Downregulation of MicroRNA-145 Caused by Hepatitis B Virus X Protein Promotes Expression of CUL5 and Contributes to Pathogenesis of Hepatitis B Virus-Associated Hepatocellular Carcinoma

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Key Words
MiR-145 • CUL5 • HBX • HCC • Cell cycle • Apoptosis

Abstract
Background: Hepatitis B viral infection-induced hepatocellular carcinoma (HCC) is a major threat to human health in China. Hepatitis B virus X protein (HBX), an HBV protein, has been reported to be involved in regulating the cellular activities of the host cells and is responsible for HCC oncogenesis. Methods and Results: In this study, we performed real-time PCR in tumor tissue samples collected from 53 HCC patients (25 HBV-positive cases and 28 HBV-negative cases) to screen the candidate miRNAs that have previously been reported to be aberrantly expressed in HBV-associated HCC and found that miR-145 was significantly downregulated. The following computational analysis identified CUL5 and RAB5C as virtual targets of miR-145, whereas only CUL5 was verified as a validated target gene of miR-145 in liver cells via luciferase reporter assay. In line with this result, we found that both the mRNA and protein expression levels of CUL5 were significantly higher in HBV-positive than in HBV-negative HCC. An in vitro experiment demonstrated a significant decrease in the expression of miRNA-145, a substantial increase in the mRNA and protein expression of CUL5, and an enhanced proliferation of HBX over-expressing HepG2 cells compared with the control. In HepG2.2.15, we found significant decreases in both the expression of CUL5 and the cell growth rate of H cells transfected with 60 nM miR-145 mimics compared with the scramble controls. Conclusion: HBV infection promotes cell growth, at least partially, through the HBX-induced downregulation of miRNA-145 expression, which is responsible for the oncogenesis of HBV-associated HCC.
Cellular Physiology and Biochemistry

Introduction

Hepatocellular carcinoma (HCC) is the leading cause of primary malignant liver tumors, is the third most common cause of cancer-associated death worldwide, and represents a major and global threat to human health [1]. Several risk factors, including infection with hepatitis C virus (HCV), hepatitis B virus (HBV), alcoholism and other liver diseases substantially contribute to the development of HCC [2]. Among these risk factors, HBV is the most common cause of HCC worldwide and is responsible for the increasing incidence of the disease [1, 2].

HBV-encoded X protein (HBX), a 17-kD protein encoded by the 'X' open reading frame of the hepatitis B oncogenic viral genome, is a key regulator in the development of HBV-associated HCC, as evidenced by its ability to transactivate oncogenes or tumor suppressive genes that are functionally associated with cellular activities such as apoptosis, signaling, and cell growth [3-5]. In vivo and in vitro experiments demonstrated that HBX is indispensable for in vivo infection and that it promotes the replication of the virus via facilitating mitochondrial calcium uptake. HBX also significantly contributes to HCC tumorigenesis due to its critical role in the initiation of neoplastic transformation [6-8]. In line with this, HBX transgenic mice showed a significantly elevated incidence of hepatitis, steatosis, and dysplasia, as well as HCC in the liver [9-11]. HBV DNA was exclusively integrated into the genome of the HBV-associated HCC tumor tissues in individuals who were positive for the HBX transcript but negative for the HBV surface antigen (HBsAg), thereby suggesting the role of HBV in genome integration and malignancy transformation [12]. Several studies showed that HBX can promote proliferation, motility and invasion in human hepatocytes by up-regulating MEKK2, MIG, MMP-9, I KKα and Capn4 [13-17]. HBX has also been shown to be involved in controlling tumor growth via modifying cell cycle progression by regulating cell cycle regulatory protein in a calcium-dependent manner [18, 19]. Aberrantly expressed microRNAs (miRNAs) are also increasingly believed to be functionally involved in the control of various biological processes that are involved in cancer development, such as tumorigenesis, metastasis and proliferation, by negatively regulating the expression of oncogenes or tumor suppressive genes [20]. The role of miRNAs in the pathogenesis of HCC has been extensively investigated, and the dysregulation of miRNAs appear to contribute to HCC development [20, 21].

miRNAs have recently been reported to mediate HBX-induced cell proliferation and invasion in HCC [22–26]. Peng et al. reported on differentially expressed miRNAs using a microarray analysis of the expression profiles of HepG2 and HepG2.2.15 cells, which represent a well-known HBX-secreting HCC cell line [27]. In this study, we screened the candidate miRNAs that were previously reported to be aberrantly expressed [27] to identify the miRNA (and its target gene(s)) that mediate the effect of HBX in HCC development.

Materials and Methods

Patient samples

A total of 53 tumor tissue samples were collected from 53 histologically confirmed HCC patients who underwent surgical resection at the Second Hospital of Hebei Medical University, Department of Hepatobiliary Surgery. Among them, 25 patients were positive and 28 patients were negative for HBV infection. The specimens were immediately stored at -80°C for future use. Clinicopathological data were obtained from patient records and are described in Table 1. The patients who received radiotherapy or chemotherapy prior to surgery were excluded from the study. Informed consent was obtained from each patient, and the study protocol was approved by the Institute Research Ethics Committee at Hebei Medical University.

Cell Culture and Transfection

The human HCC cell lines HepG2 and HepG2.2.15 were obtained from the ATCC (American Type Culture Collection, Manassas, VA, USA) and maintained in Dulbecco’s modified Eagle’s medium supplemented with
10% fetal bovine serum containing penicillin-streptomycin antibiotics (Invitrogen, Carlsbad, CA, USA) at 37 °C. For transfection with plasmid DNA or oligos, miR-145 mimics were purchased from Ambion (Austin, TX, USA), and the full length of the coding sequence of HBX was amplified and inserted into pcDNA 3.0. The cells were seeded in 6-well plates at a density of 1x10^6 cells/well. After 24 hours, the transfection was conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), per the manufacturer’s instructions. The cell lysates were harvested and analyzed via western blot and total RNA isolation. All transfection experiments were repeated at least three times.

**RNA isolation and real-time PCR**

The total RNA of cells (or liver tissues) was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Real-time PCR was performed to determine the expression levels of miR-145, CUL5, RAB5C and U6 (an internal control) using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions. The expressions of specific genes or miRNAs were normalized using U6, and the expression levels were expressed via the comparative Ct method using $2^{\Delta\Delta Ct}$.

**Luciferase reporter gene assay**

The wild type full length 3'-untranslated regions (3'-UTRs) of human CUL5 and RAB5C were amplified via PCR. Then, the amplified fragment was inserted into psiCHECK-2 vector and the sequence accuracy was confirmed via direct Sanger sequencing. Subsequently, the predicted “seed sequences” of miR-145 in the 3'-UTR of CUL5 and RAB5C were replaced with the complementary sequences (in RAB5C, 50-56 bp in 3'-UTR of RAB5C, “ACUGGAA” were replaced with “UGACCUG”; in CUL5, 574-580 bp in 3'-UTR of CUL5, “AACUGGA” were replaced with “UUGACCU”) using the QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer’s protocol. Verification was performed via direct Sanger sequencing.

For the luciferase assay, HepG2 cells were seeded on 24-well plates and transfected with 100 ng of luciferase reporter vectors (both wild type and mutant) and 30/60 nM of miR-145 mimics or scramble controls. At 48 hours after transfection, firefly and Renilla luciferase activities were determined using the Dual-Luciferase Reporter Assay (Promega, Fitchburg, WI, USA).

**Cell growth assay**

Cells were seeded into 96-well plates at a density of 3x10^4 cells per well, and cell proliferation was evaluated 48 h after the transfection using the Cell Counting Kit-8 (CCK-8) (Dojindo, Kumamoto, Japan) following the manufacturer’s instruction. The experiments were carried out at least three times in triplicate wells.

**Cell cycle and apoptosis analysis**

At 48 h after transfection, HepG2 cells were digested, washed with phosphate-buffered saline (PBS), and fixed in 70% ethanol. Cell cycle analyses were performed using propidium iodide (PI) staining. A
FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) was used to determine the G0/G1, S, and G2/M fractions.

For apoptosis analysis, the cells were harvested and resuspended in PBS 48 h post-transfection and then fixed in ethanol under room temperature overnight. The cells were washed with PBS and resuspended in staining solution (50 mg/ml propidium iodide, 1 mg/ml RNase A and 0.1% Triton X-100 in PBS, all purchased from Invitrogen, Carlsbad, CA, USA). The stained cells were then analyzed for apoptosis via FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Western blot

The cell or tissue samples were collected and lysed in lysis buffer containing 50 mmol/l Tris-HCl (pH 8.5), 150 mmol/l NaCl, 0.2 g/l NaN3, 0.1 g/l SDS, 100 μg/ml phenylmethylsulfonylfluoride, 1 μg/ml aprotinin, 10 ml/l NP-40, and 5 g/l sodium deoxycholate (all purchased from Sigma-Aldrich, St. Louis, MO). A total of 30-50 μg of protein was loaded onto sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and the separated protein was then transferred to polyvinylidene fluoride membranes, following by being blocked in 5% non-fat milk for 2 h. Subsequently, the membrane was incubated with primary antibodies raised against HBX (1:1,000), CUL5 (1:1,500), β-actin (1:10,000) and RAB5C (1:2,000) (obtained from Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at 37˚C and incubated overnight at 4˚C, followed by a 1-h incubation with the appropriate horseradish peroxidase-conjugated monoclonal secondary antibody. The bands were visualized using an enhanced chemiluminescence (ECL) detection system (Thermo Fisher Scientific Inc., Rockford, IL, USA).

Statistical analysis

The statistical analysis was performed using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). The data are expressed as the means (M) ± standard deviation (SD). The gene expression levels were compared using the independent Student’s t test between two groups or via one-way ANOVA if more than two groups were involved.

Results

In this study, we performed real-time PCR on tumor tissue samples collected from 53 HCC patients (25 HBV-positive cases and 28 HBV-negative cases) to screen the candidate miRNAs that have previously been reported to be aberrantly expressed in HBV-associated HCC [27], including miR-152, -143, -192, -19b, -18b, -148a, -345, -145, -122, -210, -18a and -20b. This was performed to identify the miRNA and its target gene(s) that mediate
the effect of HBX in the development of HCC. We identified that miR-145 was significantly downregulated in tumor tissue samples collected from HBV-positive HCC compared with the HBV-negative HCC, as shown in Fig. 1. Due to the reduction in the expression of miR-145 in HBV-related HCC, we hypothesized that the target genes that mediate the effect of miR-145 in the development of HBV-related HCC should be oncogenes and that the downregulation of miR-145 released the physiological inhibition of the oncogenes and promoted tumorigenesis. We subsequently searched the online miRNA database (www.targetscan.org) and identified two potential targets of miR-145, RAB5C and CUL5, both of which showed perfect matches with mature miR-145 and highly conserved among species.

To validate the regulatory association between miR-145 and the predicted targets, we subcloned both the wild type and mutant full length 3'-UTR of CUL5 and RAB5C into psiCHECK and performed luciferase reporter assay in HepG2 cells. We found that the
cells cotransfected with wild-type CUL5 3’-UTR and miR-145 had a significantly lower luciferase activity than the scramble controls, whereas the cells transfected with mutant CUL5 3’UTR and miR-145 showed comparable luciferase activity as the controls (A); While both the wild-type and mutant RAB5C 3’-UTR had similar luciferase activity compared with the scramble controls (B).

Fig. 4. Both mRNA (A) and protein (B and C is densitometry analysis of western blot results of B) expression levels of CUL5 were significantly higher in HBV-positive HCC than in HBV-negative HCC.
and transfected it into HepG2 cells. HBX expression was assessed in HBX-transfected cells. The HBX protein was expressed at high levels in HBX-transfected HepG2 cells (Fig. 5A); however, no expression was detected in the empty vector-transfected cells. Next, the effect of HBX over-expression on the intracellular expression of miRNA-145 and CUL5 was studied in HepG2 cells. The cells were transfected with HBX or empty vector, and the cells were collected either for real time PCR or western blot 48 hours after transfection. A significant decrease was observed in the expression of miRNA-145 and a substantial increase in the mRNA and protein expression of CUL5 in HBX over-expressing HepG2 cells compared with empty vector-transfected cells, respectively (Fig. 5B-D). Additionally, the proliferation was analyzed in HBX over-expressing cells using a CCK-8 assay as described in the Materials and Methods section. The proliferation of HepG2 cells transfected with HBX plasmid markedly increased (Fig. 6A) compared with the cells transfected with control plasmid (empty vector). To explore the underlying molecular mechanism, we performed a flow cytometry analysis and found that the overexpression of HBX significantly increased the G2/M fraction, decreased the G0/G1 fraction (Fig. 6B), and significantly suppressed apoptosis (Fig. 6C and D) compared with the cells transfected with empty vector.

We subsequently investigated the effect of the overexpression of miR-145 on the expression of CUL5 and the proliferation and cell cycle status in HepG2.2.15. There was a significant decrease in the expression of CUL5 in the HepG2.2.15 cells transfected with 60 nM miR-145 mimics (Fig. 7). The proliferation of HepG2.2.15 cells transfected with miR-145 mimics significantly decreased (Fig. 8A) compared with the cells that were transfected with the scramble controls. The flow cytometry analysis showed that the overexpression of miR-145 significantly decreased the G2/M fraction, increased the G0/G1 fraction (Fig. 8B), and significantly induced apoptosis (Fig. 8C and D) compared with the cells that were transfected with the scramble controls.
Fig. 6. A. The proliferation of Hep G2 cells transfected with HBX plasmid markedly increased; B. The overexpression of HBX significantly increased fraction of G2/M and decrease G0/G1 fraction as compared with the cells transfected with empty vector; C. The apoptosis status of the Hep G2 cells transfected with empty vector; D. The apoptosis status of the Hep G2 cells transfected with HBX.

Fig. 7. A significant decrease in the mRNA (A) and protein (B) expression of CUL5 in the Hep G2.1.15 cells transfected with miR-145 mimics 60 nM.
Discussion

It has been estimated that less than 2% of the human genome involves protein-coding sequences and that more than 90% of the human genome is transcribed into non-coding RNA (ncRNA), which includes miRNAs. miRNAs are believed to be an essential regulator in a wide range of human biological processes such as proliferation, apoptosis, cell cycle, and metastasis [28]. miRNAs may function as oncogene- or tumor-suppressive genes depending on the target genes. Alterations in miRNA expression have been intensively studied in HCC and have been linked to the molecular mechanism underlying HCC development. The HBX protein is highly conserved among the subtypes of the virus and is universally shared by all mammalian members of Hepadnaviridae [29]. The interactions between HBX and miRNAs have been repeatedly investigated. Zhang et al. reported that HBX overexpression increased the expression of fucosyltransferase by suppressing the expression of miR-15b [30]. The expression of miR-205 was found to be significantly reduced in the liver of HBX-transgenic mice, and the ectopic expression of HBX can abrogate the effect of miR-205 as a tumor suppressive gene [31]. Another study by Cui et al. showed that miR-224 expression was significantly upregulated in HBX-transgenic mice and that the introduction of miR-
224 promoted tumor formation in HCC via targeting Smad4 [32]. HBX-mediated miRNA expression alternation may function as an essential regulator in hepatocarcinogenesis [33]. miRNAs have recently been reported to mediate HBX-induced cell proliferation and invasion in HCC [22–26]. Peng et al. reported many miRNAs that were differentially expressed between HepG2 and HepG2.2.15 cells based on a microarray analysis of expression profiles [27]. In this study, we performed real-time PCR in tumor tissue samples collected from 53 HCC patients (25 HBV-positive cases and 28 HBV-negative cases) to screen candidate miRNAs including miR-152, -143, -192, -19b, -18b, -148a, -345, -145, -122, -210, -18a and -20b. miR-145 was significantly downregulated in the tumor tissue samples collected from HBV-positive HCC compared with HBV-negative HCC (Fig. 1).

MicroRNA-145 (miR-145), a 22-nt miRNA, has been mapped to chromosome 5q in the human genome and functions as a tumor suppressor in variety of cancers such as lung adenocarcinoma, bladder cancer, and colon cancer [34-36]. miR-145 was significantly downregulated in various cancer types [37] and showed an inhibitory effect on the proliferation of human cancer cells [38]. In HCC, the expression of miR-145 was significantly downregulated and was associated with poor histological grade and prognosis in HCC [39]. In this study, we searched the online miRNA database (www.targetscan.org) and identified two potential targets of miR-145, RAB5C and CUL5, both of which showed a perfect match with mature miR-145 and were highly conserved between species (Fig. 2). The following luciferase assay validated CUL5, rather than RAB5C, to be a target of miR-145 in HepG2 cells, as shown in Fig. 3. Additionally, both the mRNA and protein expression levels of CUL5 were significantly higher in HBV-positive HCC than in HBV-negative HCC; the expression levels of RAB5C were comparable between the two groups (data not shown).

As a member of the Cullin-RING E3 ubiquitin family, Cullin-5 (CUL5) has been reported to be functionally involved in numerous cellular activities including the cell cycle and apoptosis [40, 41]. CUL5 is connected to SOCS-box proteins and an E2 ubiquitin-conjugating enzyme by binding to adaptor proteins such as elongins [42]. The complex of CUL5, elongin A and the elongin BC complex can efficiently polyubiquitinate the poly II subunit, Rpb1, to influence the cell cycle [43, 44]. In addition to its role in controlling the cell cycle, CUL5 was also reported to be involved in regulating apoptosis by modulating the phosphorylation of mitogen-activated protein kinase (MAPK) and induce p53 mRNA and protein expression [45]. The activation of tumor suppressor gene p53, which is caused by the reduction of CUL5, is closely related to the cell apoptosis status [46], indicating that CUL5 may be associated with the apoptosis of human cells. In this study, we transfected HepG2 cells with pcDNA 3.0 with or without HBX and found a significant decrease in the expression of miRNA-145 and a substantial increase in the mRNA and protein expression of CUL5 in HBX over-expressing HepG2 cells compared with empty vector-transfected cells. The proliferation of HepG2 cells transfected with HBX plasmid markedly increased compared with cells transfected with empty vector. Additionally, the flow cytometry analysis showed that the overexpression of HBX significantly increased the G2/M fraction, decreased the G0/G1 fraction, and significantly suppressed apoptosis compared with the cells transfected with empty vector. We also found a significant decrease in the expression of CUL5 in the HepG2.2.15 cells transfected with 60 nM miR-145 mimics. The proliferation of HepG2.2.15 cells transfected with miR-145 mimics significantly decreased compared with cells transfected with the scramble controls, and the flow cytometry analysis showed that the overexpression of miR-145 significantly decreased the G2/M fraction, increased the G0/G1 fraction, and significantly induced apoptosis compared with the cells that were transfected with the scramble controls.

In summary, our data show that HBX at least in part induces cell proliferation via suppressing miRNA-145, which in turn releasing the inhibition of CUL5. Identifying miRNAs that are key initiators of cancer development represents an important step in understanding the molecular mechanism of tumorigenesis, as well as the development of new therapeutic or preventive targets.
Disclosure Statement

There is no conflict of interest.

References


