BORIS, brother of the regulator of imprinted sites, is aberrantly expressed in hepatocellular carcinoma

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Abstract

Background

BORIS is a novel member of the cancer/testis antigen gene family. These genes are normally expressed only in spermatocytes but abnormally activated in different malignancies. To explore the expression of BORIS in hepatocellular carcinoma (HCC) and its correlation with clinicopathologic features and prognosis of HCC

Materials and Methods

In this study, we investigated BORIS expression in HCC cell lines and 105 primary HCC clinical surgical specimens. We further examined the correlation of BORIS with Liver Stem Cells Marker (CD90) in HCC tissues by histochemical doublestaining. The correlation of BORIS with clinicopathologic features and prognosis of HCC was analyzed.

Results

The expression of BORIS was found in SMMC-7721, BEL-7402 and Huh-7, but not in hep-G2. The positive expression rate of BORIS was significantly higher in the HCC tissues than in the paracancer tissues (P=0.001). BORIS expression was correlated with the tumor size (P=0.001), CD90 (P=0.001), satellite nodule (P=0.001), and Serum AFP (P=0.036). Kaplan-Meier survival curves showed that patients with positive expression of BORIS had lower overall survival rate (P=0.003).

Conclusions

Our data indicated that BORIS may be a novel favorable prognostic indicator and a candidate therapeutic target against HCC.
Keywords: BORIS, Hepatocellular carcinoma, CD90, Expression, Prognosis

Introduction

HCC is a common malignant tumor especially in East Asia, resulting in more than 250,000 deaths each year in China (1-3). In spite of enormous efforts to improve clinical treatment, the overall survival of patients with HCC remains unsatisfactory because of a high incidence of recurrence and metastasis. Recent research efforts on stem cells have shed light on new directions for the eradication of cancer stem cells (CSCs) in HCC (4-6). This crucial early role for epigenetic alterations in cancer is in addition to epigenetic alterations that can substitute for genetic variation later in tumour progression(7). Numerous recent findings in the molecular biology of cancer suggest that the neoplastic phenotype arises and is maintained through genome-wide modifications associated with epigenetic changes (8-9). BORIS is an unique epigenetically acting, tumor-promoting, transcription factor expressed in different types of human and mouse cancer cells has been recently described (10).

BORIS gene was first described as a DNA-binding protein that shares 11 zinc finger (ZF) domains with CCTC-binding factor (CTCF), but differs from this molecule at the N and C Termini (11). Lobanenkov etal originally identified the CCCTC-binding factor (CTCF) as a transcription factor regulating cmyc expression (12,13), which was subsequently found to act as a tumor suppressor (14–16). BORIS as the same protein family with CTCF have aroused increasing attention since its decreasing methylation
of DNA. BORIS protein is absent in nonmalignant male tissues with the exception of testis, and is totally absent in females. However, BORIS transcripts were detected in more than half of the cancer cell lines (10). Several collaborating laboratories have initiated studies of BORIS expression in a variety of primary cancers (17,18). As yet we have not found any reports about expression of BORIS in hepatocellular carcinoma.

Based on above discoveries, in the present study, we investigated BORIS expression in a total of 105 tissue samples of HCC and kinds of liver cancer cell lines by immunohistochemical, PCR and Western blot, and demonstrated that BORIS expression was a reliable indicator for disease diagnosis and the poor prognosis of HCC. The expression of cancer stem cell marker CD90 is related to the expression of BORIS in HCC tissue.

Materials and methods

Tissue samples

HCC tissue samples (n=105) were obtained from patients who underwent surgical treatment without prior radiotherapy or chemotherapy treatment at the west china hospital between 2005 and 2006 (chengdu, China). normal liver Samples (n=20) and cirrhosis samples (n=16) were collected from individuals without cancer and were used as control. Clinical information on gender, Age, serum HBsAg, serum AFP, cirrhosis, satellite nodule and tumor capsule of patients were received from the west china Hospital. All samples were obtained with previous written consent. The histologic cell types were assigned according to the criteria of the WHO
Cell lines and culture

Different human liver cell lines, Hep-G2 and Huh-7 were obtained from the American Type Culture Collection (ATCC). BEL-7402 and SMMC-7721 cell lines were obtained from the Committee of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM or RPMI-1640 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C with 5% CO₂.

Immunohistochemical and Histochemical Double Staining

The embedded tissues were cut into 4-μm-thick sections for histological studies by hematoxylin-eosin staining. BORIS protein was detected with a specific rabbit anti-human polyclonal antibody (Abcam, Cambridge, UK). Paraffin-embedded tumor sections were boiled in a cooker for 4 min in citrate buffer. Then slides were treated with 3% hydrogen peroxide 20–30 min before blocking with 3% BSA. The primary antibody (dilution, 1/50) was incubated with the tumor sections overnight at 4°C. For negative control, the primary antibody incubation was replaced by 3% blocking solution in every set of slides stained. Sections were washed in PBS three times and incubated with goat-anti-rabbit IgG (Jackson ImmunoResearch, West Grove, USA) for 1 h at room temperature. After further washing the samples were covered with Vectashield mounting medium (Vector Laboratories, Burlingame, USA) and stored at -20°C. Negative control slides were probed with normal goat serum under the same experimental conditions.
To determine the relationship between the expression of BORIS and cancer stem cell marker CD90, we performed Histochemical Double staining for BORIS (Abcam, Cambridge, UK) and CD90 (DAKO, Glostrup, Denmark) on the same sections using Envision Doublestain System (DAKO, Glostrup, Denmark). Deparaffinization and antigen retrieval were performed as described above. After quenching the endogenous peroxidase activity with peroxidase blocking reagent (DAKO, Glostrup, Denmark), tissue sections were incubated with mouse monoclonal anti-CD90 antibodies for one hour at room temperature, followed by rinsing with washing buffer. Then the tissue sections were subjected to the second staining for BORIS with the sequential steps of quenching the, incubation with anti-BORIS antibodies, incubation with anti-goat IgG antibodies and detection of the phosphatase activity in accordance with the manufacturer’s instructions (Envision Doublestain System, DAKO, Glostrup, Denmark).

Immunofluorescence detection of BORIS and CD90 derived from liver cancer tissues frozen section. Antibodies were conjugated to Alexa Fluorescent dyes (Invitrogen). Fluorescent images were acquired at 2-mm Z-axis intervals using a confocal microscope (Leica TCS SP5 II)

**Immunohistochemical staining and assessment of BORIS levels in HCC and paracancer tissues**

Immunohistochemical analysis was done by staining normal (n=20), cirrhosis (n=16), paracancer tissues (n=34), HCC (n=58) with the Vectastain Elite ABC standard kit (Vector Laboratories, Burlingame, CA) as suggested by the manufacturer.
Immunohistochemical staining was evaluated by using the immunoreactivity score (IRS) as previously described (19). In brief, the percentage of BORIS-positive cells was divided into four categories (<10%, 11-50%, 51-80%, and >80%, with corresponding scores of 1, 2, 3, and 4, respectively), whereas the staining intensity was given a value between 0 (no detectable immunostaining) and 3 (strong immunostaining). The IRS (0-12) was then calculated by multiplying the score values. Scoring was done in a blinded fashion by two independent scorers, with each slide read twice.

**Reverse transcription-PCR**

To determine the BORIS transcription in human HCC cell lines, total RNA was extracted from cells with TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Total RNA was reverse-transcribed with 25 units of MMLV reverse transcriptase (Promega) and OligodT as primer (Takara). The resulting cDNAs were amplified with the following oligonucleotide sequences: BORIS, 5-CCCATTGTGCCACCATTCA-3 (Forward), BORIS, 5-AGCATGCAAGTGGCGCA-3 (Reverse). actin transcription was used as an invariant endogenous control. The primer sequences were as follow:actinF: 5-TCATCACCATTGGCAATGAG-3 (Forward),actinR, 5-CACTGTGTTGGCGTACAGGT-3 (Reverse)

**Western blot analysis**

For western blot analysis, lysates from cells were prepared according to Klenova et al (20) with modifications. Lysates from testis tissues were prepared as follows. Tissue
was homogenized in the lysis buffer at the ratio 500mm$^3$ tissue/100 ml buffers. The homogenate was kept on ice for 30 min, filtered through gauze and centrifuged for 15 min, at 4$^\circ$ and 13 000 r.p.m. Samples containing high concentration of lipids were additionally precipitated with acetone. The supernatant was discarded, pellet dried at room temperature and re-suspended in SDS loading/lyses’ buffer. Western blot assay was conducted as described previously with the anti-BORIS antibody (Abcam) or anti-a-tubulin antibody (Sigma). Detection was performed with enhanced chemiluminescence reagent (Amersham Biosciences, now GE Healthcare, and Buckingham, UK) according to the manufacturer’s instructions.

**Statistical analysis**

Data were expressed as (mean ± standard deviation) or median with range. SPSS program for Windows (version 15.0, SPSS Inc, USA) was used for statistical analysis. Comparisons of BORIS tumor expression with clinical and pathologic features were evaluated by using chi-square tests. Overall survival analyses were estimated by using the Kaplan–Meier method. The student’s $t$-test was used to detect differences between groups. All tests performed were two-tailed. A difference was considered significant if $p<0.05$.

**Results**

**BORIS expression in hepatocellular carcinoma cell lines**

BORIS expression was investigated in different human liver cell lines by means of RT-PCR and Western blot method. As shown in (Figure1), We found that testis (positive control), BEL-7402, SMMC-7721, and Huh-7 constitutively express BORIS
mRNA and protein. However, BORIS can not be found in Hep-G2 and normal liver tissue.

**Figure 1** Detection of BORIS mRNA and protein in 4 human liver cancer cell lines by RT-PCR (A) and Western blot (B). Expression of BORIS mRNA and protein in SMMC-7721 (Lane 2), BEL-7402 (Lane 3) and Huh-7 (Lane 4), but not in hep-G2 (Lane 5). Human testicular tissues (Lane 1) were used as positive control, normal liver (Lane 6) were used as nonmalignant negative control.

**BORIS expression in HCC clinical samples**

In order to screen the state of BORIS expression in vivo, we investigated BORIS protein expression by immunohistochemistry, in a total of 105 primary HCC, 20 normal liver and 16 cirrhosis tissues. BORIS expression of 58 cases (55.2%) localized in cytoplasm and nucleolus was found in HCC surgical resection samples whereas the remaining 47 cases (44.8%) displayed almost undetectable BORIS expression. BORIS expression was not detected in cirrhosis and normal liver tissues. Both did not show positive staining. The analysis of relationship between the expression of BORIS and various clinicopathological parameters was listed in Table 1. The expression of BORIS was significantly correlated with serum AFP (p = 0.036), tumor size (p = 0.001) and satellite nodule (p = 0.001), but not with age, gender, serum HBsAg, cirrhosis and tumor capsule (Table 1). On the other hand, we calculated that the IRS values for 34 BORIS positive samples (2.43±0.51) in Paracancer tissues were lower than 58 BORIS positive samples (5.22±0.53) in HCC tissues (p = 0.01) (Figure 2).

**Table 1. Correlation between BORIS protein expression and clinicopathological features of hepatocellular**
<table>
<thead>
<tr>
<th>Features</th>
<th>BORIS Negative n=47</th>
<th>BORIS Positive n=58</th>
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<td>Age (years)</td>
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<tr>
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<tr>
<td>≥50 n=54</td>
<td>23</td>
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<tr>
<td>Gender</td>
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<tr>
<td>Serum HBsAg</td>
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<tr>
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<tr>
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<tr>
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<td>Yes, n=88</td>
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<td>&lt;5 n=42</td>
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</tr>
<tr>
<td>≥5 n=63</td>
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<td>Satellite nodule</td>
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**Figure 2** The IRS values for 34 BORIS positive samples (2.43±0.51) in Paracancer tissues were lower than 58 BORIS positive samples (5.22±0.53) in HCC tissues (p=0.01). We found the relationship between the expression of BORIS and CD90. Control, combined “Healthy donors” and “cirrhosis” groups.
BORIS Present in HCC Associated with CD90 Expression

We performed histochemical double staining for BORIS and DC90 in 105 primary HCC specimens. CD90 expression of 70 cases (66.7%) localized in plasma membrane and cytoplasm, BORIS expression of 58 cases (55.2%) localized in nucleolus and cytoplasm (Figure3). 49 cases (46.7%) have double staining in 105 specimens, the expression of BORIS was correlated with CD90 (P=0.001, Table1). We also found BORIS Present in HCC tissue associated with the expression of CD90 by Multiple staining fluorescence(Figure3).

Figure3 Detection of BORIS and CD90 protein in human normal liver(A and B), HCC (D-J)and Testicular tissues,positive control (C) by IHC and Immunofluorescence.

Prognostic significance of BORIS expression

To elucidate the prognostic role of BORIS in HCC, overall survival rates were estimated by Kaplan–Meier survival curves (Figure4). the expression of BORIS was associated with the poor prognosis of patients with HCC (P = 0.003, log-rank test). Overall survival rate and 5-year survival rate of BORIS group were lower than negative group [46.8 vs. 20.7% (overall survival rate) and 38.3 vs. 15.6% (5-year survival rate), respectively p < 0.05, Figure4]. On the other hand, the Satellite nodule tends to be higher in the positive-BORIS group (34/58 cases, 58.6%) than in the negative-BORIS group (10/47 cases, 21.3% P=0.001).

Figure4 Kaplan–Meier survival curves for Positive-BORIS group versus Negative-BORIS group in 105 patients with HCC showed a highly significant
Discussion

A number of recent studies have found that epigenetic changes play an important role in the process of cancer. In epithelial ovarian cancer the BORIS/CTCF expression ratio is also associated with increased stage and decreased overall and progression free survival (21). It has been suggested that the expression of BORIS displaces CTCF in the genome and leads to proliferation of cancer cells. Anahit et al demonstrated that the BORIS protein was always expressed in pancreas carcinoma (22), but the expression level was usually negative in the normal tissue; V D’Arcy et al showed that normal glands of breast were all negative for BORIS, whereas all types of breast carcinoma expressed this protein with very high incidence (23). The results of these studies including ours are similar in that they show BORIS expression to be more intense in the carcinoma nest than in normal or benign lesions. However, BORIS, as a regulatory protein of DNA demethylation, has not been reported in the role of liver tumors. This is the first study on the expression of BORIS in HCC. In the investigation of the protein level for a large number of cases, expression of BORIS as compared to no cancerous tissues was observed in 55.2% of the cases. Our study also showed that the expression of BORIS in HCC is significantly related to cancer stem cell marker CD90 in HCC tissues.

In the present study, we investigated the BORIS mRNA and protein expression in HCC cell lines or primary HCC tissue, and evaluated the correlation of BORIS expression and clinical outcome of HCC patients in HCC tissue study, we
found that BORIS expression of HCC tissue was obviously higher than that in normal tissue, which suggests that BORIS may be involved in the genesis and development of liver cancer, and it might be used in clinical practice as an indicator of liver cancer. On the other hand, we further found BORIS expression in different human liver cell lines, which suggests that BORIS is likely to be associated with malignant liver cells.

In the present study, our results showed that BORIS was very frequently expressed in HCC as compared to no cancerous tissue. We obtained more informative results probably because of the larger number of cases examined by IHC analysis. We found that the expression of BORIS is also correlated with high proliferating activity and poor overall survival rate. Our study also showed that the expression of BORIS in HCC is significantly related to cancer stem cell marker CD90 in HCC tissue. The research efforts on BORIS and CD90 have shed light on new directions for the eradication of cancer stem cells. The expression of BORIS in stem cell could provide theory evidence for treating liver cancer taking BORIS as target point. Kaplan–Meier survival analysis showed that the expression of BORIS significantly correlated with shorter survival time of HCC patients ($p = 0.017$). More ever, the expression of BORIS also showed a tendency to correlate with high tumor metastasis rate in the liver (Table1). The present study made it clearer that BORIS strongly reflects the biological aggressiveness of this carcinoma and plays an important role in its progression. These results also suggested that the HCC patients with BORIS expression very probably have a relapse and have poor prognosis, and BORIS may become a newly useful prognostic indicator for the HCC. Thus,
inhibiting BORIS activity might contribute to the application of adjuvant therapy
intervention for HCC.

In summary, our study has shown for the first time that BORIS is expressed in HCC
tissues and its expression significantly correlates with a poor prognosis of HCC.
Furthermore, we demonstrated The expression of cancer stem cell marker CD90 is
related to the expression of BORIS in HCC tissue and hepatocellular Carcinoma cell
lines the expression of BORIS in stem cell could provide experimental basis and
theory evidence for treating liver cancer taking BORIS as target point.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
Bl, YQand KFC designed and directed the study. YQ,BL , KFC, QHW, ZC, YGW
performed the majority of the experiments and composition of the manuscript.
GY, ,WIH, and YGW were responsible for data collection and analysis, and reviewing
and scoring the degree of immunostaining of sections. All authors have read and
approved the final manuscript.

Acknowledgments
This research was supported by grants from the National Natural Science Foundation
of China (No.81172372) and Sichuan University Young Teachers in start-up funding
(NO:2011SCU11046).

We thank Shu Wang Duan and Ling Wei for his critical review and language editing
of this manuscript as well as Yan He and Qun Ying Li for his technical support.
References


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Figure 1

(A) Boris

(B) Boris

Figure 1
Figure 2

Mean BORIS IRS Value

- Nomal liver (n=20)
- Cirrhosis (n=16)
- Paracancer (n=34)
- HCC (n=58)

p=0.01
Figure 4

Cumulative Survival

Overall survival (months)

p = 0.003