Original Article
Down-regulation of BubR1 inhibits growth and induces apoptosis in hepatitis B virus-related hepatocellular carcinoma cells

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Abstract: BubR1 is a critical component of the mitotic spindle checkpoint and has been reported overexpressed in many types of tumor. Hepatitis B virus (HBV) infection is acknowledged as the main cause of hepatocellular carcinoma (HCC). In this study, we aimed to explore the biological role of BubR1 in HBV-related hepatocellular carcinoma cells, examine the interaction between BubR1 and HBV X protein (HBx), and uncover the underlying mechanism. BubR1 expression was knocked down in HBV-related HepG2.2.15 cells using a small interfering RNA (siRNA) technology. Down-regulation was confirmed using Real-time PCR and Western blot analysis. Effects of BubR1 down-regulation on the proliferation, colony formation, apoptosis and cell cycle progression were assessed, respectively. The expression changes of apoptosis-related proteins and multiple signaling pathways after silencing BubR1 were observed by Western blot. Co-immunoprecipitation and immunofluorescence assays were performed to examine the interaction between BubR1 and HBx. The expression of BubR1 in HBV-related HCC cells (HepG2, SMMC7721 and Huh7). BubR1 down-regulation inhibited proliferation and colony formation, induced S phase cell cycle arrest and increased apoptosis in HepG2.2.15 cells. At the protein level, BubR1 silencing decreased the levels of phosphorylation of ERK1/2 and NF-κB, meanwhile it increased the expression of Bax and caspase-3. In addition, we also found that BubR1 binds to HBx and co-localizes with HBx at the nucleus in HepG2.2.15 cells. Down-regulation of BubR1 inhibits growth and induces apoptosis in HBV-related hepatocellular carcinoma cells, which is mediated, at least partially, through the NF-κB and MAPK/ERK1/2 signaling pathways. Therefore, BubR1 may serve as a promising therapeutic target for HBV-related HCC.

Keywords: BubR1, HBV-related hepatocellular carcinoma, HBV X protein, small interfering RNA, signaling pathways

Introduction

Hepatocellular carcinoma (HCC) is the third most common cause of death from cancer worldwide, ranking as the fifth most common cancer in men and the seventh in women [1]. Since HBV is the most frequent underlying cause of HCC, HCC risk is remarkably increased in patients with higher levels of HBV replication [2]. HBx protein, encoded by the HBV X gene, is a multifunctional protein that can modulate cellular transcription, protein-degradation, cell cycle, and apoptotic pathways [3]. Many studies have suggest that the HBx protein is important for HBV replication [4-6], and Melegari et al confirmed that HBx stimulates HBV replication via its transactivation function [7]. Taking together, HBx activities significantly contribute to the development of HBV-related HCC.

The BubR1 gene is a functional orthologue of the yeast mitotic checkpoint gene Mad3 [8]. The BubR1 protein, a critical component of the mitotic spindle checkpoint encoded by the BubR1 gene, plays an important role in preventing premature separation of sister chromatids and ensuring proper chromosome segregation [9, 10]. Suijkerbuijk et al indicated that BubR1 dysfunction may cause chromosome segregation errors, resulting in chromosomal instability (CIN) and tumor occurrence [9]. In several human cancers, such as lung carcinoma [10-12], gastric carcinoma [13], renal carcinoma [14] and bladder carcinoma [15], BubR1 expres-
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Expression has been found up-regulated, thereby contributing to tumor growth. Our preliminary study indicates that BubR1 is overexpressed frequently in hepatocellular carcinoma (HCC), especially in HBV-related HCC [16]. Additionally, our clinical work also confirms that the up-regulation of BubR1 is significantly associated with tumor size, tumor grade and poor prognosis, suggesting the important role for BubR1 in hepatocellular carcinoma progression [16].

Many studies have shown that a multiple of signaling pathways can be activated in HBV-related HCC, such as nuclear factor-κB (NF-κB) [17] and Mitogen-activated protein kinases (MAPK) [18]. Specifically, NF-κB acts as a critical role in cancer development and provides a mechanistic link between inflammation and cancer [19]. And MAPK is known to regulate diverse cellular programs including proliferation, differentiation, apoptosis and stress response [20]. Currently, four different MAPKs have been identified: extracellular signal-regulated kinase 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK), p38, and ERK5. ERK1/2 pathway is mainly involved in cell growth and differentiation, while JNK and p38 pathways participate in stress reactions such as inflammation and apoptosis [21].

Until now, it is still unclear about the biological functions of BubR1 in HBV-related HCC. In this study, we aimed to investigate the effects of BubR1 silencing on the proliferation and apoptosis in HBV-related HCC cells, explore the interaction between BubR1 and HBx and clarify the underlying mechanism.

Materials and methods

Cell lines and culture

Human hepatocellular carcinoma cells (HepG2, SMMC7721, Huh7 and HepG2.2.15) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The HBV-unrelated hepatocellular carcinoma cells (HepG2, SMMC7721 and Huh7) were routinely cultured in Dulbecco’s modified Eagle’s medium (DMEM, Solarbio, Shanghai, China) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 100 μg/mL streptomycin and 100 U/mL penicillin. The HBV-unrelated HepG2.2.15 cells were maintained in DMEM with 10% FBS and 380 μg/ml antibiotic G-418 sulfate (Solarbio, Shanghai, China). All cells were incubated at 37°C with 5% CO₂.

RNA interference (RNAi)

Three siRNAs targeting BubR1 were designed and synthesized by Invitrogen (Shanghai, China). The non-targeting siRNA (Invitrogen) served as negative control was also designed. Cells were cultured and transfected using Lipofectamine 2000 (Invitrogen) when they were 70% confluent. Transfection was performed according to manufacturer’s instructions with 50 nM concentration of siRNAs. Transfected cells had been incubated for 24 h or 48 h before the extraction of RNA or protein. Sequences of specific BubR1-siRNAs and negative control siRNA Targeting were as follows: siRNA-BubR1-1 Positive-sense strand: 5’-GUC-CCUACCAGAGUU AAGUTT-3’. Anti-sense strand: 5’-ACUUAAUCUGGUAGGGACCTT-3’. siRNA-BubR1-2 Positive-sense strand: 5’-CUCACCAACAG-UGAAAUATT-3’. Anti-sense strand: 5’-AUAAUUA CACUGUGUUGGT-3’. siRNA-BubR1-3 Positive-sense strand: 5’-GGUGCUCUCUGAA-AUUATT-3’. Negative control, positive-sense strand: 5’-UUCUCCGAACGUGUCAGU-3’. Anti-sense strand 5’-ACGUGAGU CGAACCC-3’.

Real-time PCR analysis

Total RNA was extracted from cells using Trizol reagent (Invitrogen) according to manufacturer’s instructions. Then RT-PCR was performed using the PrimeScript RT reagent Kit (Takara, Japan). Amplification of BubR1 cDNA in the RT-PCR assay was achieved with the primers: forward 5’-GCACCGACAATTCCAAGCTC-3’ and reverse 5’-TGTGCTTCGTTGTGGTACAGA-3’ (annealing temperature: 53°C). As a loading control, GAPDH was amplified in a parallel reaction, with the following primers: forward 5’-TGGCCGAGGCTCAAGCTG-3’ and reverse 5’-TGATGCTTCGTTGTGACACT-3’. The RT-PCR products were separated by electrophoresis in 1.5% agarose gel and the imaging was performed using UVP imaging device.

Western blot analysis

Cells were lysed in lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and complete protease inhibitors. BCA protein assays (Beyotime, Shanghai, China) of cell lysates were performed to determine the protein concentration and ensure equal loading of
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lanes. Lysate samples were separated by 10%-12% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, USA). The membranes were then blocked with 5% BSA for 2 h and incubated with the primary antibodies at 4°C overnight. After incubation with horseradish peroxidase-conjugated secondary antibodies for 2 h, the blots were visualized by chemiluminescence (Beyotime, Shanghai, China), the intensity of each band was analyzed and quantitated by Image J software. The primary antibodies applied to the Western blots were: rabbit anti-BubR1 (ab183496, Abcam, 1:10000), mouse anti-IgG (Proteintech, 1:10000), mouse anti-β-actin (Proteintech, 1:15000). Rabbit anti-ERK1/2 (1:1,000), rabbit anti-Phospho-ERK1/2 (1:1,000), rabbit anti-p38 (1:1,000), rabbit anti-Phospho-p38 (1:1,000), rabbit anti-JNK (1:1,000), rabbit anti-Phospho-JNK rabbit anti-NFκB (1:1,000), and rabbit anti-phospho-NFκB (1:1,000) were purchased from Cell Signaling Technology (Danvers, USA). Rabbit anti-Bax (1:500) and rabbit anti-Caspase3 were purchased from ImmunoWay Biotechnology (Newark, USA).

Cell proliferation assay

Cells were seeded in 96-well microplates at a density of 4×10³ cells per well. MTT was added to each well and incubated for 4 h at 37°C, then discard the supernatant and add dimethyl sulfoxide (DMSO) to dissolve the MTT formazan. The absorbance was measured at a wavelength of 490 nm. Cell proliferation was measured daily over a 3-day period.

Colony formation assay

Cells were seeded in 6-well plates at a density of 1,000 cells per well. After incubation for 14 days, discard the supernatant and wash the cells twice by PBS. Then cells were fixed in 10% methanol for 15 min and stained with Giemsa for 20 min. Colonies consisting of >50 cells were scored and the rate of colony formation was calculated.

Flow cytometry analysis

Cells were seeded in 6-well plates at a density of 1×10⁶ cells per well. Cells were collected 24 h after transfection. Cell cycle assay and apoptosis assay were performed according to manufacturer’s instructions using the Cell Cycle Analysis Kit (MultiSciences, Hanzhou, China), Annexin-V/FITC kit (Bestbio, Shanghai, China), respectively. Finally, cells were analyzed on a FACScan flow cytometer (BD Biosciences).

Co-Immunoprecipitation assay

Cell lysates were prepared by extracting cells with RIPA buffer (Beyotime, Shanghai, China) and incubated with various primary antibodies (mouse anti-HBx monoclonal antibody, rabbit anti-BubR1 monoclonal antibody, and mouse anti-IgG) at 4°C overnight. The immune complexes were precipitated using protein A+G Sepharose (7 seabiotech, China) and analyzed by western blotting.

Immunofluorescence assay

Cells were seeded on coverslips, fixed with 100% methanol for 30 minutes. Fixed cells were blocked with 5% bovine serum albumin (BSA) for 30 min, followed by incubation with primary antibodies (mouse anti-HBx monoclonal antibody (1:100) and rabbit anti-BubR1 monoclonal antibody (1:100) at 4°C overnight and probed with fluorescein-conjugated antibody (1:100, Earthox, USA). The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Fluorescence images were captured under confocal laser microscopy (Leica, Germany).

Statistical analysis

Data are presented as means ± standard deviation (SD). Student’s t-test was used for comparison between two groups and one-way analysis of variance (ANOVA) was used for comparison among multiple groups. All statistical calculations were performed with SPSS 19.0 software and statistical significance was at P<0.05.

Results

BubR1 is overexpressed in HBV-related HCC cells

We first examined the expression of BubR1 in a panel of human hepatocellular carcinoma cell lines (HepG2, SMMC7721, Huh7 and HepG2.2.15). As shown in Figure 1A and 1B, HepG2.2.15 cells exhibited the highest BubR1 mRNA and protein expression levels among the four HCC cell lines, suggesting that the expression of BubR1 in HBV-related HCC cells
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(HepG2.2.15 cells) was higher than in HBV-unrelated HCC cells (HepG2, SMMC7721 and Huh7). Given the higher endogenous BubR1 expression levels, HBV-related HepG2.2.15 cells were chosen for subsequent experiments.

**BubR1 expression is knocked down by siRNA in HepG2.2.15 cells**

Next, HepG2.2.15 cells were transfected with three siRNAs targeting BubR1 (BubR1-1, BubR1-2, BubR1-3) and scrambled control siRNA using lipofectamine 2000. Significant down-regulation of the BubR1 mRNA and protein levels was observed in 24 h (Figure 2A) and 48 h (Figure 2B) after transfection. RT-PCR and Western blot consistently showed three siRNAs (BubR1-1, BubR1-2 and BubR1-3) reduced the BubR1 expression levels with different efficacy in HepG2.2.15 cells. BubR1-3 was used for subsequent analyses due to the highest efficiency in BubR1-3 transfected cells.

**Down-regulation of BubR1 inhibits proliferation and colony formation in HepG2.2.15 cells**

The MTT assay showed that the proliferation rate of BubR1-3 transfected cells was significantly lower than control cells, suggesting that BubR1 silencing effectively reduced the proliferation rate of HepG2.2.15 cells (P<0.01; Figure 3A, 4A). The colony formation assay showed that down-regulation of BubR1 by BubR1-3 significantly suppressed colony formation of HepG2.2.15 cells compared to cells transfected with control siRNA (P<0.01; Figure 3B, 4B). Down-regulation of BubR1 induces cell cycle arrest and apoptosis in HepG2.2.15 cells cell cycle analysis indicated that BubR1 silencing caused an inhibition of cell cycle progression and an S phase arrest (Figure 3C). Specifically, in BubR1-3 transfected cells, the percentage of cells in S phase significantly increased, whereas cells in G1 phase decreased compared to control cells (Figure 4C). Quantification of apoptosis by annexin-V/PI double labeling indicated that apoptotic index of BubR1-3 transfected cells was remarkably higher than control cells (Figure 3D, 4D).

**BubR1 regulates multiple signaling pathways associated with tumor progression**

To explore the mechanism of BubR1 activities, we examined the activation of MAPKs and...
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Figure 3. BubR1 silencing inhibits the proliferation of HepG2.2.15 cells. A: MTT assay showing that transfection with BubR1-3 significantly reduced the proliferation of HepG2.2.15 cells compared to transfection with control siRNA. **P<0.01 versus control. B: Quantitative colony formation rates of HepG2.2.15 were calculated as percentages of total seeded cells. **P<0.01 versus control. C: Cell cycle distribution in Normal group, Control-siRNA group and BubR1-3 group of HepG2.2.15 cells stained with PI. D: Representative flow cytometric analysis of early apoptosis of HepG2.2.15 stained with annexin-V and PI. Early apoptotic cells were increased in BubR1-3 transfected cells compared with control cells.
NF-κB after silencing BubR1 by Western blot. There were significant decrease in levels of phosphorylated ERK1/2 and NF-κB protein in BubR1-3 transfected cells compared to the control cells with total protein amounts of ERK1/2 and NF-κB little affected (Figure 5). However, there were no significant differences in phosphorylated JNK and p38 protein expression between BubR1-3 transfected cells and control cells. We also found that the protein levels of the two pro-apoptotic regulators, Bax and Caspase-3, were markedly up-regulated in BubR1-3 transfected cells compared to the control cells (Figure 5).

**Interaction between BubR1 and HBV X protein (HBx)**

In co-Immunoprecipitation assay, cell lysates were immunoprecipitated with either an anti-mouse IgG antibody or a mouse anti-HBx antibody, followed by western blotting using a rabbit anti-BubR1 antibody. BubR1 was detected in immune complexes obtained with the anti-HBx antibody, but not with anti-mouse IgG (Figure 6A). The reciprocal immunoprecipitation experiment using anti-BubR1 antibody also confirmed the binding of HBx and BubR1 in HepG2.2.15 cells.

Further immunofluorescence assay was performed to explore the distribution interaction between BubR1 and HBx. As shown in Figure 6B, distribution of the two proteins was observed both in cytoplasm and nucleus. Notably, a portion of BubR1 co-localized with HBx at the core of nucleus in HepG2.2.15 cells. Co-localization of HBx and BubR1 further confirmed the interaction between HBx and BubR1.
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Discussion

Elevated expression of BubR1 has been reported in many human cancers, including lung cancer [10, 11], gastric cancer [13], renal cancer [14], bladder cancer [15], breast cancer [22] and esophageal cancer [23]. Our preliminary study also indicates that BubR1 is overexpressed in hepatocellular carcinoma (HCC), especially in HBV-related HCC, and the up-regulation of BubR1 predicts a poor overall survival in patients with HCC [16]. As gene expression is regulated at multiple levels [24-30], how HBV affect global gene expression of the host remain largely unknown. In this study, We found out that HBV-related HepG2.2.15 cells exhibit the highest BubR1 mRNA and protein expression levels among the four HCC cell lines, suggesting that BubR1 over-expression may be regulated by HBV infection at the transcriptional level.

To uncover the role of BubR1 in the development of HBV-related HCC, we employed a loss-of-function approach to assess the effects of BubR1 down-regulation on the growth and survival of HepG2.2.15 cells. SiRNA mediated down-regulation of BubR1 significantly inhibited proliferation and colony formation, indicat-
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BubR1 is required for HCC cells growth. Besides suppressive proliferation, induction of cell cycle arrest is also an important mechanism for controlling tumor growth. Our results additionally demonstrate that BubR1 down-regulation caused a significant decrease in the percentage of G0/G1-phase cells, and a corresponding increase in the percentage of S-phase cells, indicative of an S-phase cell cycle arrest. Recently a study suggests that HBx inhibits S phase entry, progression beyond G1 phase and into S phase inhibits HBV replication [31]. And inhibition of HBV replication in S phase was previously observed in HepG2.2.15 cells [32]. Then we hypothesized that S-phase cell cycle arrest induced by BubR1 knock-down may result in an inhibition of HBV replication, opposed to the effects of HBx in cell cycle progress.

In addition to induction of cell cycle arrest, BubR1 down-regulation was also found to promote apoptosis in HepG2.2.15 cells. In concordance with annexin-V/PI double labeling analysis, Western blot analysis revealed that the Bax and Caspase-3 protein level were increased in BubR1-3 transfected cells compared to the control cells. Bax, a pro-apoptotic regulator belongs to the Bcl-2 protein family, is expressed abundantly and selectively during apoptosis, promoting cell death [33]. Caspase-3 is a crucial executioner of cell apoptosis in caspase signaling [34]. These observations strongly suggest that the increased apoptosis after BubR1 silencing in HepG2.2.15 cells is associated with alteration of the Bcl-2 family and the caspase signaling.

Many studies have shown that both NF-κB and MAPK/ERK1/2 pathway can be activated by HBx in HBV-related HCC [17, 18]. Herein, we found significant decrease of phosphorylated NF-κB and ERK protein in BubR1-3 transfected cells compared to control cells. Since NF-κB acts as a critical promoter of tumor, inhibition of NF-κB activity by chemical reagents has yielded anti-inflammatory and anticancer effects [35]. Additionally, NF-κB activity is known to promote proliferation [36] and suppress apoptosis [37-39], the significant decreased cell proliferation and increased apoptosis in our study may be partially regulated by inhibition of NF-κB activity after BubR1 silencing. Besides NF-κB, MAPK/ERK1/2 is also inhibited after BubR1 silencing, while JNK and p38 MAPK pathways are not affected. MAPK/ERK 1/2 is mainly involved in cell proliferation and aberrant activation of the ERK pathway has been shown to be a critical feature to many types of human tumors [40, 41]. Kohno et al.

Figure 6. Interaction between TLR4 and HBx. A: HBx interacts with BubR1. Lysates of HepG2.2.15 were immunoprecipitated (IP) with either anti-IgG, anti-HBx or anti-BubR1 antibody. The precipitates were analyzed by western blotting (WB) with anti-BubR1 antibody (top) and with anti-HBx antibody (bottom). B: HBx co-localizes with BubR1 at the nucleus in HepG2.2.15 cells. HepG2.2.15 cells were fixed with 100% methanol and immunofluorescence staining was carried out using rabbit anti-BubR1 (FITC) and mouse anti-HBx (TRITC) antibodies with DAPI staining (blue). Arrows in the inset indicate nucleus co-localization in HepG2.2.15 cells.
suggest that inhibition of the ERK pathway is an attractive strategy for cancer treatment [42]. Given the regulatory role in the activation of the NF-kB and MAPK/ERK1/2 pathways, we assume that the anticancer effects of BubR1 down-regulation is mediated, at least partially, through the NF-kB and MAPK/ERK1/2 signaling pathways in HBV-related HCC.

HBx is a multifunctional protein and plays a critical role in the development of HCC [43, 44]. Specifically, HBx can activate hepatoma cell proliferation [45], prevent apoptosis of hepatoma cells [46], induce quiescent hepatocytes to stall in G1 phase of the cell cycle [47] and regulate multiple intracellular signaling pathways. Having confirmed the expression of BubR1 in HBV-related HepG2.2.15 cells was higher than that in HBV-unrelated HCC cells, we hypothesized that BubR1 may interacts with HBx. Kim S et al demonstrates that HBx can interact with the BubR1 and interfere with the binding of BubR1 to CDC20, resulting in mitotic slippage and an accumulation of aberrant chromosomes [48]. In this study, we consistently found that BubR1 binds to HBx and co-localizes with HBx at the nucleus in HepG2.2.15 cells. Combining with other results, we hypothesized that BubR1 may act synergistically with HBx to promote the progress of HBV-related HCC. Further experimental data are needed to elucidate the exact mechanisms of how HBx affects on BubR1 and the biological significance of their binding.

In summary, our results indicated that BubR1 is required for the growth and survival of HBV-related HCC cells. And the tumor-promotion effect of BubR1 is mediated, at least partially, through the NF-kB and MAPK/ERK1/2 signaling pathways. Thus, BubR1 may serve as a promising therapeutic target for HBV-related HCC.

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Disclosure of conflict of interest

None.

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