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Circulating B7-H4 in serum predicts prognosis in patients with hepatocellular carcinoma

S.A. Zhang, Z.X. Wu, X. Zhang, Z.Y. Zeng and D.L. Li

Department of Hepatobiliary Medicine, Fuzhou PLA General Hospital, Fuzhou Fujian, China

Corresponding author: D.L. Li
E-mail: Dongliang_lifzzy@163.com

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ABSTRACT. B7-H4 is member of the B7 family that negatively regulates the immune response, which are important for fine-tuning of the tumor microenvironment. Dysregulation of B7-H4 expression has been associated with tumor progression. However, expression level of B7-H4 in hepatocellular carcinoma (HCC) tissues is still a controversial topic. In addition, whether serum B7-H4 expression of HCC patients has any clinical value is unknown. We compared serum levels of B7-H4 in patients with HCC and healthy controls by using the ELISA method. Association between serum B7-H4 expression level and clinical parameters of HCC was further investigated. Log-rank test and Kaplan-Meier method were employed to evaluate the overall survival rate of HCC patients. Univariate and multivariate analysis of prognostic factors were performed with the Cox regression model. Our results showed that HCC patients had significantly higher serum B7-H4 level as compared with healthy controls ($P < 0.001$). In addition, serum B7-H4 expression was correlated with HCC clinical parameters including serum AFP expression and TNM stage. HCC patients in the higher serum B7-H4 expression group had a poorer 5-year overall survival rate ($P = 0.028$). Moreover, serum B7-H4 expression was shown to be an independent prognostic factor for HCC ($P = 0.034$). The findings from this study suggest that serum B7-H4 is an independent prognostic indicator

for HCC and may be a promising biomarker for early diagnosis as well as disease prognosis of HCC.

Key words: Hepatocellular carcinoma; Prognostic value; Serum B7-H4

INTRODUCTION

Hepatocellular carcinoma (HCC), the most common type of primary liver cancer, has become the third largest cause of cancer-related deaths in the world (Venook et al., 2014). Chronic infections of HBV/HCV viruses are the major causes of HCC. The overall incidence rate of HCC remains high in East Asia, especially in the east coast areas of China (Luo et al., 2005). In HCC, frequent tumor metastasis and high relapse rates are the major causes of high mortality. Moreover, many patients with HCC are not diagnosed until the disease is in its advanced stages and is no longer treatable. Therefore, HCC has a poor prognosis of HCC with relatively low 5-year survival rates (approximately 10%) (Altekruse et al., 2009). Successful detections of HCC at early stages is crucial for providing effective therapies for this aggressive and malignant disease. In the past few decades, screening for sensitive and specific tissue or body fluids biomarkers which may be useful for early diagnosis of cancer has become an important task for both clinicians and cancer biologists.

B7-H4 is a highly evolutionarily conserved transmembrane protein. It shares approximately 25% amino acid homology in the extracellular portion with other B7 family members. In addition, previous study showed that human and mouse B7-H4 share 87% amino acid identity (Smith et al., 2014). B7-H4 mRNA is widely distributed in human peripheral tissues while its protein has limited expression in healthy individuals, indicating that expression of B7-H4 is tightly controlled at the translational level (Choi et al., 2003). B7-H4 plays important roles in regulating the tumor microenvironment as it is shown to be an inhibitor of both innate immunity and T-cell responses (Yi et al., 2009). Dysregulation of B7-H4 has been reported in a variety of cancers such as breast cancer, cervical cancer, lung carcinoma, and colorectal cancer (Tringler et al., 2005; Sun et al., 2006; Liu et al., 2014; Zhao et al., 2014). Simon and his colleagues found that the expression level of serum B7-H4 was significant higher in patients with ovarian cancer as compared with healthy controls (Simon et al., 2006). Recently, higher serum B7-H4 level was also reported in gastric cancer patients, and was closely correlated with many important clinic-pathological parameters of gastric cancer, further supporting its potential value for early diagnosis and prediction of disease prognosis (Shi et al., 2014).

Previous study showed that there was no significant difference in B7-H4 expression level between liver cancer tissues and normal tissues (Qian et al., 2011). However, whether B7-H4 expression in blood specimens of HCC patients has any clinical significance and prognostic value remains poorly unknown. In the current study, we examined circulating B7-H4 levels in blood samples from patients with HCC and healthy controls. Furthermore, we evaluated the correlation between B7-H4 expression levels and clinic-pathological characteristics of HCC.

MATERIAL AND METHODS

Study population

This study was approved by the ethics committee of Fuzhou PLA General Hospital. Written informed consent was obtained from all patients and healthy volunteers recruited in the study. Blood samples were collected at the Department of Hepatobiliary Medicine, Fuzhou PLA General

Hospital (116 HCC patients and 60 healthy controls). No patients received surgery, chemotherapy, or radiotherapy prior to blood sample collection. The health status of all healthy subjects was confirmed by medical checkups, which included routine physical examination and standard laboratory tests. Various methods such as pathological examinations, serum α-fetoprotein (AFP) expression levels, and imaging examinations were employed to confirm HCC diagnosis. TNM clinical stages were classified according to the 6th edition TNM classification of the American Joint Committee on Cancer. Clinical information of HCC patients such as age, gender, tumor size, TNM stage, liver cirrhosis, venous infiltration, tumor nodes, serum AFP expression, Hepatitis B virus infection and Hepatitis C virus infection are indicated in Table 1.

Sandwich ELISA

Serum levels of B7-H4 were determined according to previous protocols [11, 12]. High-binding polystyrene plates were coated with capture mAb Clone H74 (eBioscience, San Diego, United States). Blood samples (30 μL) collected from each patient and healthy volunteer were added to the plates. Diluted biotinylated secondary mAb (eBioscience) was used to detect immobilized antigen. Streptavidin-conjugated horseradish peroxidase (DingGuo, Beijing, China) was employed to detect the signals.

Statistical analysis

Statistical analyses were performed using the SPSS 21.0 (Chicago, Ill., USA) and GraphPad Prism 5 (GraphPad Software Inc., CA, USA) softwares. Mann-Whitney U tests were used to compare expression levels of serum B7-H4 between HCC patients and healthy controls. Association between serum B7-H4 expression level and clinicopathological parameters was evaluated by chi-square test. Overall survival curve was generated using the Kaplan-Meier method and analysis. Univariate and multivariate Cox's proportional hazard models were employed to identify prognosis factors for determining survival time. $P < 0.05$ was determined to be statistically significant.

RESULTS

Expression level of serum B7-H4 is up-regulated in HCC patients

Our ELISA results showed that the expression level of serum B7-H4 in patients with HCC (71.53 ± 21.67 ng/mL) was significantly higher compared to healthy controls (40.75 ± 14.91 ng/mL) ($P < 0.001$, Figure 1).

Association between serum B7-H4 expression level with clinicopathological parameters of HCC

The median serum level of B7-H4 (70.55 ng/ mL) was used as the cut-off value for subgroup classification (high expression group or low expression group). Serum B7-H4 expression level was found to be associated with AFP expression ($P = 0.012$) and TNM stage ($P = 0.003$). However, there was no correlation between serum B7-H4 expression and gender ($P = 0.655$), age ($P = 0.723$), tumor size ($P = 0.104$), cirrhosis status ($P = 0.067$), venous infiltration ($P = 0.263$), tumor nodes ($P = 0.166$), HBV infection ($P = 0.537$), and HCV infection ($P = 0.581$) (Table 1).

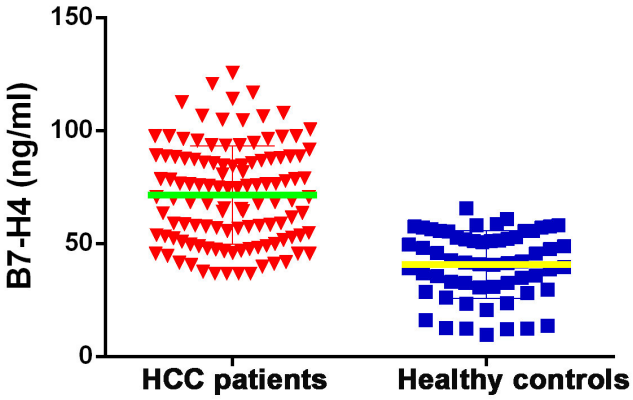

Figure 1. The expression level of serum B7-H4 is up-regulated in patients with HCC.

Table 1. The association between serum B7-H4 expression level and HCC clinicopathological parameters.

Variables	N (%)	Serum B7-H4 expression		P value
		Low (%)	High (%)	
Gender				
Male	68 (58.62)	28 (24.14)	40 (34.48)	0.655
Female	48 (41.38)	24 (20.69)	24 (20.69)	
Age				
<60	47 (40.52)	22 (18.97)	25 (21.55)	0.723
≥60	69 (59.48)	30 (25.86)	39 (33.62)	
Tumor size (cm)				
<5	55 (47.41)	29 (25.00)	26 (22.41)	0.104
≥5	61 (52.59)	23 (19.83)	38 (32.76)	
TNM				
I-II	56 (48.28)	33 (28.45)	23 (19.83)	0.003
III-IV	60 (51.72)	19 (16.38)	41 (35.34)	
Cirrhosis				
No	65 (56.03)	34 (29.31)	31 (26.72)	0.067
Yes	51 (43.97)	18 (15.52)	33 (28.45)	
Venous infiltration				
No	58 (50.00)	29 (25.00)	29 (25.00)	0.263
Yes	58 (50.00)	23 (19.83)	35 (30.17)	
Tumor nodes				
Single	52 (44.83)	27 (23.28)	25 (21.55)	0.166
Multiple	64 (55.17)	25 (21.55)	39 (33.62)	
Serum AFP (μg/L)				
<400	51 (43.97)	31 (26.72)	20 (17.24)	0.012
≥400	65 (56.03)	21 (18.10)	44 (37.93)	
HBV infection				
No	30 (25.86)	12 (10.34)	18 (15.52)	0.537
Yes	86 (74.14)	40 (34.48)	46 (39.66)	
HCV infection				
No	98 (84.48)	45 (38.79)	53 (45.69)	0.581
Yes	18 (15.52)	7 (6.03)	11 (9.48)	

Survival analysis

Log-rank test and Kaplan-Meier method were used to examine the association between serum B7-H4 level and the overall survival rate of HCC patients. Our results showed that high serum B7-H4 expression was associated with poorer 5-year survival rates (P = 0.028) (Figure 2).

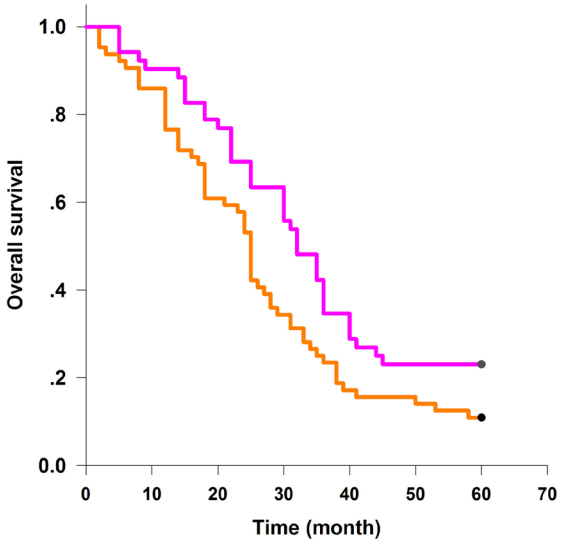

Figure 2. High serum B7-H4 expression level was associated with poor overall survival.

Univariate analysis and multivariate analysis of prognostic factors in HCC

Univariate analyses showed that serum AFP expression (P = 0.021), TNM stage (P = 0.005) and serum B7-H4 expression level (P = 0.008) were significant prognostic indicators for HCC.

In the multivariate analysis model, TNM stage (P = 0.013) and serum B7-H4 expression level (P = 0.034) were independent prognostic factors for HCC. However, serum AFP expression was shown to not be an independent prognostic factor (P = 0.148) (Table 2).

Table 2. Univariate and multivariate analyses of prognostic factors in HCC.

Variable	Univariate P value	Multivariate P value	HR (95%CI)
Serum AFP (μg/L)			
≥400 *vs* <400	0.021	0.148	1.35 (0.92-1.84)
TNM stage			
III-IV *vs* I-II	0.005	0.013	3.21 (1.36-4.43)
Serum B7-H4 expression			
High *vs* low	0.008	0.034	2.05 (1.17-3.18)

DISCUSSION

Great advances have been made in the area of early diagnosis and surgical techniques. In addition, some novel treatment strategies such as molecular targeted therapy and gene therapy have also been developed to fight against HCC (el Tazi et al., 2011; Duan et al., 2013). However, prognosis of many HCC patients remains poor. Therefore, investigations into biomarkers that are beneficial to early diagnosis and predictor of prognosis are urgently needed. This will allow us to not only detect HCC at a very early clinical stage, but also to monitor treatment responses in clinical practice. In the present study, we observed that serum B7-H4 expression in patients with HCC was significantly higher than that of healthy controls. Furthermore, serum B7-H4 expression

was associated with serum AFP expression and TNM clinical stage. We also found that HCC patients in the higher serum B7-H4 expression group suffered from poorer 5-year overall survival rate. Moreover, multivariate analysis result revealed that serum B7-H4 expression level may be an independent prognostic factor for HCC.

The B7 family plays an essential role in regulation of immune responses, which is closely associated with cancer initiation and progression. The concrete role of B7-H4 in cancer development remains controversial. Leung et al. (2013) reported that host B7-H4 not only dampens antitumor T cells responses, but also inhibits tumor function of myeloid-derived suppressor cells. Wang et al. (2013) showed that B7-H4 overexpression was associated with lower numbers of tumor-infiltrating CD8 (+) T lymphocytes and reduced IFN-γ production, suggesting that B7-H4 may be important in dampening anti-tumor immunity of CD8 (+) T cell in the tumor microenvironments. Conversely, Rahbar et al. (2015) revealed that B7-H4 expression could inhibit breast cancer growth, and was required for antitumor immune responses in mouse models. Interestingly, B7-H4 gene polymorphism was also reported to be associated with sporadic breast cancer in a Chinese Han population (Zhang et al., 2009).

Consistent with our study, overexpression of serum B7-H4 has been reported in various cancers such as ovarian and gastric cancer. Simon et al. (2006) showed that expression level of B7-H4 was up-regulated in both tissue lysates and serum of patients with ovarian cancer. They also found that serum B7-H4 had great potential for early detection of ovarian cancer, as it had similar diagnostic performance for early-stage ovarian cancer as the traditional cancer biomarker CA125 (Simon et al., 2007). In agreement with this, Oikonomopoulou et al. (2008) reported that serum B7-H4 may be a promising biomarker for prediction of ovarian cancer prognosis and chemotherapy response. Recently, Shi et al. (2014) found that serum B7-H4 expression level was significantly higher in gastric cancer patients as compared to healthy controls. Moreover, it is closely correlated with various important clinic-pathological parameters such as tumor size, lymph node metastasis, tumor invasion depth, TNM stage, and survival rate.

Serum B7-H4 concentrations were increased in patients with bladder urothelial carcinoma and its expression was associated with histology grade (Liu et al., 2014). Similar findings were also reported which confirmed the association between tissue B7-H4 expression level and various types of cancer. Zhu et al. (2014) showed that tissue B7-H4 expression level in human thyroid cancer tissues was significantly up-regulated and associated with cancer progression related parameters such TNM stages, extrathyroidal extension, and overall survival. Liu et al. (2014) reported that positive cervical cancer tissue B7-H4 expression was correlated with poor overall survival and may be used as an independent prognostic factor for cervical cancer. However, no association was found between its expression and any of the clinicopathological parameters. Krambeck et al. (2006) revealed that B7-H4 expression in renal cell carcinoma tissue was associated with adverse clinical and pathologic features. In addition, patients with positive B7-H4 expression suffered higher risk of death. However, Qian et al. (2011) reported that there was no significant difference in B7-H4 expression between the liver cancer tissues and normal tissues. This differed from our findings, which showed that serum B7-H4 was elevated in HCC patients. It is possible that HCC cancer cells could secrete soluble B7-H4 into the body fluids, and that there is no definite consistent correlation between the expression pattern of serum protein and tissue protein. In addition, immunohistochemistry may be not sensitive enough to detect minor differences in B7-H4 expression level between liver cancer tissue and normal tissue. Lastly, the sample size in the study was relatively small, and further and larger scale experiments are needed to definitively determine whether tissue B7-H4 expression is enhanced in HCC patients.

CONCLUSION

Our study showed that serum B7-H4 is an independent prognostic factor for HCC, may be a promising biomarker for early diagnosis as well as being a predictor of disease prognosis for HCC patients.

Conflicts of interest

The authors declare no conflict of interest.

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REFERENCES

Altekruse SF, McGlynn KA and Reichman ME (2009). Hepatocellular carcinoma incidence, mortality, and survival trends in the United States from 1975 to 2005. *J. Clin. Oncol*. 27: 1485-1491.

Choi IH, Zhu G, Sica GL, Strome SE, et al. (2003). Genomic organization and expression analysis of B7-H4, an immune inhibitory molecule of the B7 family. *J. Immunol.* 171: 4650-4654.

Duan F and Lam MG (2013). Delivery approaches of gene therapy in hepatocellular carcinoma. *Anticancer Res*. 33: 4711-4718.

Krambeck AE, Thompson RH, Dong H, Lohse CM, et al. (2006). B7-H4 expression in renal cell carcinoma and tumor vasculature: associations with cancer progression and survival. *Proc. Natl. Acad. Sci. USA*. 103: 10391-10396.

Leung J and Suh WK (2013). Host B7-H4 regulates antitumor T cell responses through inhibition of myeloid-derived suppressor cells in a 4T1 tumor transplantation model. *J. Immunol.* 190: 6651-6661.

Liu W, Shibata K, Koya Y, Kajiyama H, et al. (2014). B7-H4 overexpression correlates with a poor prognosis for cervical cancer patients. *Mol. Clin. Oncol*. 2: 219-225.

Liu WH, Chen YY, Zhu SX, Li YN, et al. (2014). B7-H4 expression in bladder urothelial carcinoma and immune escape mechanisms. *Oncol. Lett*. 8: 2527-2534.

Luo RH, Zhao ZX, Zhou XY, Gao ZL, et al. (2005). Risk factors for primary liver carcinoma in Chinese population. *World J. Gastroenterol*. 11: 4431-4434.

Oikonomopoulou K, Li L, Zheng Y, Simon I, et al. (2008). Prediction of ovarian cancer prognosis and response to chemotherapy by a serum-based multiparametric biomarker panel. *Br. J. Cancer* 99: 1103-1113.

Qian Y, Shen L, Cheng L, Wu Z, et al. (2011). B7-H4 expression in various tumors determined using a novel developed monoclonal antibody. *Clin. Exp. Med*. 11: 163-170.

Rahbar R, Lin A, Ghazarian M, Yau HL, et al. (2015). B7-H4 expression by nonhematopoietic cells in the tumor microenvironment promotes antitumor immunity. *Cancer Immunol. Res*. 3: 184-195.

Shi H, Ji M, Wu J, Zhou Q, et al. (2014). Serum B7-H4 expression is a significant prognostic indicator for patients with gastric cancer. *World J. Surg. Oncol.* 12: 188.

Simon I, Zhuo S, Corral L, Diamandis EP, et al. (2006). B7-H4 is a novel membrane-bound protein and a candidate serum and tissue biomarker for ovarian cancer. *Cancer Res*. 66: 1570-1575.

Simon I, Liu Y, Krall KL, Urban N, et al. (2007). Evaluation of the novel serum markers B7-H4, Spondin 2, and DcR3 for diagnosis and early detection of ovarian cancer. *Gynecol. Oncol.* 106: 112-118.

Smith JB, Stashwick C and Powell DJ Jr (2014). B7-H4 as a potential target for immunotherapy for gynecologic cancers: a closer look. *Gynecol. Oncol.* 134: 181-189.

Sun Y, Wang Y, Zhao J, Gu M, et al. (2006). B7-H3 and B7-H4 expression in non-small-cell lung cancer. *Lung Cancer*. 53: 143-151

Tazi el M, Essadi I, M'Rabti H, Touyar A, et al. (2011). Systemic treatment and targeted therapy in patients with advanced hepatocellular carcinoma. *N. Am J Med. Sci*. 3: 167-175.

Tringler B, Zhuo S, Pilkington G, Torkko C, et al. (2005). B7-H4 is highly expressed in ductal and lobular breast cancer. *Clin. Cancer Res*. 11: 1842-1848.

Venook AP, Papandreou C, Furuse J and de Guevara LL (2014).The incidence and epidemiology of hepatocellular carcinoma: a global and regional perspective. *Oncologist* 15 (Suppl) 4: 5-13.

Wang X, Wang T, Xu M, Xiao M, et al. (2014). B7-H4 overexpression impairs the immune response of T cells in human cervical carcinomas. *Hum. Immunol.* 75: 1203-1209.

Yi KH and Chen L (2009). Fine tuning the immune response through B7-H3 and B7-H4. *Immunol. Rev*. 229: 145-151.

Zhang J, Zhang M, Jiang W, Wang L, et al. (2009). B7-H4 gene polymorphisms are associated with sporadic breast cancer in a Chinese Han population. *BMC Cancer* 9: 394.

Zhao LW, Li C, Zhang RL, Xue HG, et al. (2014). B7-H1 and B7-H4 expression in colorectal carcinoma: correlation with tumor FOXP3(+) regulatory T-cell infiltration. *Acta. Histochem*. 116: 1163-1168.

Zhu J, Chu BF, Yang YP, Zhang SL, et al. (2013). B7-H4 expression is associated with cancer progression and predicts patient survival in human thyroid cancer. *Asian Pac. J. Cancer Prev.* 14: 3011-3015.