HBx protein-induced upregulation of microRNA-221 promotes aberrant proliferation in HBV-related hepatocellular carcinoma by targeting estrogen receptor-α

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Abstract. Hepatitis B virus X protein (HBx) plays an important role in the development of hepatocellular carcinoma (HCC). Emerging evidence has shown the association between aberrantly expressed miR-221 and cancer development; however, little is known concerning its potential role in hepatitis B virus (HBV)-related HCC. In the present study, functional studies demonstrated that HBx leads to the promotion of cell proliferation and cell growth viability. Obviously overexpressed miR-221 was found in HBx-transfected cells compared with the mock counterparts. Suppression of miR-221 significantly inhibited HCC cell proliferation. Western blot analysis indicated that estrogen receptor-α (ERα) was downregulated in HCC tissues and cell lines. Bioinformatic analysis combined with validation experiments identified ERα as a direct target of miR-221. The present study suggests that miR-221 modulates HCC cancer cell proliferation by suppressing ERα, functioning as a tumor promoter. Moreover, our data imply that miR-221 has potential as an miRNA-based therapeutic target for HBV-related HCC.

Introduction

Hepatocellular carcinoma (HCC) is a major health concern worldwide and is the main cause of death among cirrhotic patients (1). It is one of the most lethal malignancies in the world with more than half a million new cases diagnosed annually (2). Hepatocarcinogenesis is a complex and multistep process, which is associated with many risk factors (3). Epidemiological studies have demonstrated that hepatitis B virus (HBV) infection is one of the key risk factors for HCC (4), particularly in eastern Asia.

Hepatitis B virus X (HBx) protein, a 17-kDa protein encoded by the HBV genome, plays a critical role in the progression of HBV-related HCC (5,6). In addition, HBx protein can interfere with several signaling pathways that are associated with cell proliferation and apoptosis. However, the exact role of HBx protein in the progression of HCC remains to be determined. Investigation of the mechanism is important to clarify the tumorigenic pathway and is helpful for developing new biomarkers for early detection and accurate diagnosis.

MicroRNAs (miRNAs) are a class of ~21-25 nucleotide non-coding RNAs, which mediate the post-transcriptional regulation of mRNAs by interfering with mRNA stability or protein translation (7). They have been identified as being involved in biological processes, including cell proliferation, apoptosis as well as cell differentiation (8,9). Deregulation of miRNAs has been found to be essential in cancer development and progression (10). Growing evidence reveals that many miRNAs are involved in HCC tumorigenesis, including miR-221 (11,12). miR-221 upregulation indicates the activation of stellate cells and the progression of liver fibrosis (13).

Previous data indicate that estrogen receptor-α (ERα) has protective effects on HCC (14). The relationship between miR-221 and ERα was studied by Zhao *et al* (15). They found that miR-221 negatively regulates ERα expression by interaction with the 3'-untranslated region (3'UTR) of ERα in breast cancer. To date, however, the role of miR-221 in HBV-related hepatocarcinogenesis and the molecular mechanisms by which miR-221 modulates malignant phenotypes of HCC cells remain largely unknown.

In the present study, we found that upregulation of miR-221 was unregulated by HBx protein in HCC. The overexpression of miR-221 was found to induce cell proliferation. Finally, an inverse correlation between the expression of miR-221 and ERα was found in HCC. Our findings will help to determine the oncogenic role of miR-221 in hepatocarcinogenesis and the molecular mechanism of HBx protein in the progression of HCC.

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Materials and methods

Tissue specimens and cell culture. HepG2 and MCF-7 cells were purchased from the American Type Culture Collection. The HepG2.215 cell line, which persistently produces HBV due to the integrated HBV genome, was kindly provided by Professor Ailong Huang (Key Laboratory of Molecular Biology on Infection Diseases of the Ministry of Education, Chongqing Medical University). The cells were cultured in minimum essential medium (MEM; HyClone) supplemented with 10% (v/v) FCS, 2 mmol/l glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin-streptomycin solution in a 5% CO_2 incubator at 37˚C.

Fourteen formalin-fixed and paraffin-embedded (FFPE) hepatic tissue specimens were obtained from patients chronically infected with HBV at the First Affiliated Hospital of Chongqing Medical University and were confirmed as HCC by histopathological evaluation. All of the tissue specimens were obtained after consent was obtained by the patients.

Construction of the Ad-HBx adenovirus and the infection of HepG2 cells. Both the Ad-HBx and the control Ad-EGFP adenoviruses were constructed with the AdEasy system. HepG2 cells were plated into a 6-well plate in 2 ml of complete medium at a density of $1.5x10^5$ cells/well and incubated overnight until 30-50% confluency. The Ad-HBx and Ad-EGFP adenoviruses were respectively infected into HepG2 cells at an MOI of 50. The infection efficiency was evaluated by observation of EGFP expression under a fluorescence microscope after 48 h.

Semi-quantitative reverse-transcription PCR. After incubation for 48 h, cells infected with the adenovirus were harvested. Total RNA was then extracted using TRIzol reagent (Invitrogen) and quantified by spectrophotometry at OD260. First-strand cDNA was synthesized using oligo(dT)$_{12-18}$ primers and SuperScript II RNase H-Reverse Transcriptase (both from Invitrogen) according to the manufacturer's recommendations. The target cDNA was amplified using Platinum *Taq* DNA polymerase (Invitrogen) for 28-30 cycles. PCR was performed with the following primers: P1 and P2 for HBx, P3 and P4 for GAPDH: P1, 5'-ACCGACCTTGAGGCCTACTT-3'; P2, 5'-GC TTGGCAGAGGTGAAAAAG-3'; P3, 5'-GAGTCAACGGAT TTGGTCGT-3'; and P4, 5'-TTGATTTTGGAGGGATCT CG-3'. Each sample was then analyzed by electrophoresis, and the resulting gel was photographed using a gel documentation system (Bio-Rad).

miRNA isolation and quantitative reverse transcription-polymerase chain reaction. Total RNA was isolated from HepG2 cells using the mirVana PARIS kit (Applied Biosystems, Inc.) according to the manufacturer's instructions. Quantification of miR-221 was performed using the TaqMan miRNA assay kit (Applied Biosystems, Inc.) according to the manufacturer's instructions. U6 small nuclear RNA was used as an internal control for determining the relative miRNA expression level, followed by detection with the Applied Biosystems 7500HT Sequence Detection System (P/N: 4329002) (both from Applied Biosystems). In relation to the expression of small nuclear U6 RNA, the expression level of miR-221 for each RNA sample was calculated, reflected by the value of ΔCt (Ct of miR-221 - Ct of U6).

Western blot analysis. M-PER mammalian protein extraction reagent (Cell Signaling) was employed to lyse the cells. Proteins were resolved on 10% SDS-PAGE gels and transferred to PVDF membranes. The membranes were blocked with 5% BSA, and incubated with antibodies against ERα (R&D Systems) and GAPDH according to the standard western blot protocol. Blots were developed using SuperSignal West Pico chemiluminescent substrate (Pierce), imaged and analyzed using the Bio-Rad Gel Imaging System (Bio-Rad).

Cell transfection. The miR-221 mimic or the miR-221 inhibitor (both from Qiagen, Valencia, CA, USA) was transfected (4.5 μM) with HiPerFect Transfection Reagent (Qiagen) according to the manufacturer's procedures. Negative control premiR (Qiagen)was also transfected into the HepG2 cells as the control.

Cell growth assay. HepG2 cells ($4x10^3$/well) were plated in 96-well plates, and then the cells were transfected (final miRNA concentration of 3 nM), or infected. Cell proliferation was documented every 24 h for 3 days using a cell counting kit (Dojindo), and absorbance at 450 and 610 nm was evaluated using a SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Flow cytometry for cell cycle and apoptosis analysis. For cell cycle analysis, the cells from each well were harvested 48 h after transfection, and then the transfected cells were fixed with 70% cold ethanol for at least 1 h after collecting and washing. Cells were stained with propidium iodide, and cell cycle distribution was examined by a fluorescence-activated cell sorting (FACS) flow cytometer, and DNA histograms were analyzed with modified software.

Luciferase reporter assay. The 3'UTR of the candidate gene was cloned and inserted into the downstream of the luciferase gene in the pGL3/luciferase vector. The mutant 3'UTR of the candidate gene was cloned using the wild-type 3'UTR as a template and inserted into pGL3/luciferase as described for the wild-type 3'UTR. The cells were co-transfected with miRNA mimics and the wild-type or mutant 3'UTR of the candidate gene. Luciferase activity was determined 48 h after transfection using the Dual-Luciferase Reporter Assay system (Promega).

Statistical analysis. All data are expressed as mean ± standard deviation (SD) from at least 3 separate experiments. Statistical significance was determined with the paired Student's t-test, except as noted for analyses of microarray data, which were examined with Fisher's exact tests. P-value <0.05 was considered to indicate a statistically significant result. All statistical analyses were performed with SPSS software (version 17.0).

Results

Ad-HBx-transfected HCC cells. HepG2 cells were infected with Ad-HBx core or Ad-GFP at an MOI of 50, and the
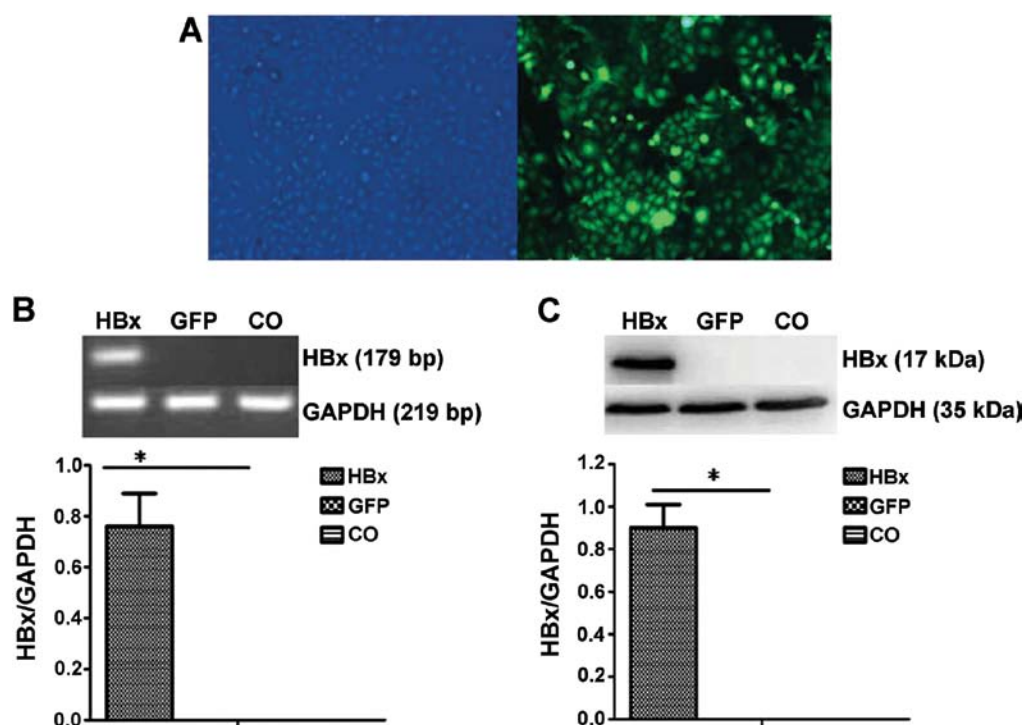
Figure 1. Ad-HBx-infected HCC cells. HepG2 cells were infected with Ad-HBx or the control Ad-GFP adenovirus. (A) The infection efficiency was evaluated by observation of GFP expression under a fluorescence microscope. (B) The expression of HBx mRNA was determined by RT-PCR analysis in the HepG2 cells. GAPDH was used as an internal control. (C) The expression of HBx protein was determined by western blot analysis in the HepG2 cells. GAPDH was used as an internal control. CO, control; HBx, hepatitis B virus X protein.
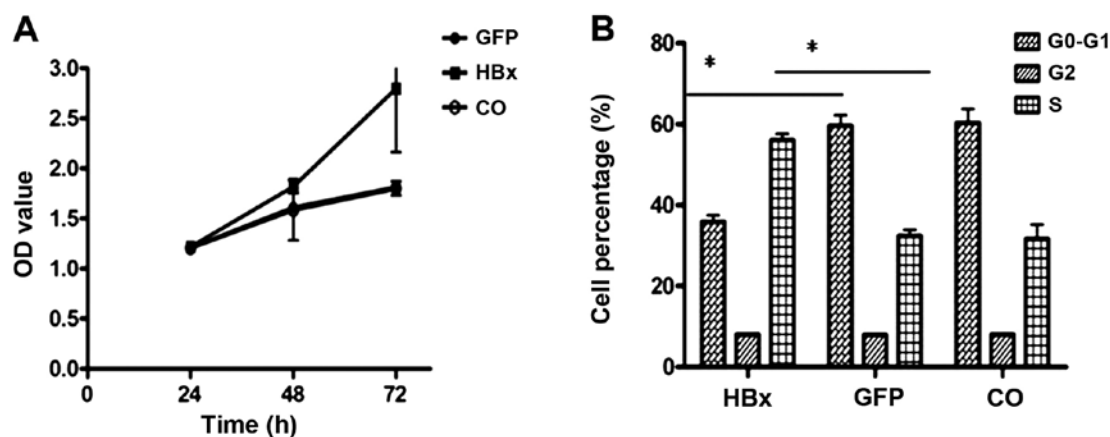

Figure 2. HBx promotes HCC cell proliferation. (A) Cell growth curve. (B) FACS cell cycle analysis revealed an S phase accumulation of the HepG2 cells 48 h after infection of Ad-HBx compared with the Ad-GFP control. *P<0.05. CO, control; HBx, hepatitis B virus X protein.

infection efficiency was evaluated by observation of EGFP expression under a fluorescence microscope (Fig. 1A). We used semi-quantitative reverse transcription-PCR to confirm the infection efficacy of HBx (Fig. 1B). As shown in Fig. 1C, the efficient transient expression of the HBx protein was noted in the HepG2 cells after Ad-HBx infection at 48 h.

HBx promotes HCC cell proliferation. After HepG2 cells were infected with Ad-HBx and Ad-GFP for 48 h, cell proliferation assays indicated significant growth promotion in the Ad-HBx-infected cells (Fig. 2A). To further investigate the effect of HBx overexpression on cell cycle distribution, cells were

analyzed for cell cycle status using propidium iodide staining and flow cytometry. An obvious accumulation of cells in the S phase was detected after HBx overexpression compared with the control (Fig. 2B).

HBx downregulates ERα expression in HBV-related HCC. To determine whether HBx protein alters ERα expression, we detected the ERα protein level after the transient infection of Ad-HBx into HepG2 cells. Our results confirmed that ERα expression was inhibited by HBx (Fig. 3A and B).

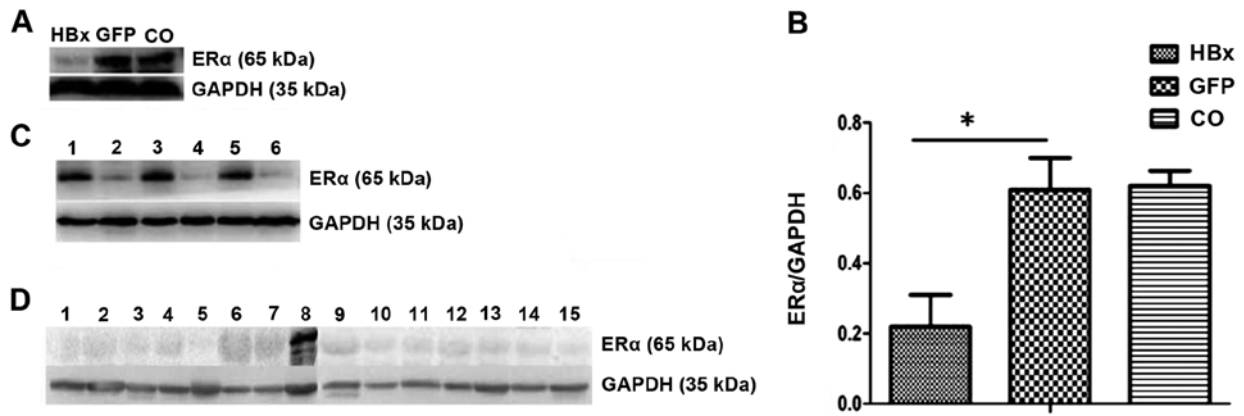HepG2.215 is a cell line that is derived from HepG2. The only difference between them is that HepG2.215 cells are

Figure 3. HBx downregulates ERα expression in HBV-related HCC. (A and B) Expression of ERα was determined by western blotting in HepG2 cells infected with the Ad-HBx or Ad-GFP adenovirus. GAPDH was used as an internal control. (C) Expression of ERα was determined by western blotting in HepG2 and HepG2.215 cells. GAPDH was used as an internal control. Lanes 1, 3 and 5, HepG2 cells; lanes 2, 4 and 6, HepG2.215 cells. (D) Expression of ERα was determined by western blotting in 14 HBV-related HCC tumor specimens. MCF-7 cells were used as a positive control. GAPDH was used as an internal control. Lanes 1-7, tumor specimens; lane 8, MCF-7 cells; lanes 9-15, tumor specimens. *P<0.05. CO, control; HBx, hepatitis B virus X protein; ERα, estrogen receptor-α.
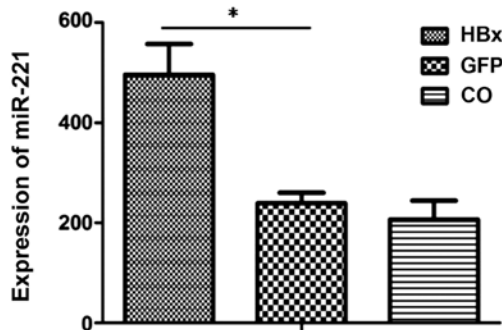

Figure 4. HBx unregulates miR-221 in the HepG2 cells. Expression of miR-221 was determined by qRT-PCR in HepG2 cells infected by the Ad-HBx or Ad-GFP adenovirus. *P<0.05. CO, control; HBx, hepatitis B virus X protein.

able to produce infectious viruses through the HBV genome integrated in the cell chromosome (16-18). The results suggest that the expression of ERα was obviously higher in the HepG2 cells than that in the HepG2.215 cells (Fig. 3C).

Additionally, in accordance with the results obtained in the cancer cells, ERα was validated to be downregulated in HBV-related HCC tumor tissues. MCF-7 breast cancer cells were used as a positive control (Fig. 3D).

HBx unregulates miR-221 in HepG2 cells. We aimed to ascertain whether the miR-221 level is affected by HBx overexpression. As shown in Fig. 4, the level of miR-221 was significantly higher in the HepG2-HBx cells than that in the HepG2-GFP control group or HepG2-mock control group.

HBx promotes cell growth and G1-S transition via upregulation of miR-221 in HepG2 cells. To test the hypothesis that the HBx protein influences the proliferation of HepG2 cells via its upregulation of miR-221, we evaluated the effects of transient transfection of the miR-221 inhibitor and miR-221 mimics on cell growth using MTT and flow cytometry. Upon the construction of cell growth curves, we found that the miR-221 mimic

promoted the growth of HepG2 cells compared to the control cells (Fig. 5A). However, the miR-221 inhibitor significantly inhibited cell growth (Fig. 5B). To test the hypothesis that the HBx protein promotes G1/S transition via its upregulation of miRA-221, we performed gain-of-function and loss-of-function studies of miR-221, and evaluated the effects on the cell cycle profile. We observed profound promotion of G1/S transition by the miR-221 mimics (Fig. 5C), while the miR-221 inhibitor significantly inhibited G1/S progression (Fig. 5D). Overall, these data suggest that miR-221 promotes HCC cell proliferation.

ERα is a direct target of miR-221. Online bioinformatic analyses showed that the 3'UTR of ERα-encoded mRNA contains a sequence that is partially complementary to miR-221 (Fig. 6A). Cells were transiently transfected with miR-221 mimics or the inhibitor. The luciferase reporter assay indicated that miR-221 led to inhibition of the wild-type 3'UTR by 47.01%, yet it had no effect on the luciferase intensity controlled by the mutant 3'UTR (Fig. 6B). To further determine whether miR-221 affects ERα expression in the HCC intracellular environment, we analyzed changes in ERα expression in HepG2 cells after transient transfection of miR-221 mimics or the inhibitor. Western blot analysis showed that the miR-221 inhibitor upregulated ERα expression while the miR-221 mimics markedly inhibited its expression (Fig. 6C and D). Taken together, these results indicate that miR-221 negatively regulates ERα by directly targeting its 3'UTR.

Discussion

miRNAs have been shown to exhibit regulatory functions in numerous cellular processes, including cell growth, differentiation and apoptosis. Moreover miRNAs were demonstrated to induce hepatocyte apoptosis (16), liver fibrosis (17) and hepatocarcinogenesis (18).

Previous reports suggest that miR-221 is upregulated in various types of cancers (19), including HCC (20). Meanwhile,
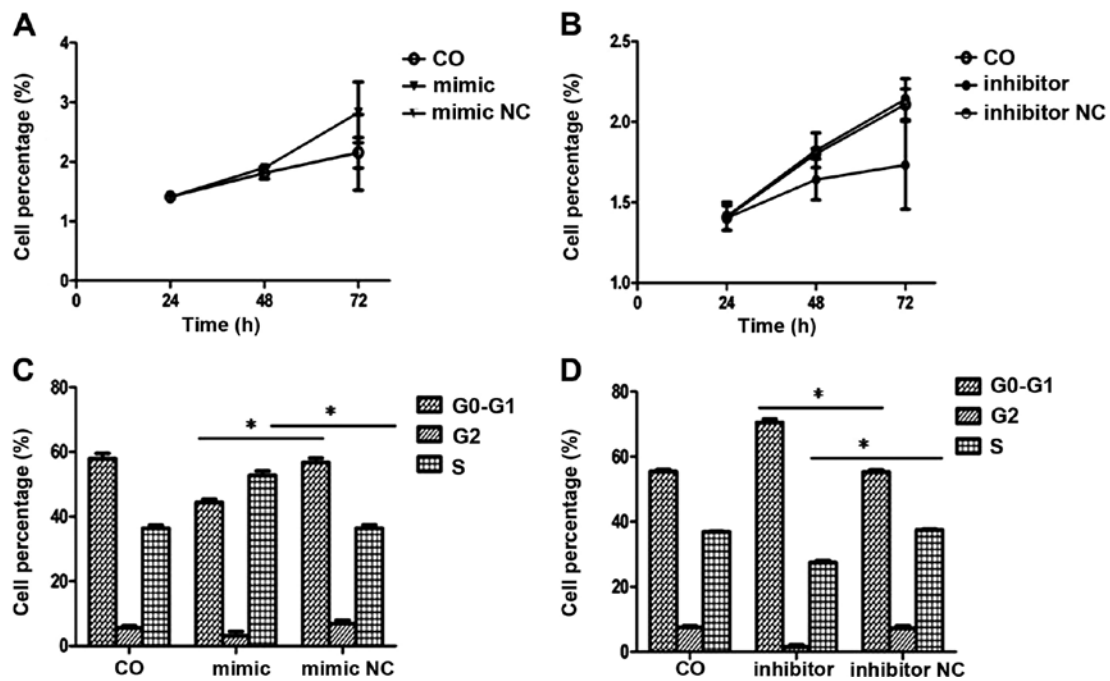
Figure 5. HBx promotes cell growth and G1-S phase transition via its upregulation of miR-221 in HepG2 cells. HepG2 cells were transfected with miR-221 mimics or the miR-221 inhibitor. (A) CCK-8 assay showed growth promotion in HepG2 cells transfected with the miR-221 mimics. (B) CCK-8 assay showed growth inhibition in HepG2 cells transfected with the miR-221 inhibitor. (C) A FACS cell cycle analysis showed that the miR-221 mimic increased the percentage of cells in the S phase but inhibited the percentage of cells in the G0/G1 phase. (D) FACS cell cycle analysis showed that the miR-221 inhibitor increased the percentage of cells in the G0/G1 phase but decreased the percentage of cells in the S phase. *P<0.05. CO, control; mimic, miR-221 mimic; mimic NC, miR-221 mimic negative control; inhibitor, miR-221 inhibitor; inhibitor NC, miR-221 inhibitor negative control. HBx, hepatitis B virus X protein.
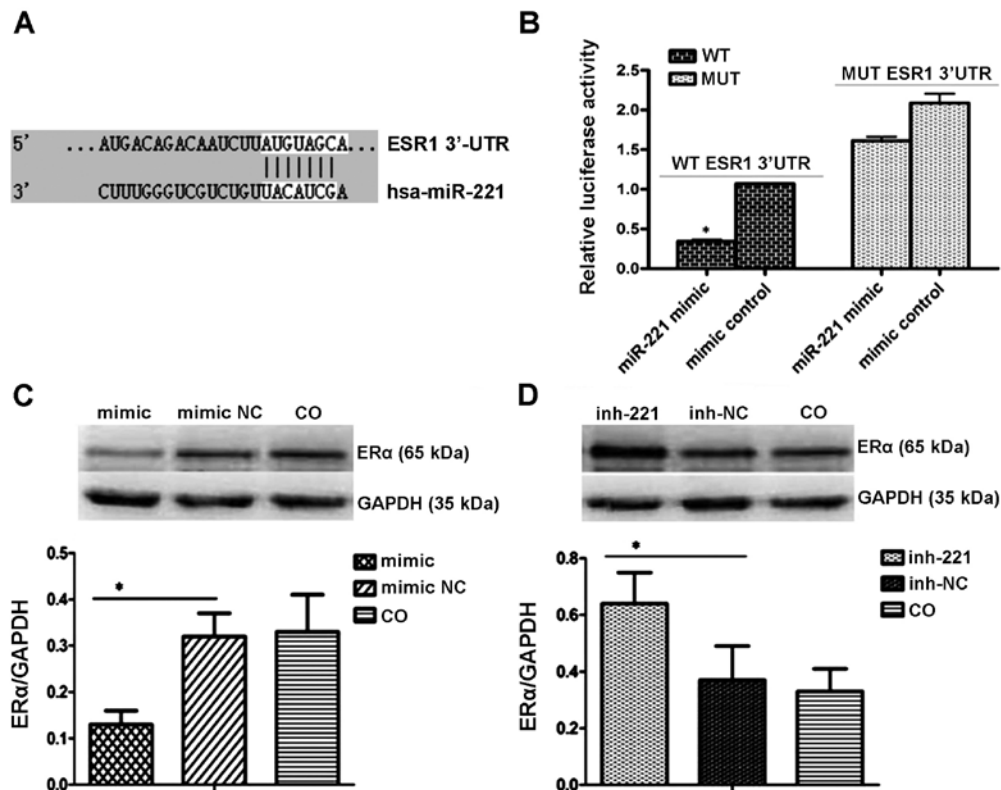

Figure 6. ERα is a direct target of miR-221. (A) The predicted duplex formation between human ERα and miR-221 is shown, and the 3'UTR of human ERα contains a conserved binding site for miR-221 in different species. (B) The effect of miR-221 on luciferase intensity controlled by the wild-type or mutant 3'UTR of ERα was determined by luciferase assay. *P<0.01. (C) The effect of the miR-221 mimic on the protein levels of ERα was determined by western blotting in HepG2 cells. (D) The effect of miR-221 inhibitor on the protein levels of ERα was determined by western blotting in HepG2 cells. Mimic, miR-221 mimic; mimic NC, scrambled oligonucleotides as the negative control for miR-221 mimics; CO, HepG2-MOCK; inh-221, miR-221 inhibitor; inh-NC, scrambled oligonucleotides as the negative control for miR-221 inhibitor. ERα, estrogen receptor-α; 3'UTR, 3'-untranslated region; HBx, hepatitis B virus X protein.

overexpression of miR-221 was shown to promote cancer cell proliferation by inhibiting the expression of cell cycle controllers CDKN1B/p27 (21) and CDKN1C/p57 (22). In addition, miR-221 silencing was found to inhibit HCC cell proliferation and promote survival (23).

In the present study, we investigated the role and the functional target of miR-221 in HBV-related HCC.

Our data indicated that HBx overexpression promoted cell proliferation and upregulated miR-221. Suppression of miR-221 significantly inhibited HCC cell growth. All of these results were supported by data from other reports. For example, miR-221 was found to promote cell proliferation of breast cancer (24). MiR-221 was also found to be essential for the EMT phenotype, migration and growth of pancreatic cancer cells (25). Moreover, the level of miR-221 consistently reflects the stage of acute myeloid leukemia, serving as a tumor promoter (26).

As for the mechanism of miRNA regulation in cancer, it is crucial to validate target genes. Our data demonstrated that HBx protein stimulated miR-221 but inhibited ERα expression. Further studies showed that miR-221 directly targeted the 3'UTR of ERα. MiR-221 was also shown to reduce the protein levels of ERα, resulting in increased proliferation activity in hepatoma cell lines. These results suggest a new mechanism by which to suppress the estrogen signaling pathway, which has been long known to protect against HCC development (27-29).

Previous report found that ERα was significantly downregulated in HCCs, both by immunohistochemical staining or by receptor binding assay (30-32). Obviously downregulation of ERα was found in HCC cancer tissues compared with their non-tumor counterparts. Thus, ERα suppression promotes the proliferation of HCC cells (33).

We demonstrated that ERα expression was downregulated in both HBV-related HCC cell lines and tumor specimens, which is consistent with the result from a recent publication by Liu *et al* (33). Studies have demonstrated that the methylation of a CpG island at the promoter of ESR1 is one of the main contributors to ERα downregulation in HCC (34).

Estrogens are well accepted as cancer-promoting agents in both the breast and the uterus (35). How they function in a contradictory manner to protect individuals against HCC is intriguing. We presume that estrogens through tissue-specific selective ER modulators (36), may exert opposite effects on HCC and breast cancer. This can be addressed in future studies by overexpressing miR-221 specifically in the liver or in the breast of transgenic mice.

In conclusion, the present study indicates that HBx increases miR-221 expression, and miR-221 promotes cell proliferation through the suppression of ERα, functioning as a tumor promoter. Our data imply that the overexpression of miR-221 may serve as a novel biomarker for HBV-related HCC and may also be a potential target for an miRNA-based molecular therapeutic strategy.

Acknowledgements

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