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Expression of BC047440 protein in hepatocellular carcinoma and its relationship to prognosis

Lu Zheng, Ping Liang, Jing Li, Xiao-Bing Huang, Wei-Wei Wang, Liang Wang, Huan Feng

Department of Hepatobiliary Surgery, Second Affiliated Hospital of Third Military Medical University, Chongqing 400037, P. R. China

[Abstract] Background and Objective: BC047440 is a new gene related to cancer growth and proliferation. Due to the lack of specific antibodies, how BC047440 protein influences the liver cancer growth is unclear. This study aimed to determine the relationship between BC047440 protein expression and clinicopathologic parameters of hepatocellular carcinoma (HCC), and to evaluate the prognostic value of BC047440 for HCC patients. **Methods:** We prepared the polyclonal antibodies of BC047440, and used Western blot and immunohistochemical staining to detect BC047440 expression in 68 specimens of HCC. The correlations of BC047440 expression to clinicopathologic features and prognosis of HCC patients were analyzed. **Results:** The polyclonal antibodies could effectively recognize endogenous BC047440 in HCC tissues. The positive rate of BC047440 protein was significantly higher in HCCs than in adjacent tissues (44.1% vs. 23.5%, $P < 0.05$); the rate was significantly higher in patients with larger tumor ($P < 0.05$) and portal vein invasion ($P < 0.01$). The HCC patients with high BC047440 expression showed a significantly poorer prognosis than those with low BC047440 expression ($P < 0.05$). **Conclusion:** BC047440 can promote the growth and invasion of HCC.

Key words: Liver neoplasm, polyclonal antibody, prognosis

Hepatocellular carcinoma (HCC) is a common malignant cancer. In China, the incidence of HCC increases gradually and the mortality of HCC accounts for 18.8% in all types of cancer^[1]. In China, 230 000 patients with HCC died every year, which accounts for 53% in the whole world. Although the study of HCC has improved in the past ten years, the 5-year survival rate of the patients with HCC is still as low as 5% in the entire world.

Recently, genomics and proteomics have been improved rapidly. A new hypothesis for the initiation of HCC considers that the development of HCC is a multi-factor involved process, including virus infection, carcinogenic substance, activation of oncogenes, inactivation of tumor suppressor genes, the dysregulation of cell apoptosis and proliferation^[2,3]. Usually, DNA continuously exposes to various mutagens during the whole life. During DNA

replication, mistakes may occur. Once the DNA mutation can not be repaired, tumor cells will be developed. Hence, many malignant tumors, including HCC, are common genetic diseases. With the development of molecular technologies, finding and cloning new tumor associated genes become possible. Human genome is composed of 3×10^9 base pairs, which encode about 30 000 to 50 000 genes. Thirty tumor suppressor genes and more than 100 oncogenes have been identified.

While screening HCC-associated genes, a new HCC-associated expressed sequence tag (EST) has been found by suppression subtractive hybridization (SSH) and its full cDNA sequence has been further cloned by RACE. According the GenBank searching, the sequence of this new gene was homologous to BC04740. Semi-quantitative RT-PCR confirmed that the expression of this gene was higher in HCC tissues than in adjacent liver tissues. In addition, the full length of BC047440 cDNA was cloned from human HCC tissues. After blocking BC047440 expression in HepG2 cells by a vector-based hairpin small interference RNA against BC047440, cell growth was decreased and cells were arrested at G₁ phase^[4-7]. Due to the deficiency of specific antibody, the effects of BC047440 on HCC

Correspondence to: Jing Li; Tel: +86-23-68774506;
Fax: +86-23-68755606; Email: xqyylj@tom.com

This paper was translated from Chinese into English by CJC Medical Translation and edited by Wei Liu.

Received: 2010-05-31; Accepted: 2010-08-30

Grant: National Natural Science Foundation of China (No. 30570843).

development and progression are unknown yet. To further clarify the function of BC047440, we constructed a prokaryotic expression vector to induce the expression of BC047440 fusion protein. After purification, the protein was served as an antigen to immunize rabbit, resulting in the generation of rabbit anti-human BC047440 antibody. We used the purified antibody to examine the expression of BC047440 in HCC specimens. Moreover, we evaluated the relationship between BC047440 expression and the development and progression of HCC.

Materials and Methods

Materials

Tissue samples

Sixty-eight specimens of HCC and their corresponding adjacent tissues were collected from the Second Affiliated Hospital of the Third Military Medical University between 2004 and 2009 and were confirmed by pathologic examination. These patients were 52 men and 16 women, with a median age of 40.5 years (range, 28–74 years). All lesions (including satellite lesions) observed by preoperative contrast CT and intraoperative B ultrasound were completely resected, with a resection margin at 2 cm from tumor. Liver function was evaluated according to the CHILD classification: 51 patients were grade A and 17 were grade B. Liver cancer tissues and adjacent liver tissues at 1.5 cm from tumor margin were collected immediately after operation. No cancer cells were detected at the resection margin. Eight cases of normal liver tissues (hepatic hemangioma) were served as negative control.

Animals

One rabbit (1.8 kg and 5 months old) was purchased from the Animal Center of Third Military Medical University.

Reagents

Purified BC047440 fusion protein was prepared as described previously^[8]. Freund's complete adjuvant and Freund's incomplete adjuvant were from Gibco BRL. HRP-conjugated goat anti-rabbit IgG was purchased from Boster company. SP immunohistochemistry kit was from Biosynthesis Biotechnology Co., Ltd.

Methods

Preparation of rabbit anti-human BC047440 antibody

Antibody was generated as previously described^[9]. In brief, recombinant BC047440 fusion protein (500 µg) in Freund's complete adjuvant was injected into the muscle of the back leg and injected subcutaneously into the back in the rabbit. Two weeks later, recombinant BC047440 fusion protein (250 µg) in Freund's incomplete adjuvant was

injected into the rabbit to strengthen immunity. Thereafter, the second injection was repeated every 10 days for three times. Two milliliter blood was taken from the ear vein of the rabbit to prepare serum and detect the titer of the antibody. The blood was taken from the heart to prepare antibody. The titer of the antibody in blood was detected by indirect ELISA. The specificity of the antibody was identified by Western blot analysis.

Immunohistochemistry

BC047440 antibody was diluted (1:200) according to the SP method. The paraffin slices were dewaxed in ethanol and washed with PBS for three times (5 min/time). Slices were immersed into 3% hydrogen peroxide in formaldehyde for 20 min at room temperature, and then washed with PBS for three times (5 min/time). Non-immune serum was used to block the slices for 20 min at room temperature. BC047440 antibody was added and incubated for 60 min. Slices were washed with PBS for three times (5 min/time). Secondary antibody was added and incubated for 20 min at room temperature. Slices were washed with PBS for three times (5 min/time). DAB was freshly prepared and added to slices for 5 min at room temperature. Once positive results with clear background were observed by microscope, the staining procedure was terminated. Finally, slices were washed, stained with hematoxylin, dehydrated with ethanol and mounted. PBS was used as a negative control. Positive slices were used as a positive control. The cells with brown particles in cytoplasm were regarded positive. The expression intensity was evaluated as follows: the number of positive cells < 2% was considered as (-), 2%–10% as (+), 11%–50% as (++), and 51%–100% as (+++)^[10].

Western blot analysis

Tissues were lysed in 500 µL of lysis buffer on ice for 20 min, then centrifuged for 2 min. The supernant was taken and preserved at -20°C. Protein concentration was determined by the Bradford method. The protein (50 µg/well) was subjected to 120 g/L SDS-PAGE for electrophoresis and transferred to PVDF membrane. The membrane was blocked in 50 g/L non-fat milk at 4°C overnight. BC047440 antibody (dilution: 1:1000) was added and incubated for 2 h at room temperature. After washing with 0.05% Tween-PBS for three times, HRP-conjugated secondary antibody (dilution: 1:2000) was added and incubated for 2 h. Membrane was washed with 0.05% Tween-PBS for three times (10 min/time). The results were shown by ECL detection. The gray value of target band was examined. GAPDH was used as internal control. The relative expression of target protein = absorbance of target protein / absorbance of GAPDH.

Statistical analysis

The relation between protein expression and

clinicopathologic features of the patients with HCC was analyzed by χ^2 test. Variance analysis was used to detect the differences between groups. Survival was analyzed by the Kaplan-Meier method. The log-rank method was applied for significance analysis. $P < 0.05$ was considered as significant.

Results

Titer and specificity of the polyclonal antibody

Polyclonal antibody was generated from purified BC047440 fusion protein. The titer was determined by indirect ELISA. After four times of immunization of the rabbit, the titer of BC047440 antibody reached 1:256 000. Western blot analysis showed that the purified BC047440 fusion protein displayed a specific binding band at 23 kDa,

whereas no band of non-induced protein was detected. BC047440 expression in HCC tissues

As shown by immunohistochemistry, the prepared polyclonal antibody recognized endogenous BC047440 in HCC tissues effectively. Positive staining was observed in 30 of 68 specimens of HCC, 16 of 68 specimens of corresponding adjacent liver tissues, and none of 8 specimens of normal liver tissues (Figure 1). In HCC cells, BC047440 was mainly stained in the cytoplasm and membrane with the positive rate of 44.1%. In the adjacent tissues, BC047440 was expressed in cytoplasm with the positive rate of 23.5%. The expression of BC047440 was significantly higher in HCC tissues than in the adjacent and normal tissues ($P < 0.05$). In addition, Western blot analysis showed that BC047440 was highly expressed in HCC tissues (Figure 2). The average expression level of BC047440 was 0.23 ± 0.02 in normal liver tissues, 1.45 ± 0.13 in HCC tissues, and 0.40 ± 0.04 in their corresponding adjacent tissues ($P < 0.01$).

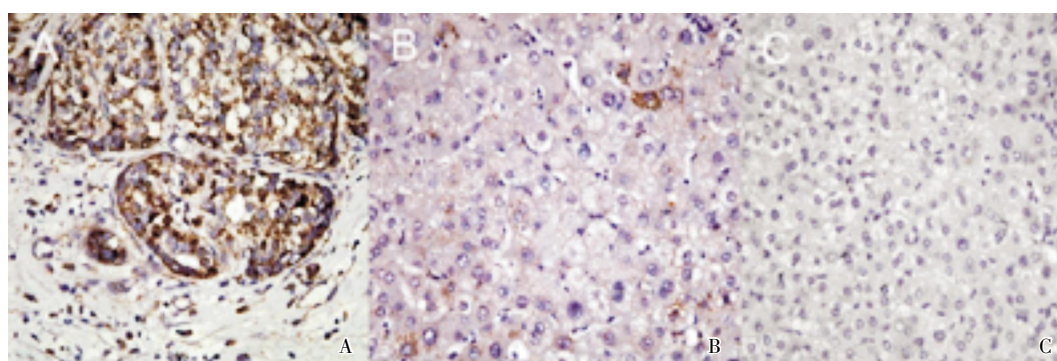


Figure 1 Immunohistochemical staining of BC047440 in HCC tissues, the adjacent liver tissues and normal liver tissues (HE $\times 400$)

A, BC047440 protein is highly expressed in cytoplasm in HCC tissues; B, BC047440 protein is lowly expressed in adjacent tissues; C, no expression of BC047440 protein was detected in normal liver tissues.

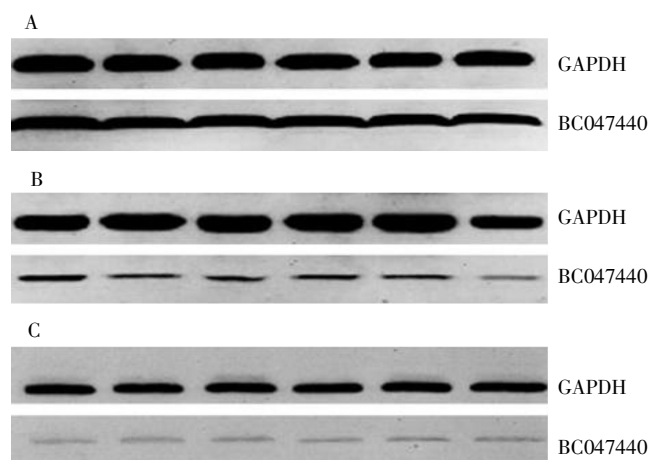


Figure 2 BC047440 protein expression in HCCs detected by Western blot

A, HCC tissues; B, adjacent tissues; C, normal liver tissues.

The correlations between BC047440 and clinicopathologic features of patients with HCC are shown in Table 1. No significant correlations were found between the expression of BC047440 and sex, HBV infection, liver cirrhosis, and AFP level ($P > 0.05$). On the other hand, BC047440 expression was correlated with tumor diameter and invasion ($P < 0.05$ and $P < 0.01$). Moreover, we found that the average age of patients with positive BC047440 expression was younger than those with negative BC047440 expression (35.3 years vs. 45.6 years, $P < 0.05$), and that the positive rate of BC047440 was higher in patients younger than 40 years than in patients older than 40 years.

BC047440 expression and prognosis

The specimens were divided into two groups according to BC047440 staining: 30 in positive group and 38 in negative group. The survival curves (Figure 3) showed that the survival was significantly shorter in positive staining group than in negative staining group [(851.67 \pm 83.90) days vs. (1161.25 \pm 83.60) days, $\chi^2 = 4.77$, $P = 0.029$], suggesting that BC047440 expression was associated with prognosis.

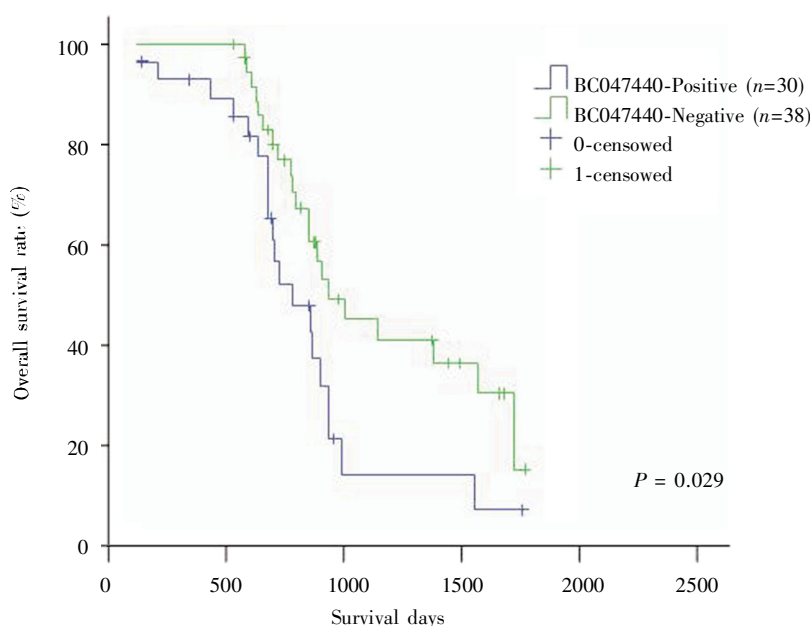


Figure 3 Kaplan-Meier survival curves for BC047440-positive expression group ($n = 30$) and BC047440-negative expression group ($n = 38$)
BC047440-positive patients with HCC have significantly poorer prognosis than BC047440-negative patients (log-rank test, $\chi^2 = 4.77$, $P = 0.029$).

Discussion

Previously, we isolated an EST fragment which was highly expressed in HCC by SSH, and obtained the full length (1476 bp) of cDNA by rapid amplification of cDNA ends (RACE). We found that the sequence of this EST fragment was homologous to a new gene (GenBank accession number: BC047440) with unknown function [4,5]. BC047440 locates at 20q11.22 and encodes a protein which is composed of 200 amino acids with a molecular weight of 22.6 kDa. Prosite analysis showed that BC047440 contains several potential kinase modification sites, suggesting that it is a functional protein. We analyzed the functional region of

BC047440 and found that BC047440 belongs to the COMMD gene family and encodes a transcriptional factor [5]. Our previous work showed that BC047440 promotes the proliferation and invasion of HCC [6,7]. However, the detailed roles of BC047440 in the development and progression of HCC are unknown yet due to the lack of specific antibody.

We have constructed a prokaryotic expression vector of BC047440 and induced the expression of BC047440 fusion protein. Based on the fusion protein, we generated a polyclonal antibody of BC047440 in the present study. After purified the serum, indirect ELISA test was applied to determine the titer of antibody (1:256 000). The antibody showed immunologic activities including recognition of both fusion protein and BC047440 in tumor tissues through

Table 1 Correlation of BC047440 expression to clinicopathologic features of hepatocellular carcinoma (HCC)

Characteristic	No. of cases	BC047440 [cases (%)]		<i>P</i>
		Positive	Negative	
Sex				NS
Male	52	20 (38.5)	32 (61.5)	
Female	16	10 (62.5)	6 (37.5)	
Age (years)				< 0.01
≤ 40	38	27 (71.1)	11 (28.9)	
> 40	30	3 (10.0)	27 (90.0)	
Mean ± SD (40.8 ± 9.6)		35.3 ± 4.9	45.6 ± 10.1	< 0.05
HBsAg				NS
Positive	45	21 (46.7)	24 (53.3)	
Negative	23	9 (39.1)	14 (60.9)	
AFP level (μg/L)				NS
< 400	30	14 (46.7)	16 (53.3)	
> 400	38	16 (42.1)	22 (57.9)	
Cirrhosis				NS
No	40	18 (45.0)	22 (55.0)	
Yes	28	12 (42.9)	16 (57.1)	
Tumor diameter (cm)				< 0.05
≤ 5.0	25	6 (24.0)	19 (76.0)	
5.1–10.0	30	14 (46.7)	16 (53.3)	
> 10.0	13	10 (76.9)	3 (23.1)	
Differentiation				NS
Well-moderate	33	15 (45.5)	18 (54.5)	
Poor and undifferentiated	35	15 (42.9)	20 (57.1)	
Vein invasion				< 0.01
Absent	25	6 (24.0)	19 (76.0)	
Present	43	24 (55.8)	19 (44.2)	

Western blot analysis and immunohistochemistry staining. Therefore, the antibody can be used for further studies.

To clarify the roles of BC047440 in the development of HCC, we analyzed the association between BC047440 expression and clinicopathologic features of patients with HCC and found that BC047440 expression was not related to HBV infection and liver cirrhosis, indicating that BC047440 may be not associated with the initiation of HCC [11–13]. In addition, the expression of BC047440 was not related to AFP level, which was found to affect the growth of HCC through cell apoptosis signaling pathway [14–16]. Our previous study showed that BC047440 was not related to HepG2 cell apoptosis [17]. Here, we further confirmed that BC047440 was not involved in AFP-mediated cell apoptosis of HCC. However, BC047440 expression was associated with the size and portal vein invasion of HCC, indicating that BC047440 promoted the proliferation and invasion of HCC. Moreover, silencing the expression of BC047440 inhibited cell proliferation, arrested HepG2 cells at S phase, and reduced the invasion rate of HepG2 cells, in consistent with the present study showing that BC047440 was associated with invasion of HCC. More importantly, we found that high BC047440 expression was related to low age of patients, suggesting that age may be an impact factor for BC047440

expression. Previous studies show that elder patients with HCC have a worse prognosis compared to younger ones [18–20]. Thus, BC047440 expression may be served as a potential factor for prognosis of HCC. To verify the hypothesis, we followed up 68 patients with HCC after operation and analyzed the association between BC047440 expression and the prognosis of HCC. Our results showed that high expression of BC047440 was related to poor prognosis.

In conclusion, we found that both BC047440 mRNA and protein were highly expressed in HCC. BC047440 may be involved in the proliferation and invasion of HCC. A prospective study is undergoing to further clarify the prognostic value of BC047440 in HCC and to provide basis for prognosis prediction and clinical comprehensive treatment selection.

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