>3</sup> and MINGDONG ZHAO<sup>1</sup>

<sup>1</sup>Department of Radiology, Binzhou Affiliated Hospital of Binzhou Medical University, Binzhou, Shandong 256603; <sup>2</sup>Department of Pathology, Yu Huang Ding Hospital, Yantai, Shandong 264000; <sup>3</sup>Department of Immunology, Binzhou Medical University, Yantai, Shandong 264003, P.R. China

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**Abstract.** In order to determine the effect of B7-H4 on the development of human hepatocellular carcinoma (HCC), the expression levels of B7-H4 were evaluated using reverse transcription-polymerase chain reaction and flow cytometry in HL-7702 and Huh7 cells. B7-H4 protein expression levels were analyzed using western blotting and immunohistochemistry in HCC tissues collected from patients and from a mouse tumor model. Soluble B7-H4 (sB7-H4), interferon- $\gamma$  (IFN- $\gamma$ ), and interleukin-4 (IL-4) in blood serum were assessed using ELISA in patients with HCC and mice injected with tumor cells. B7-H4 was expressed in HCC cell lines, mouse tumor tissues and HCC patient tissues. However, B7-H4 was not detected in HL-7702 cells or normal human liver tissues. The expression level of B7-H4 was positively correlated with tumor-node-metastasis (TNM) stage, lymph node metastasis, and differentiation degree in patients with HCC. sB7-H4 levels in blood serum samples collected from patients with HCC and tumorigenic mice were higher compared with healthy controls. Expression levels of IFN- $\gamma$  were reduced, and IL-4 levels were increased in blood serum samples of patients with HCC and tumorigenic mice compared with healthy controls. sB7-H4 expression levels were negatively correlated with IFN- $\gamma$  levels, and with the ratio of IFN- $\gamma$  to IL-4. Additionally, sB7-H4 was positively correlated with IL-4 levels in mouse tumor tissues,

serum samples obtained from tumorigenic mice and human HCC patients. Notably, the levels of sB7-H4 and IL-4 were positively correlated and IFN- $\gamma$  was negatively correlated with the TNM stage of patients with HCC. In addition, sB7-H4 and IL-4 expression levels increased and levels of IFN- $\gamma$  and the ratio of IFN- $\gamma$ /IL-4 decreased as a function of time post tumor implantation in the mouse model. The present study determined that aberrant expression of B7-H4 contributed to HCC development. B7-H4 may be a potential target for therapy and diagnosis of HCC.

## Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world accounting for ~600,000 mortalities each year (1-3). HCC is the third most common cause of cancer mortality and is more prevalent in developing countries, particularly those located in Eastern and South-Eastern Asia compared with the developed world (4,5). In China, the age-standardized incidence rate of HCC is 37.9 per 100,000 for men and 14.2 per 100,000 for women (6). Tumor resection, liver transplantation, radiofrequency (thermal) ablation, percutaneous ethanol injection and transarterial chemoembolization are the primary treatment options (7-9); however, these treatments are expensive. Therefore, additional investigation of the pathogenesis of HCC with the goal of identifying effective and affordable methods of treatment is required.

The B7 family of T cell co-stimulatory and co-inhibitory molecules is vital for the regulation of adaptive immune responses. B7-H4 (also termed B7x or B7S1) has been determined to be involved in the downregulation of antigen-specific immune responses as it inhibited T cell proliferation, cell cycle progression, and cytokine production (10-12). In mice, B7-H4 transcripts were ubiquitously expressed; however, no protein expression was detected (11-13). A previous study did not detect B7-H4 protein expression in normal human tissues (14). However, B7-H4 has been overexpressed in various human tumors, including esophageal squamous cell carcinoma (15), pancreatic cancer (16), gastric cancer (17,18), colorectal carcinoma (19) and lung cancer (20). A previous study also reported that B7-H4 is expressed in patients with liver cancer (21);

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*Correspondence to:* Dr Mingdong Zhao, Department of Radiology, Binzhou Affiliated Hospital of Binzhou Medical University, 661 Huang-He Road, Binzhou, Shandong 256603, P.R. China  
E-mail: mingdongzhao@126.com

Dr Xuemei Hu, Department of Immunology, Binzhou Medical University, 346 Lai-Shan Road, Yantai, Shandong 264003, P.R. China  
E-mail: xue-mei-hu@163.com

\*Contributed equally

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however, whether the level of B7-H4 may be correlated with HCC pathogenesis remains to be elucidated.

B7-H4 may inhibit the function of human T cells (22). Th1 and Th2 CD4<sup>+</sup> T cells are crucial for effective immune protection (23). Th1 cells mediate antitumor reactivity through the secretion of cytokines, including interferon- $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor- $\alpha$ . Th2 cells downregulate antitumor immunity by secretion of cytokines, such as interleukin (IL)-4, IL-6 and IL-10 (24,25). A previous study determined that the Th1/Th2 balance may be disrupted in patients with HCC (26). Therefore, it is possible that aberrant expression of B7-H4 may be associated with the Th1/Th2 imbalance and contribute to HCC pathogenesis. The present study investigated the association between the expression levels of B7-H4 and HCC development.

## Materials and methods

**Cell culture.** The H22 murine hepatoma cell line was obtained from the Department of Medicine & Pharmacy Research Center of Binzhou Medical University (Binzhou, China). The HL-7702 normal human normal liver cell line and the Huh7 human hepatoma cell line were obtained from Cell Research Institute of the Chinese Academy of Sciences (Shanghai, China) and were cultured in Dulbecco's modified Eagle's medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 20% fetal bovine serum (Hyclone; GE Healthcare Life Sciences), 2 mM L-glutamine, 2 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid, 100 mg/ml streptomycin and 100 U/ml penicillin in 5% CO<sub>2</sub> at 37°C.

**Establishment of tumor model.** C57BL/6 mice were purchased from Weitong Lihua Experimental Animal Technical Co., Ltd. (Beijing, China) In total 30 mice were tumor-bearing mice and 18 were used as controls. Female and male mice (6-7 weeks old; 20-22 g) were housed at a ratio of 1:1 in a cage. All mice were housed six per cage at 20-24°C in a specific pathogen-free environment with a 12 h light/12 h dark cycle. Water and feed were sterilized by high pressure steam sterilization. All the animal experiments were performed following the ethical standards formulated by the Institutional Animal Experimental Ethics Committee of Binzhou Medical University.

H22 cells (1x10<sup>6</sup>) were injected into the abdominal cavities of 6-7 week old C57BL/6 mice (n=5). After 10 days, the mice were euthanized by cervical dislocation and a syringe was used to extract the ascites. The ascites were diluted to 5.0x10<sup>5</sup> cells/ml. Next, 0.2 ml H22 cells were subcutaneously injected into the right armpit of each mouse in order to establish tumors. After 24 h, 30 mice were randomly divided into three groups (n=10 per group). Tumors were established for 20, 30 or 40 days in the three groups. Six mice were used as controls for each time point. The healthy control group received only saline injections. The mice were sacrificed via cervical dislocation after 20, 30 and 40 days.

**Reverse transcription-polymerase chain reaction (RT-PCR).** Total mRNA was extracted from HL-7702 and Huh7 cells (1x10<sup>6</sup>) using TRIzol (TaKaRa Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol. RT

was performed using a PrimeScript RT-PCR kit (TaKaRa Biotechnology Co., Ltd.) according to the manufacturer's instructions. The following primers for *B7-H4* and  $\beta$ -*actin* were obtained from Sangon Biotech Co., Ltd. (Shanghai, China): *B7-H4*, F 5'-AGGCTTCTCTGTGTGTCTCTTC-3', R 5'-CTTGCTCTTGTGTTGCTCACTCC-3';  $\beta$ -*actin*, F 5'-TTGTTACAGGAAGTCCCTTGCC-3', R 5'-ATGCTA TCACCTCCCCTGTGTG-3'. All reactions were performed using DNA polymerase from Transgen Biotech Co., Ltd. (Beijing, China) as follows: 95°C for 10 min, 95°C for 15 sec and 60°C for 1 min for 35 cycles. PCR products were separated on 1% agarose gels and visualized by ethidium bromide staining.

**Western blot analysis.** Mouse tumor tissues were lysed using a lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) and quantified using a bicinchoninic acid protein assay. Aliquots containing 20  $\mu$ g protein were dissolved in Laemmli buffer and incubated at 95°C for 10 min. Proteins were separated on a 12% Tris-glycine SDS-PAGE (Beyotime Institute of Biotechnology), transferred onto polyvinylidene difluoride membranes (BD Pharmingen, San Diego, CA, USA) and incubated with 5% non-fat dry milk in Tris-buffered saline/0.2% Tween-20 (TBST) for 2 h at room temperature. The membranes were then incubated with anti-B7-H4 antibody (1:1,000 dilution; cat. no. ab108336; Abcam, Cambridge, UK) at 4°C overnight. Membranes were then washed three times with 1X TBST followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:5,000; dilution; cat. no. ZB-2301; BIOSS, Beijing, China) for 1 h at room temperature. Membranes were washed five times in 1X TBST and proteins were visualized using an enhanced chemiluminescence kit (Roche Diagnostics, Basel, Switzerland). GAPDH was detected with mouse anti-GAPDH antibody (1:1,000 dilution; cat. no. AB-P-R 001; OriGene Technologies, Inc., Beijing, China) as an internal control.

**Cytokine quantification.** Fresh mouse tumor tissue supernatants, and mouse and human peripheral blood cells were lysed and centrifuged at 12,000 x g at 4°C for 30 min. Cytokine levels in supernatants were determined using enzyme-linked immunosorbent assay (ELISA) kits for IL-4 (human, cat. no. BP-E10142; mouse, cat. no. BP-E20011) IFN- $\gamma$  (human, BP-E10162; mouse, cat. no. BP-E11382), and soluble B7-H4 (sB7-H4; human, cat. no. BP-E11382; mouse, BP-E20905) from Shanghai Langdon Biotechnology Co., Ltd. (Shanghai, China) following the manufacturer's protocols. Standard curves served as internal controls for the sensitivity and range of each assay. Each sample was analyzed in triplicate.

**Flow cytometry analysis.** The expression level of B7-H4 in the HL-7702 and Huh7 cell lines was determined by flow cytometry. Cells were fixed and permeabilized using BD Perm Buffer III (BD Biosciences, Franklin Lakes, NJ, USA) for 20 mins at 4°C. Samples were washed and incubated with purified mouse anti-human B7-H4 antibody (2  $\mu$ l per sample; cat. no. 562506; BD Biosciences) for 60 min and then with Alexa Fluor<sup>®</sup> 488 donkey anti-mouse IgG H&L (1:500; cat. no. ab150105; Abcam) in the dark for 30 min at 4°C. Finally, the cells were washed and resuspended in Stain buffer (BD Biosciences) and

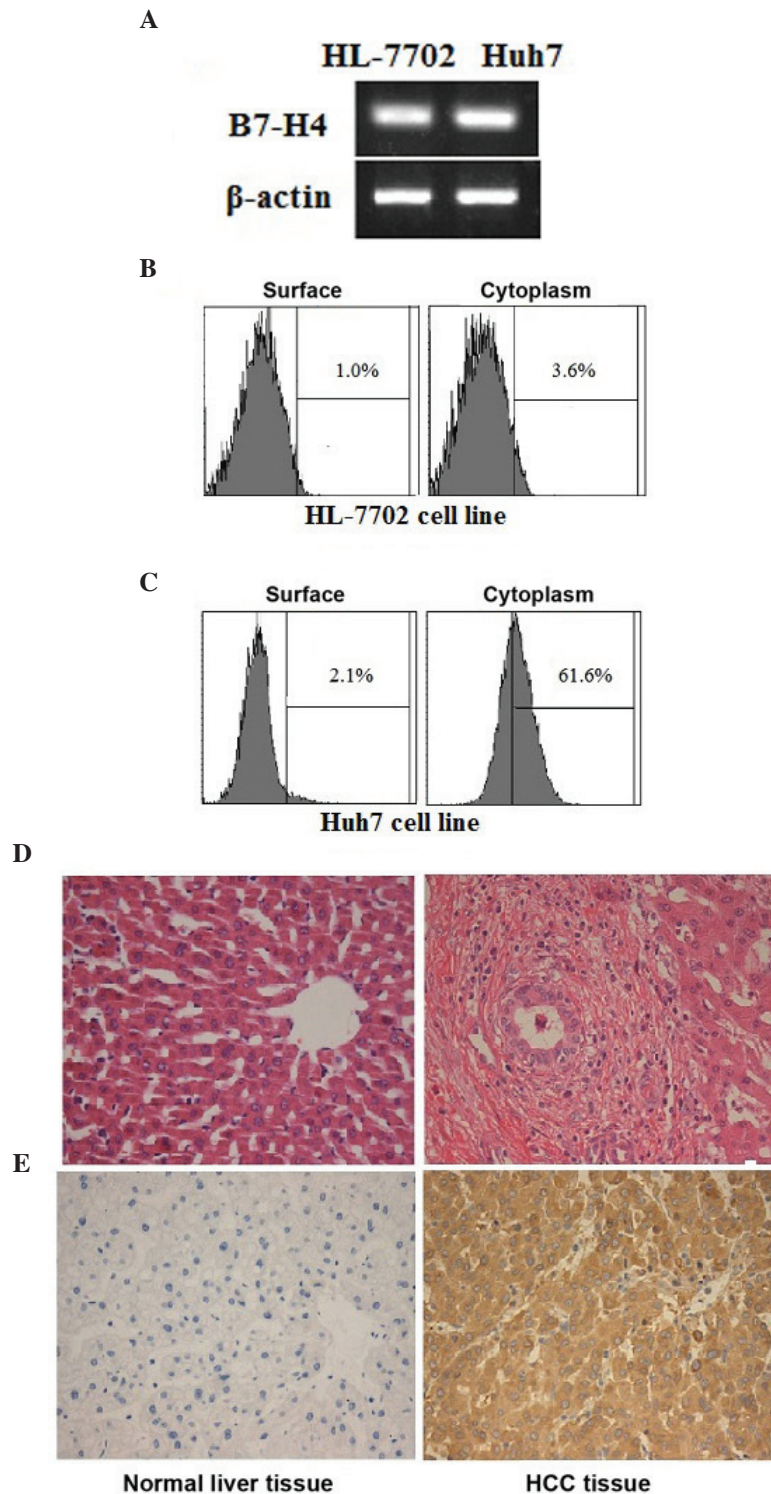


Figure 1. (A) B7-H4 mRNA was detected in HL-7702 and Huh7 cells using reverse transcription-polymerase chain reaction. (B) B7-H4 expression was not observed in HL-7702 cells. (C) B7-H4 was detected on the cell surface and in the cytoplasm of Huh7 cells. The expression levels in cytoplasm were higher compared with the cell surface. (D) H&E staining of normal human liver tissue and HCC tissues. (E) Immunohistochemistry was used to evaluate B7-H4 expression in normal liver and HCC tissues. B7-H4 expression was not detected in normal liver tissues. Intense B7-H4 staining was observed in HCC tissue. Magnification, x400. HCC, hepatocellular carcinoma; H&E, hematoxylin and eosin.

analyzed on a BD FACSCalibur. The data were analyzed with BD FACSDiva 7.0 software (BD Biosciences).

**Patients.** Patients with HCC were recruited from Yu Huang Ding Hospital (Yantai, China) from August 2013 to January 2015. The protocol for the present study was approved

by University of Binzhou Medical College Ethics Committee and informed consent was obtained from all patients. The present study included patients with HCC (n=60) and healthy controls (n=20). HCC was diagnosed on the basis of biochemistry and image findings, including sonography, computerized tomography scans, or magnetic resonance imaging scans.

Table I. Clinical and pathological features of patients with hepatocellular carcinoma.

Clinical characteristic	Number	B7-H4 expression		P-value <sup>a</sup>
		Negative	Positive	
Gender				0.895
Male	35	19	16	
Female	25	14	11	
Age				0.979
<60	29	16	13	
≥60	31	17	14	
TNM stages				0.004
I+II	28	21	7	
III+IV	32	12	20	
Differentiation degree				0.007
Well/moderate	27	20	7	
Poor	33	13	20	
Lymph node metastasis				0.002
Yes	25	8	17	
No	35	25	10	
Size of tumor (cm)				0.979
<4	29	16	13	
≥4	31	17	14	
Intravascular cancer embolus				0.802
Yes	19	10	9	
No	41	23	18	
Hepatocellular carcinoma-associated tumor antigens				
CEA (0.5 ng/ml)				0.714
<5	26	15	11	
≥5	34	18	16	
CA19-9 (0-27 U/ml)				0.895
<27	25	14	11	
≥27	35	19	16	
AFP (0-27 ng/ml)				0.176
<400	17	7	10	
≥400	43	26	17	

<sup>a</sup>Chi-square test was used to evaluate the association between B7-H4 expression and clinical/pathological features. TNM, tumor-node-metastasis; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; AFP,  $\alpha$  fetoprotein.

All samples were examined histologically and diagnosis was conducted according to the American Association for the Study of Liver Diseases guidelines (9).

**Immunohistochemical staining.** B7-H4 expression was analyzed in human normal liver tissues, HCC tissues and mouse tumor tissues using immunohistochemistry staining. The B7-H4 primary antibody used was obtained from Abcam, the secondary biotin-labeled goat-rabbit antibody and diaminobenzidine tetrahydrochloride solution were provided by Boster Biological Technology, Ltd. (Wuhan, China). All samples were fixed in formalin solution and embedded in paraffin. Sections

(5  $\mu$ m-thick) were dewaxed in xylene, dehydrated in ethanol, and incubated in 3% H<sub>2</sub>O<sub>2</sub> for 20 min. Following incubation in 5% normal bovine serum (Boster Biological Technology, Ltd.) for 20 min, slides were incubated with the primary antibody at 4°C overnight, and then with the secondary antibody at 37°C for 60 min. Negative controls were established by replacing the primary antibody with normal mouse IgG (BD Pharmingen). Slides were visualized using light microscopy.

**Evaluation of immunostaining.** Immunostaining was independently examined by two clinical pathologists. Five high-power fields were randomly selected per sample.

Table II. Serum level of sB7-H4, IFN- $\gamma$  and IL-4 are changed in different clinical stages.

Group	n	sB7-H4 (ng/ml)		IL-4 (ng/ml)		IFN- $\gamma$ (pg/m)	
		Mean $\pm$ SD	P-value <sup>a</sup>	Mean $\pm$ SD	P-value <sup>a</sup>	Mean $\pm$ SD	P-value <sup>a</sup>
I-II stages	28	41.94 $\pm$ 4.68	<0.001	600.87 $\pm$ 13.55	<0.001	247.08 $\pm$ 6.48	<0.001
III-IV stages	32	74.93 $\pm$ 16.28		796.41 $\pm$ 42.10	<0.001	163.46 $\pm$ 14.94	

<sup>a</sup>I-II stages vs. III-IV stages. sB7-H4, soluble B7-H4; IL-4, interleukin-4; IFN- $\gamma$ , interferon- $\gamma$ ; SD, standard deviation.

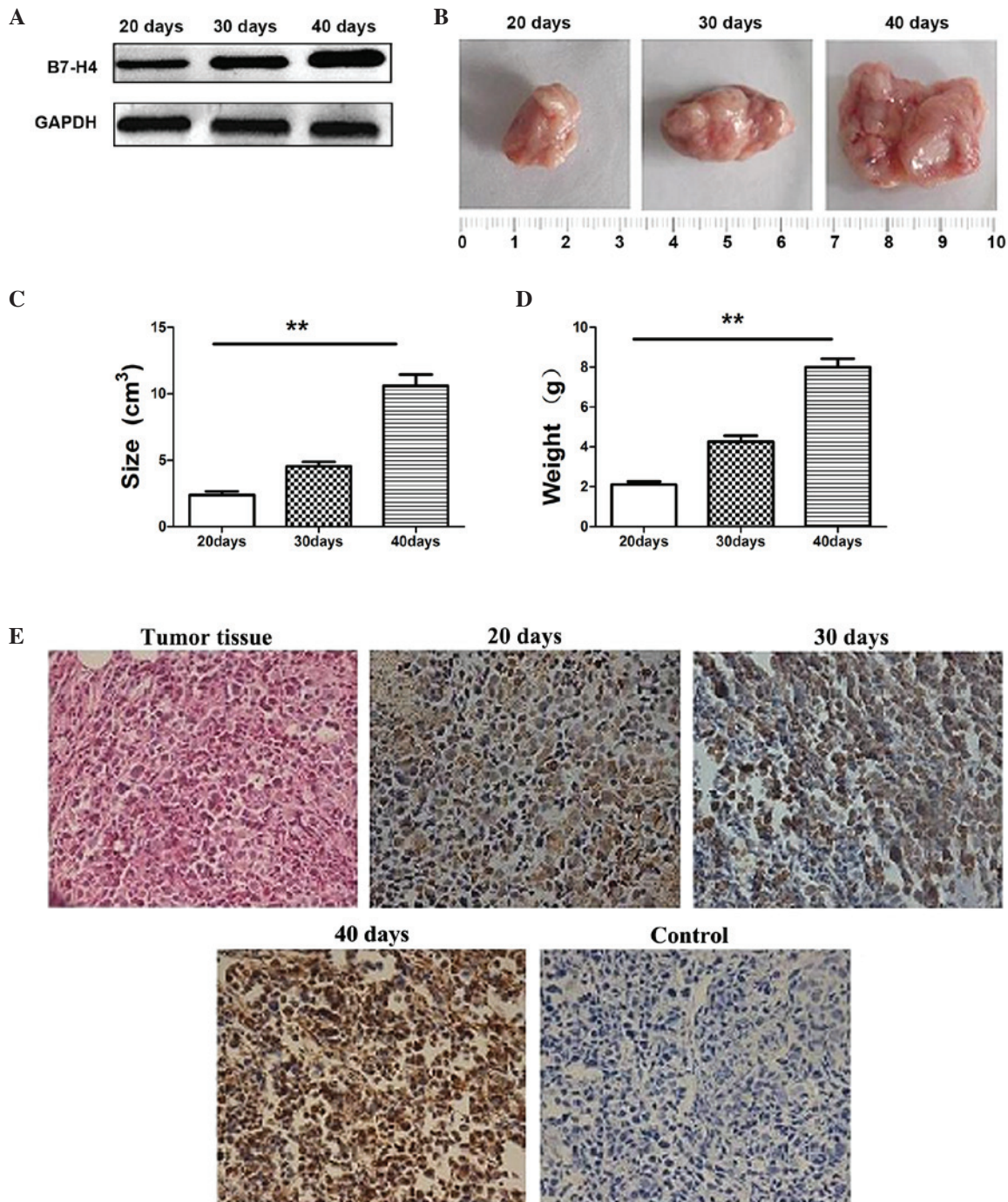


Figure 2. B7-H4 was detected in tumor tissues using western blotting at 20, 30, and 40 days following the tumor cell injection. (A) B7-H4 increased with time. (B) Tumor size also increased with time as presented in the photographs of representative tumors. (C) Quantification of tumor size increase with time. \*\*P<0.001 (D) Tumor weight increased with time. \*\*P<0.001. (E) B7-H4 immunohistochemistry and H&E staining in mouse tumor model. Representative H&E staining of mouse tumor tissue. Immunohistochemical staining was performed on normal tissue and tumor tissue at 20, 30, and 40 days to determine B7-H4 expression levels. Magnification, x400. H&E, hematoxylin and eosin.

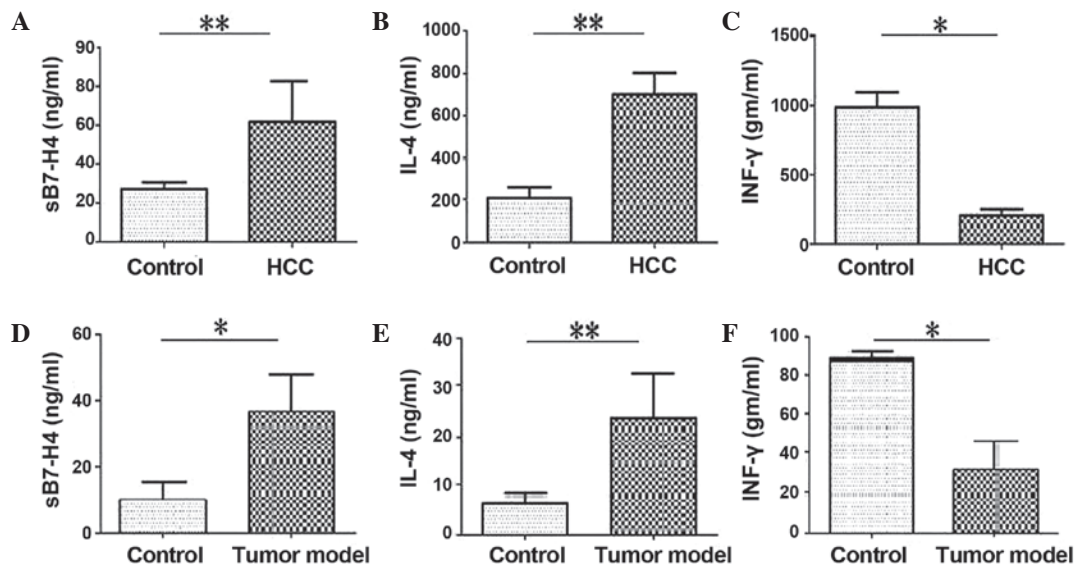


Figure 3. Analysis of sB7-H4, IFN- $\gamma$  and IL-4 in HCC patients, tumorigenic mice and healthy controls. (A) sB7-H4 (\*\* $P$ <0.001) and (B) IL-4 levels (\*\* $P$ <0.001) were increased and (C) IFN- $\gamma$  levels ( $P$ =0.017) were reduced in HCC patients compared with healthy controls. (D) sB7-H4 ( $P$ =0.018) and (E) IL-4 levels (\*\* $P$ =0.004) were increased and (F) IFN- $\gamma$  levels ( $P$ =0.012) were reduced in serum from tumorigenic mice compared with those in healthy controls. sB7-H4, soluble B7-H4; IL-4, interleukin-4; IFN- $\gamma$ , interferon- $\gamma$ ; HCC, hepatocellular carcinoma.

Staining intensity of positive tumor cells was assessed. The extent of the staining was categorized into five semiquantitative classes based on the percentages of membrane-positive tumor cells: i) 0 (<5% positive tumor cells); ii) 1 (6-25% positive tumor cells); iii) 2 (26-50% positive tumor cells); iii) 3 (51-75% positive tumor cells); and iv) 4 (>75% positive tumor cells). The intensity of staining was determined semiquantitatively on a scale of 0 to 3 as follows: i) 0 (negative); ii) 1 (weakly positive), iii) 2 (moderately positive); and iv) 3 (strongly positive). Multiplication of the intensity and the percentage scores was used to obtain the final staining scores, 0 (negative), + (1-2), ++ (3-4), and +++ (5-7).

**Statistical analysis.** For all statistical analyses, data were processed with SPSS version 17.0 statistical software (SPSS, Inc., Chicago, IL, USA). Correlation of B7-H4 protein expression with Clinical and pathological features of patients with hepatocellular carcinoma was evaluated with the Chi-square test. The correlations of the expression levels of sB7-H4, IL-4 and IFN- $\gamma$  were analyzed by Spearman correlation coefficients. Two independent sample t-test was used to analyze the significance of B7-H4 expression scores between the HCC group and the control group. For comparison of three or more groups, one-way analysis of variance was performed. Data are presented as the mean  $\pm$  standard deviation.  $P$ <0.05 was considered to indicate a statistically significant difference

## Results

**B7-H4 expression in HL-7702 and Huh7 cells.** The expression of B7-H4 in the cell lines was analyzed using RT-PCR and flow cytometry. B7-H4 transcripts were expressed in both HL-7702 and Huh7 cell lines as presented in Fig. 1A. The B7-H4 protein was not detected in HL-7702 cells (Fig. 1B). B7-H4 was detected in Huh7 cells, with the expression levels higher in the cytoplasm compared with the cell surface (Fig. 1C).

**Expression of B7-H4 in normal liver and HCC tissues.** In order to determine whether B7-H4 was differentially expressed in human normal liver and HCC tissues, immunohistochemical analysis was performed. B7-H4 protein was not expressed in human normal liver tissues; however, it was expressed in HCC tissues. B7-H4 was observed in the cytoplasm and membrane of HCC cells; however, it was not detected in the nucleus (Fig. 1D and E).

**B7-H4 expression and clinicopathological features.** Immunohistochemical analysis was used to examine B7-H4 expression in patients with HCC (Table I). The patients included 35 males and 25 females with an age range of 47 to 77 years (median, 61.9 years). A total of 27 patients had well or moderately-well differentiated cancer cells and in 33 patients cancer cells were poorly differentiated, 25 patients had lymph node metastasis. Patients were staged from I to IV based on American Joint Committee on Cancer standards. There were 28 patients in stages I and II and 32 patients in stages III and IV. Patient characteristics and clinicopathological features are summarized in Table I.

B7-H4 expression in tumor tissues was significantly positively correlated with TNM stage, differentiation degree and lymph node metastasis ( $P$ <0.05; Table I). No association between B7-H4 expression and the remaining factors, including gender, age, tumor size or hepatoma carcinoma-associated tumor antigens was identified (Table I). Therefore, this indicated that the expression of B7-H4 is associated with aggressive HCC.

**B7-H4 expression as a function of time in mouse tumor model.** The mouse liver tumor model was established by subcutaneous injection of H22 cells into mice. B7-H4 in tumor tissues was detected by western blotting (Fig. 2A). The sizes and weights of tumors were evaluated at 20, 30, and 40 days and were found to significantly increase with time ( $P$ <0.01; Fig. 2B-D). The expression of B7-H4 in tumor tissues was confirmed by

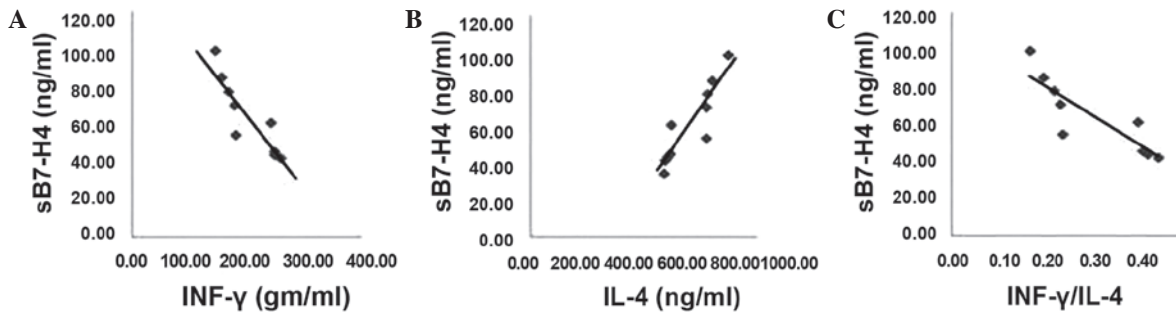


Figure 4. Correlations observed between levels of sB7-H4, IFN- $\gamma$ , and IL-4 and the ratio of IFN- $\gamma$ /IL-4 in serum collected from patients with HCC. Levels of sB7-H4 vs. (A) IFN- $\gamma$  levels (B) IL-4 levels and (C) the ratio of IFN- $\gamma$ /IL-4 in patients with HCC. sB7-H4, soluble B7-H4; IL-4, interleukin-4; IFN- $\gamma$ , interferon- $\gamma$ ; HCC, hepatocellular carcinoma.

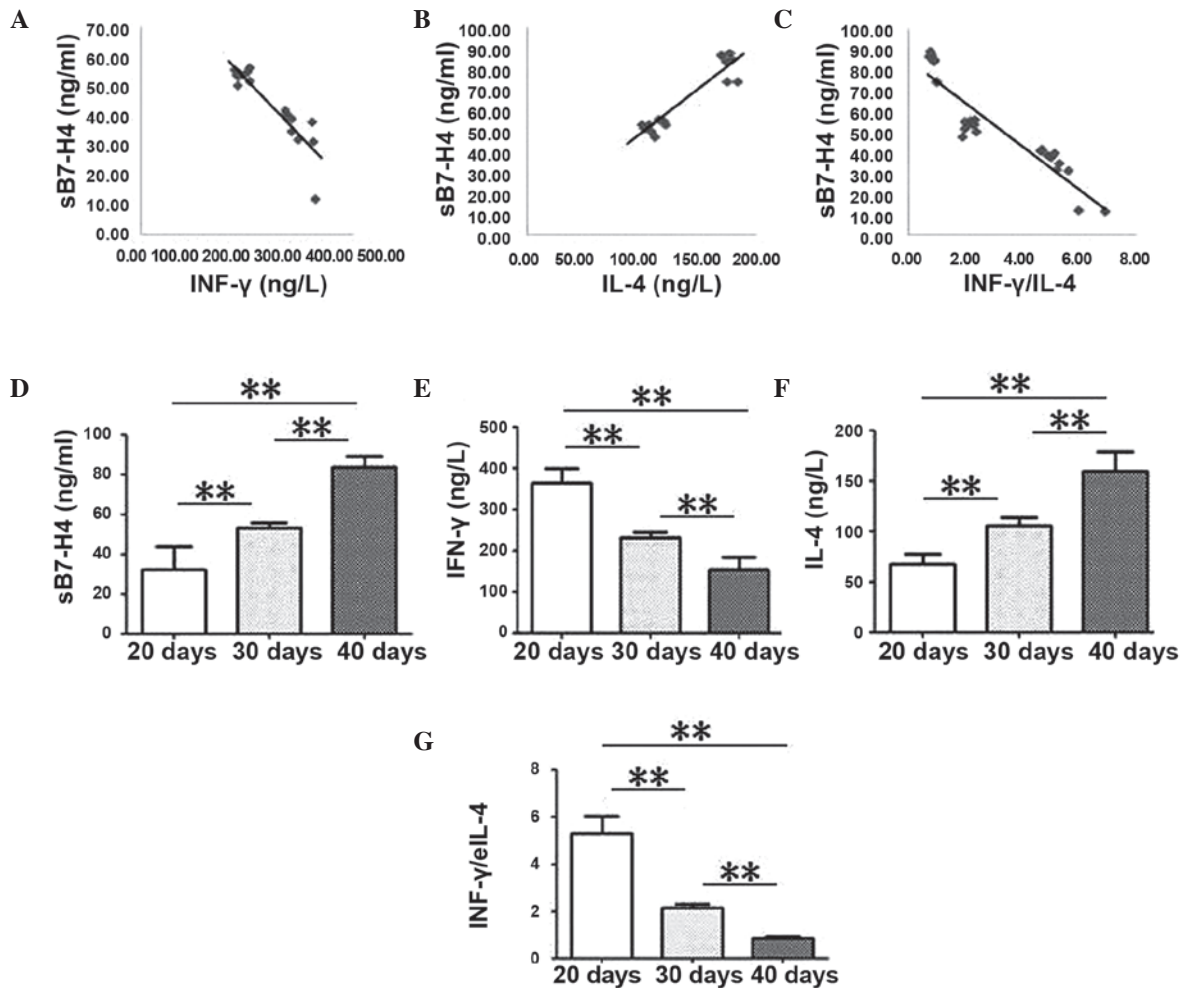


Figure 5. Correlations observed between the levels of sB7-H4, IFN- $\gamma$ , and IL-4 and the ratio of IFN- $\gamma$ /IL-4 in mouse tumor samples. (A) sB7-H4 levels correlated negatively with IFN- $\gamma$  levels in mouse tumor tissues. (B) sB7-H4 levels correlated positively with IL-4 levels in mouse tumor tissue. (C) sB7-H4 levels correlated negatively with the ratio of IFN- $\gamma$ /IL-4 in tumor tissue. Expression levels of (D) sB7-H4, (E) IFN- $\gamma$ , (F) IL-4 and (G) ratio of IFN- $\gamma$ /IL-4 at different times following the injection with tumor cells. \*\*P<0.01. sB7-H4, soluble B7-H4; IL-4, interleukin-4; IFN- $\gamma$ , interferon- $\gamma$ .

immunohistochemistry (Fig. 2E). At 20 days, B7-H4 levels were  $3.28 \pm 0.47$ ; at 30 days, levels were  $5.36 \pm 0.38$  and at 40 days, levels were  $6.18 \pm 0.32$ . These results indicated that the expression levels of B7-H4 increased as tumors increased in size and weight.

*Analysis of sB7-H4, IFN- $\gamma$  and IL-4 in HCC patients, tumorigenic mice and healthy controls.* Cytokine levels in the blood

serum were analyzed using an ELISA. The results revealed that sB7-H4 and IL-4 levels in HCC patients were significantly higher compared with healthy controls (P<0.001; Fig. 3A and B). IFN- $\gamma$  levels were significantly reduced in patients with HCC compared with the healthy control group (P=0.017; Fig. 3C). The results revealed that sB7-H4 (P=0.018; Fig. 3D) and IL-4 (P=0.004; Fig. 3E) levels in the serum of tumor-carrying mice were significantly higher compared with healthy controls.

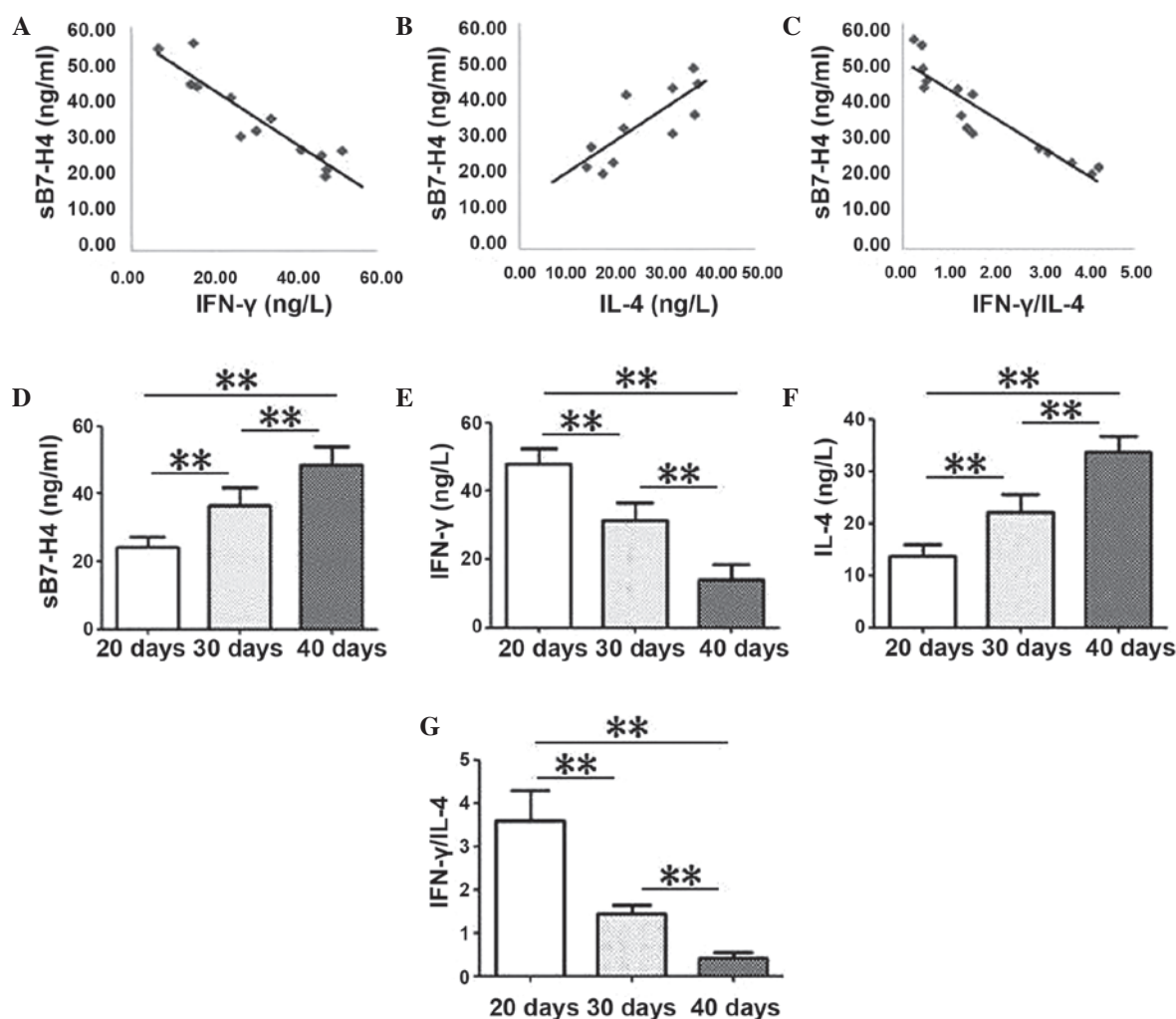


Figure 6. Correlations observed between the levels of sB7-H4, IFN- $\gamma$ , and IL-4 and the ratio of IFN- $\gamma$ /IL-4 in mouse serum samples. (A) sB7-H4 levels correlated negatively with IFN- $\gamma$  levels in tumorigenic mouse serum samples. (B) sB7-H4 levels correlated positively with IL-4 levels in tumorigenic mouse serum samples. (C) sB7-H4 levels correlated negatively with the ratio of IFN- $\gamma$ /IL-4 in mouse serum samples. Levels of (D) sB7-H4, (E) IFN- $\gamma$ , (F) IL-4, and (G) ratio of IFN- $\gamma$ /IL-4 as a function of time in tumorigenic mouse serum samples. \*\* $P < 0.01$ . sB7-H4, soluble B7-H4; IL-4, interleukin-4; IFN- $\gamma$ , interferon- $\gamma$ .

IFN- $\gamma$  levels were significantly reduced in patients with HCC compared with the healthy control group ( $P = 0.012$ ; Fig. 3F).

A Spearman's rank correlation analysis was used to identify the correlations between sB7-H4 levels and IFN- $\gamma$ , IL-4 and the ratio between IFN- $\gamma$ /IL-4 levels in blood serum samples of patients with HCC (Fig. 4). sB7-H4 levels correlated negatively with IFN- $\gamma$  and with the ratio of IFN- $\gamma$ /IL-4 ( $R = -0.888$ ;  $P = 0.001$  and  $R = -0.864$ ;  $P = 0.003$ , Fig. 4A and C). Conversely, sB7-H4 levels were positively correlated with the levels of IL-4 in the blood serum samples of patients with HCC ( $R = 0.903$ ,  $P < 0.001$ ; Fig. 4C).

Spearman's rank was also used to determine correlations between the cytokine levels in tumor samples (Fig. 5) and serum samples (Fig. 6) from the mouse tumor model. In tumor tissue samples from mice, sB7-H4 levels were negatively correlated with IFN- $\gamma$  levels and with the ratio of IFN- $\gamma$ /IL-4 ( $R = -0.919$ ,  $P < 0.001$ ; and  $R = -0.925$ ,  $P < 0.001$ , respectively; Fig. 5A and B). Negative correlations were also observed in serum samples obtained from the mice ( $R = -0.942$ ;  $P < 0.001$  and  $R = -0.923$ ;  $P < 0.001$ ; Fig. 6A and C). sB7-H4 levels were positively correlated with IL-4 levels from mouse tumor tissue ( $R = 0.951$ ;  $P < 0.001$ ; Fig. 5A) and serum samples ( $R = 0.917$ ;  $P < 0.001$ ; Fig. 6B).

IFN- $\gamma$  levels and the ratio of IFN- $\gamma$ /IL-4 significantly decreased over time in tumor tissue ( $P < 0.001$ ; Fig. 5E and G) and serum samples ( $P < 0.001$ ; Fig. 6E and G). Conversely, sB7-H4 and IL-4 levels in mouse tumor tissues ( $P < 0.001$ ; Fig. 5D and F) and serum samples ( $P < 0.001$ ; Fig. 6D and F) significantly increased with time.

*sB7-H4, IFN- $\gamma$  and IL-4 levels are altered with the clinical stage of HCC.* In order to determine whether the expression levels of sB7-H4, IFN- $\gamma$ , and IL-4 are associated with the patient's HCC clinical stage, ELISA analysis was used (Table II). sB7-H4 and IL-4 levels were increased in stage III and IV tumors compared with stage I and II tumors ( $P < 0.001$ ; Table II), whereas IFN- $\gamma$  levels were increased in stage III and IV tumors compared with stage I and II tumors ( $P < 0.001$ ; Table II). Therefore, this indicated that sB7-H4 may be a potential marker to predict tumor progression in patients with HCC.

## Discussion

HCC is a common malignant tumor with high morbidity and mortality, particularly among patients in China (27). Previous



studies have determined that B7-H4 expression may be important for tumorigenesis (15-20).

B7-H4 is a member of the B7 family of costimulatory ligands. It has a negative regulatory function in T cell-mediated immunity as it has been identified to inhibit T cell activation, proliferation and cytokine production (10-12). Notably, despite widespread *B7-H4* mRNA expression, the expression levels of the B7-H4 protein have been observed to be restricted in normal tissues (28). Previous studies have demonstrated that the B7-H4 molecule is highly expressed in various different types of human cancers, including pancreatic (16) and gastric cancer (17,18). The present study revealed that the B7-H4 protein was not expressed by the HL-7702 normal human liver cell line; however, it was expressed by the Huh7 human hepatoma cells. Immunohistochemical staining revealed that B7-H4 was expressed in HCC tissues and not in normal liver tissues. In addition, in a mouse tumor model was established using the H22 cell line. B7-H4 protein expression was observed in the tumor tissues obtained from the mice. These results indicate an association between B7-H4 expression and HCC.

Previous studies determined that B7-H4 had no prognostic value for ovarian cancer (29) or breast cancer (30). However, the expression levels of B7-H4 in renal cell carcinoma (RCC) (31), gastric cancer (17,18) and colorectal carcinoma (19) have been identified to be correlated with adverse clinicopathological features, such as advanced lymph node metastasis, tumor grade and TNM stage. Prostate carcinoma and patients with RCC, that have B7-H4-positive tumors have also been identified to be at a high risk of recurrence and had increased mortality (32). The present study determined that higher B7-H4 expression levels were observed in samples from patients with poor differentiation and lymph node metastasis and at later stages of progression. In the mouse model, the expression levels of B7-H4 increased with time. These results suggested that B7-H4 is important for the progression of HCC, B7-H4 may also be used as a molecular marker of HCC and as a novel target for HCC therapy.

A previous study determined that serum B7-H4 levels were significantly increased in patients with gastric cancer compared with healthy volunteers, additionally high sB7-H4 levels were significantly correlated with tumor size, lymph node metastasis and TNM stage in patients with gastric cancer (33). Simon *et al* (34) determined that B7-H4 expression was elevated in serum samples from ovarian cancer patients when compared with healthy controls or women with benign gynecologic diseases, including endometriosis, enlarged ovaries/edema and polycystic ovaries. Zhang *et al* (35) revealed that sB7-H4 levels in patients with HCC were significantly higher compared with those in normal controls and that sB7-H4 levels were closely associated with tumor size, tumor invasion, tumor differentiation and TNM stage (35). However, they were not associated with other characteristics, including age, gender and alanine aminotransferase levels (35). In the present study, elevated expression levels of sB7-H4 were observed in blood samples from patients with HCC compared with healthy controls, which was consistent with previous studies. Additionally, the current study determined that the levels of IL-4 were higher and those of IFN- $\gamma$  lower in serum samples from

patients with HCC compared with serum samples from healthy controls. sB7-H4 levels were negatively correlated with IFN- $\gamma$  levels and with the ratio of IFN- $\gamma$ /IL-4. However, sB7-H4 levels were positively correlated with IL-4 levels in serum samples obtained from patients with HCC. Similar results were observed in serum and tumor tissues samples obtained from the mouse model. This suggests that the expression levels of sB7-H4 may be due to an imbalance of Th1 and Th2 cells, that facilitates the development of HCC. In addition, the present study also determined that sB7-H4 and IL-4 levels were positively correlated with the TNM stage in HCC patients, whereas IFN- $\gamma$  levels were negatively correlated. Therefore, sB7-H4 may be used as a potential serum biomarker to facilitate diagnosis of HCC and may be predictive of tumor progression in patients with HCC.

The present study revealed a possible role for B7-H4 in the development of HCC. Aberrant expression of B7-H4 has been identified to correlate with the TNM stage, differentiation degree and lymph node metastasis in patients with HCC. The present study provided insight into the underlying mechanism that contributed to the progression of HCC and suggested that B7-H4 may be a promising target for immunotherapy.

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