

## Original Research Article

# Expressions of B7-H4 and B7-H3 Proteins and Effect of Sorafenib on their Expressions in Different Human Hepatoma Cell Lines

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## Abstract

**Purpose:** To study whether B7-H3 and B7-H4 proteins can be candidate drug targets for preventing and treating hepatoma.

**Methods:** Western blot assay was used to study the expressions of B7-H3 and B7-H4 proteins and the effect of sorafenib on their expressions in different human hepatoma cell lines (HepG2, Hep3B, BEL-7402, BEL-7404, BEL-7405, QGY-7701, QGY-7703, SMMC-7721, MHCC97H, MHCC97L, HCCLM3 and HCCLM6). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to study the cytotoxicity of sorafenib against different human hepatoma cell lines.

**Results:** The expressions of B7-H3 (0.26 - 0.84  $\mu$ M) and B7-H4 (0.18 - 0.78  $\mu$ M) proteins in different human hepatoma cell lines were significantly ( $p < 0.01$ ) up-regulated, compared with that of the normal human liver cell line (HL-7702) (0.09 and 0.08  $\mu$ M). Sorafenib was cytotoxic on Hep3B, BEL-7404, MHCC97H, HCCLM3 and HCCLM6 cells with half-maximal inhibitory concentration ( $IC_{50}$ ) of 14.56, 9.14, 9.46, 17.21 and 9.29  $\mu$ M, respectively. After treatment with sorafenib at concentrations of 5, 10 and 20  $\mu$ M, the expressions of B7-H3 protein in MHCC97H, HCCLM3 and HCCLM6 cells and the expressions of B7-H4 protein in Hep3B, BEL-7404 and MHCC97H cells were significantly ( $p < 0.01$ ) down-regulated, compared with that of the control.

**Conclusion:** Over-expression of B7-H3 and B7-H4 proteins is common in different human hepatoma cell lines and thus, B7-H3 and B7-H4 proteins may be regarded as candidate drug targets for preventing and treating hepatoma.

**Keywords:** Hepatoma, B7-H3, B7-H4, Sorafenib, Over-expression, Cytotoxic activity, Down-regulation

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## INTRODUCTION

Primary hepatoma is the 15th most common cancer and the 2nd leading cause of cancer mortality worldwide [1]. Primary hepatoma remains the 8th most common malignancy in women and the 5th in men according to the estimates of incidence of cancer in 2000 [2].

There were 749 thousand new cases and 695 thousand deaths from hepatoma in 2008 [3]. Therefore, finding novel therapeutic methods are urgent for preventing and treating hepatoma.

Nowadays, the therapeutic strategies of hepatoma include surgical resection [4], biotherapy [5], interventional therapy [6],

radiotherapy [7], chemotherapy [8], Chinese medicinal therapy [9] etc. The B7 protein family including B7-2, B7-1, B7-H4, B7-H3, B7-H2 and B7-H1 is a group of co-stimulators, which is related to the activation of T and B cells and immunologic functions. Therefore, the B7 protein family is widely applied to treat autoimmune diseases, transplantation immune diseases and cancers [10,11].

In the present study, western blot was used to study the expressions of B7-H3 and B7-H4 proteins in different human hepatoma cell lines (HepG2, Hep3B, BEL-7402, BEL-7404, BEL-7405, QGY-7701, QGY-7703, SMMC-7721, MHCC97H, MHCC97L, HCCLM3 and HCCLM6) and normal human liver cell line (HL-7702) [12]. Then 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to study the cytotoxicity of sorafenib against these cell lines (Hep3B, BEL-7404, MHCC97H, HCCLM3 and HCCLM6), which had the higher expressions of B7-H3 and B7-H4 proteins than other cell lines. Subsequently, the effects of sorafenib on the expressions of B7-H3 and B7-H4 proteins in Hep3B, BEL-7404, MHCC97H, HCCLM3 and HCCLM6 cells were studied using Western blot assays. The purpose was to determine whether the over-expressions of B7-H3 and B7-H4 proteins was common in human hepatoma cell lines, and B7-H3 and B7-H4 proteins could be regarded as candidate drug targets for preventing and treating hepatoma.

## EXPERIMENTAL

### Chemicals and reagents

A defined fetal bovine serum, RPMI-1640 and DMEM medium were provided by Hyclone (Logan, Utah, USA) and Invitrogen (Carlsbad, CA, USA). Dimethylsulfoxide (DMSO), primary antibodies against  $\beta$ -actin, B7-H3, B7-H4 and horseradish peroxidase (HRP)-conjugated secondary antibody were purchased from Sigma-Aldrich (Taufkirchen, Germany). Sorafenib was purchased from LKT Laboratories (St Paul, Minnesota, USA). Enhanced BCA Protein Assay kit was obtained from Beyotime (Haimen, China).

### Cell culture

The HepG2, Hep3B, BEL-7402, BEL-7404, BEL-7405, QGY-7701, QGY-7703, SMMC-7721, MHCC97H, MHCC97L, HCCLM3, HCCLM6 and HL-7702 cells were provided by American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were separately cultured in RPMI-1640 or DMEM medium supplemented with 10 %

defined fetal bovine serum, 100 U/mL of penicillin and 100 U/mL of streptomycin at 37 °C in 5 % CO<sub>2</sub>/95 % air.

### MTT assay

MTT assay was used to evaluate the anti-proliferative activities of sorafenib against Hep3B, BEL-7404, MHCC97H, HCCLM3 and HCCLM6 cells. Different human hepatoma cells were separately seeded on 96 well plates for 24 h, and then cells were treated with sorafenib at the concentrations of 0 (for control), 1.25, 2.5, 5, 10, 15, 20 and 40  $\mu$ M. After 48 h, the MTT was added into each well and the cells were cultured for another 3 h. Then the DMSO was added into each well and the optical density (OD) of the DMSO solution was measured at 570 nm using a microplate reader (Perkin-Elmer, USA) [13]. The inhibition rate of sorafenib against different human hepatoma cells were calculated as in Eq 1 [14].

$$\text{Inhibition (\%)} = \{(Ac - As)/Ac\}100 \dots\dots\dots (1)$$

where Ac and As are the absorbance of control and sorafenib samples, respectively.

### Western blot assay

The total proteins of untreated HepG2, Hep3B, BEL-7402, BEL-7404, BEL-7405, QGY-7701, QGY-7703, SMMC-7721, MHCC97H, MHCC97L, HCCLM3, HCCLM6 and HL-7702 cells were extracted. After treatment with sorafenib at concentrations of 0 (for control), 5, 10 and 20  $\mu$ M for 48 h, the total proteins of Hep3B, BEL-7404, MHCC97H, HCCLM3 and HCCLM6 were extracted. According to the manufacturer's instruction, the concentrations of total proteins were determined using an Enhanced BCA Protein Assay kit [15]. Then, equal amounts of total proteins (about 30  $\mu$ g) were separated by 12 % SDS/PAGE and moved onto PVDF membrane. After blocking with 5 % fat-free milk, the PVDF membranes were incubated with primary antibodies against  $\beta$ -actin, B7-H3, B7-H4 overnight at 4 °C and subsequently with goat anti-rabbit/HRP at room temperature for 2 h [16]. Finally, all proteins were detected by chemiluminescence. An antibody directed against  $\beta$ -actin was used to evaluate protein loading.

### Statistical analysis

All data are presented as mean  $\pm$  standard deviation. A one-way ANOVA was used to analyze the differences among different groups using Dunnett test on SPSS 19.0. When *p*-value

was less than 0.05, the differences were recognized as statistically significant.

## RESULTS

### Over-expressions of B7-H3 and B7-H4 proteins

Western blot was used to study the expressions of B7-H3 and B7-H4 proteins in HepG2, Hep3B, BEL-7402, BEL-7404, BEL-7405, QGY-7701, QGY-7703, SMMC-7721, MHCC97H, MHCC97L, HCCLM3, HCCLM6 and HL-7702 cell lines. As shown in Figure 1, the expressions of B7-H3 (0.26 - 0.84  $\mu$ M) and B7-H4 (0.18 - 0.78  $\mu$ M) proteins in all the hepatoma cell lines were significantly ( $p < 0.01$ ) up-regulated, compared with that of the normal human liver cell line (HL-7702) (0.09 and 0.08  $\mu$ M).

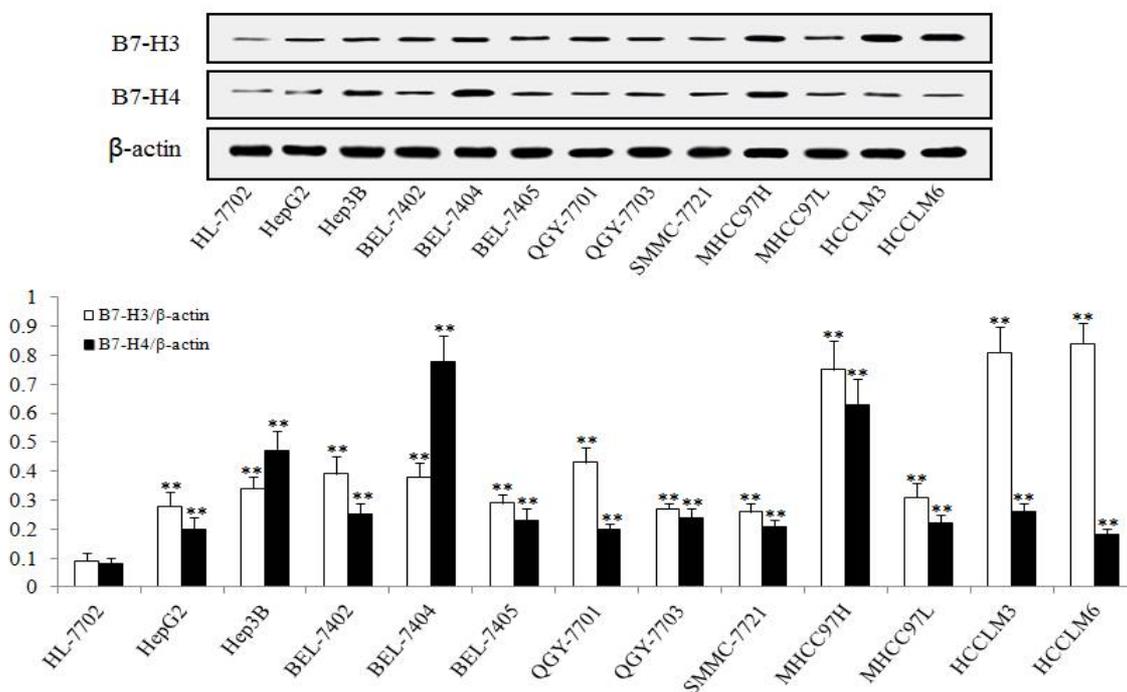
### Cytotoxicity of sorafenib against different human hepatoma cell lines

The cell lines (Hep3B, BEL-7404, MHCC97H, HCCLM3 and HCCLM6), which had the higher expressions of B7-H3 and B7-H4 proteins than other cell lines, were used to study the effect of sorafenib on the expressions of B7-H3 and B7-H4 proteins. However, before beginning the

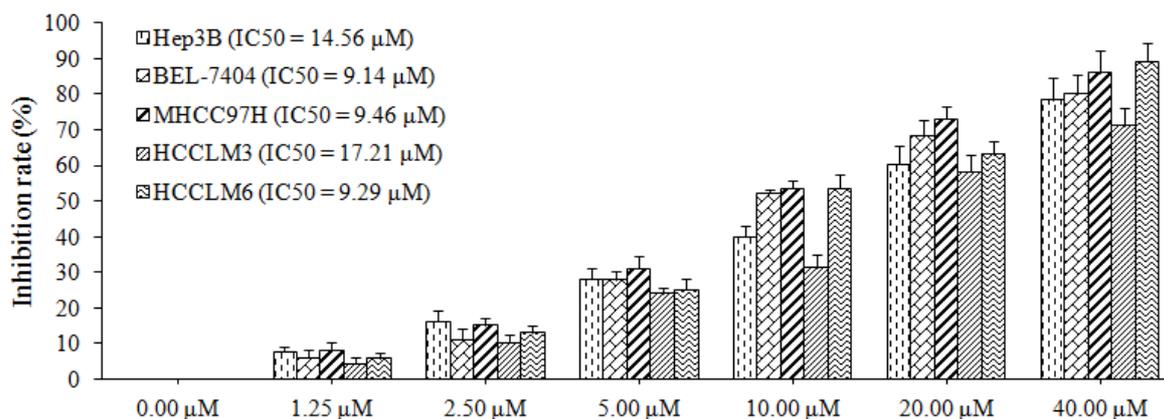
study, MTT assay was first used to determine the cytotoxicity of sorafenib against these cell lines to ensure the effectiveness of the drug. As depicted in Figure 2, sorafenib was cytotoxic to Hep3B, BEL-7404, MHCC97H, HCCLM3 and HCCLM6 with half maximal inhibitory concentration ( $IC_{50}$ ) values of 14.56, 9.14, 9.46, 17.21 and 9.29  $\mu$ M, respectively.

### Inhibitory effects of sorafenib on the expressions of B7-H3 and B7-H4 proteins

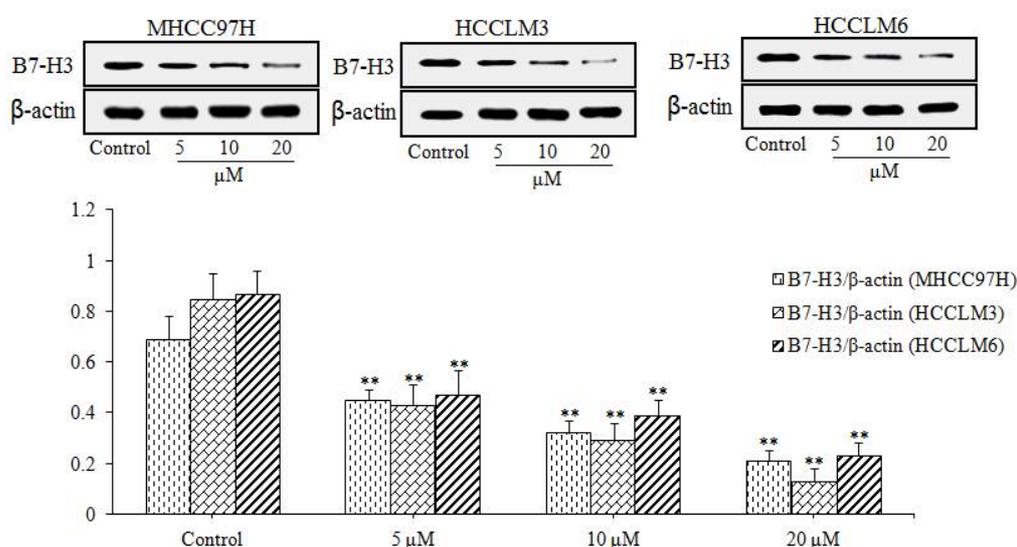
The effects of sorafenib on the expressions of B7-H3 protein in MHCC97H, HCCLM3 and HCCLM6 cells and the expressions of B7-H4 protein in Hep3B, BEL-7404 and MHCC97H cells were studied using western blot. As shown in Figure 3, after treatment with sorafenib at the concentrations of 5, 10 and 20  $\mu$ M for 48 h, the expressions of B7-H3 protein in MHCC97H, HCCLM3 and HCCLM6 cells were significantly ( $p < 0.01$ ) down-regulated, compared with that of the control. As shown in Figure 4, after treatment with sorafenib at the concentrations of 5, 10 and 20  $\mu$ M for 48 h, the expressions of B7-H4 protein in Hep3B, BEL-7404 and MHCC97H cells were significantly ( $p < 0.01$ ) down-regulated, compared with that of the control.



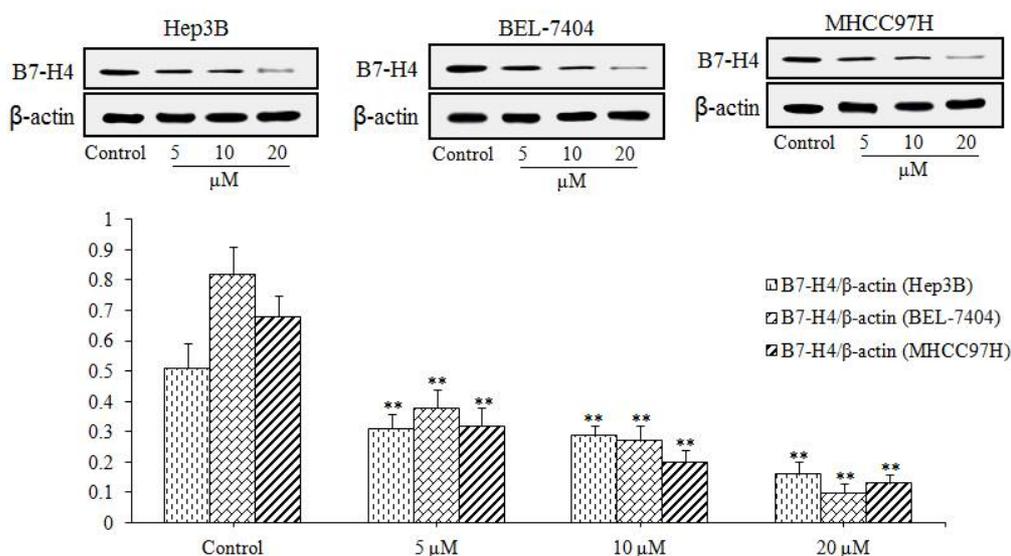
**Figure 1:** Over-expressions of B7-H3 and B7-H4 proteins in different human hepatoma cell lines (HepG2, Hep3B, BEL-7402, BEL-7404, BEL-7405, QGY-7701, QGY-7703, SMMC-7721, MHCC97H, MHCC97L, HCCLM3 and HCCLM6), \*\* $p < 0.01$ , compared with that of the normal human liver cell line (HL-7702)



**Figure 2:** Cytotoxicity of sorafenib against Hep3B, BEL-7404, MHCC97H, HCCLM3 and HCCLM6 cells



**Figure 3:** Inhibitory effects of sorafenib on the expressions of B7-H3 protein in MHCC97H, HCCLM3 and HCCLM6 cells, \*\* $p < 0.01$ , compared with that of the control



**Figure 4:** Inhibitory effects of sorafenib on the expressions of B7-H4 protein in Hep3B, BEL-7404 and MHCC97H cells, \*\* $p < 0.01$ , compared with that of the control

## DISCUSSION

In the present study, we studied the expressions of B7-H3 and B7-H4 proteins in different human hepatoma cell lines (HepG2, Hep3B, BEL-7402, BEL-7404, BEL-7405, QGY-7701, QGY-7703, SMMC-7721, MHCC97H, MHCC97L, HCCLM3 and HCCLM6) and normal human liver cell line (HL-7702) using western blot assay. Then, the cytotoxicity of sorafenib against Hep3B, BEL-7404, MHCC97H, HCCLM3 and HCCLM6 cells and the effect of sorafenib on the expressions of B7-H3 and B7-H4 proteins in these cells were studied using MTT and western blot assays.

Defect in the immune response may lead to the growth of tumor. T cell plays an important role in immune response. Co-stimulatory ligands such as CD80 and CD86 play an important role in the startup and maintenance of immune response, and their non-expressions in tumor cells has been regarded as a reason for immune evasion [17,18].

The expressions of inhibitory cofactors such as CD152 and PD-1 in immune cells, and the expression of their ligands in tumor cells are related to tumor pathogenesis [19]. Nowadays, inhibiting the interaction of inhibitory cofactors with their ligands has been successfully used to treat tumor [20]. Blocking the expressions of inhibitory cofactors in immune cells and the expressions of their ligands in tumor cells may be important in suppressing the immune evasion of tumor cells [21].

B7-H4 and B7-H3 are two major co-stimulators or ligands of inhibitory cofactors in B7 protein family, which is related to the activation of T cells and immunologic functions [22]. B7-H4 is expressed at the transcriptional level in the thymus, lung and spleen [23] while B7-H3 is widely expressed at the transcriptional level in lymphoid organs, liver, testis, uterus, lung, skeletal muscle, etc [24]. Therefore, B7-H4 and B7-H3 seem to be limited at the translational level [25]. It was reported that the B7-H4 and B7-H3 proteins were expressed in non-small-cell lung cancer [26].

Some studies indicate that B7-H4 is over-expressed at the transcriptional level in human hepatoma cell line BEL-7404 [27]. However, the expressions of B7-H3 and B7-H4 proteins in different human hepatoma and normal human liver cell lines and the effect of sorafenib on their expressions were still unknown. Because sorafenib can prevent both tumor cell

proliferation and angiogenesis, and has been successfully used to treat hepatoma [28,29], it was considered as positive drug in the present study.

The results indicate that the expressions of B7-H3 and B7-H4 proteins in all the human hepatoma cell lines were significantly up-regulated, compared with that of the normal human liver cell line (HL-7702). Sorafenib was cytotoxic to Hep3B, BEL-7404, MHCC97H, HCCLM3 and HCCLM6 with IC<sub>50</sub> values of 14.56, 9.14, 9.46, 17.21 and 9.29 μM, respectively.

After treatment with sorafenib, the expression of B7-H3 protein in MHCC97H, HCCLM3 and HCCLM6 cells and the expression of B7-H4 proteins in Hep3B, BEL-7404 and MHCC97H cells were significantly down-regulated, compared with that of the control. Although over-expression of B7-H3 and B7-H4 proteins was observed in the human hepatoma cell lines, expressions of B7-H3 and B7-H4 proteins in liver cancer tissues, the relationship between them, and the classification of liver cancer requires further study.

## CONCLUSION

Over-expression of B7-H3 and B7-H4 proteins in human hepatoma cell lines are common. Sorafenib is cytotoxic to human hepatoma cell lines and inhibit t over-expression of B7-H3 and B7-H4 proteins in human hepatoma cell lines. These findings indicate that B7-H3 and B7-H4 proteins may be regarded as candidate drug targets for preventing and treating hepatoma.

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