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Research article

# miR-203a is involved in HBx-induced inflammation by targeting Rap1a

AiRong Wu[a,1], Huo Chen[b,1], ChunFang Xu[a,1], Ji Zhou[b], Si Chen[b], YuQi Shi[a], Jie Xu[b], JianHe Gan[a,*], JinPing Zhang[b,*]

[a] Department of gastroenterology, The First affiliated Hospital of Soochow University, Suzhou 215006, China
[b] Institutes of Biology and Medical Sciences, Soochow University, Suzhou 215123, China

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ABSTRACT

Hepatitis B virus (HBV) causes acute and chronic hepatitis, and is one of the major causes of cirrhosis and hepatocellular carcinoma. Accumulating evidence suggests that inflammation is the key factor for liver cirrhosis and hepatocellular carcinoma. MicroRNAs play important roles in many biological processes. Here, we aim to explore the function of microRNAs in the HBX-induced inflammation. First, microarray experiment showed that $HBV^+$ liver samples expressed higher level of miR-203a compared to $HBV^-$ liver samples. To verify these alterations, HBx-coding plasmid was transfected into HepG2 cells to overexpress HBx protein. The real-time PCR results suggested that over-expression of HBx could induce up-regulation of miR-203a. To define how up-regulation of miR-203a can induce liver cells inflammation, we over-expressed miR-203a in HepG2 cells. Annexin V staining and BrdU staining suggested that overexpression of miR-203a significantly increased the cell apoptosis and proliferation, meanwhile, over-expression of miR-203a could lead to a decrease in G0/G1 phase cells and an increase in G2/M phase cells. Some cytokines production including IL-6 and IL-8 were significantly increased, but TGFβ and IFNγ were decreased in miR-203a over-expressed HepG2 cells. Luciferase reporter assay experiments, protein mass-spectrum assay and real-time PCR all together demonstrated that Rap1a was the target gene of miR-203a. Further experiments showed that these alterations were modulated through PI3K/ERK/p38/NFκB pathways. These data suggested that HBV-infection could up-regulate the expression of miR-203a, thus down regulated the expression of Rap1a and affected the PI3K/ERK/p38/NFκB pathways, finally induced the hepatitis inflammation.

## 1. Introduction

Hepatitis B virus (HBV) infection, a global public health problem with approximately 2 billion people who are virus carriers and more than 350 million people been chronically infected worldwide, is one of the major causes of cirrhosis and hepatocellular carcinoma [1,2] The outcome of HBV infection is depended on a complex interplay between host factors and HBV encoded proteins [3,4]. 15–40% people with chronic hepatitis B develop liver cirrhosis and hepatocellular carcinoma due to chronic inflammation-induced liver cell destruction and regeneration [5,6]. Accumulating evidence suggests that the HBV X protein (HBx) is a diversified regulatory protein and it interacts with host components and initiates the inflammation and finally develops to liver cirrhosis and cancer [7].

MicroRNAs (miRNAs), a class of the endogenous non-coding small RNAs with 18–22 nucleotides in size, are post-transcriptional regulators that binds to the 3′-untranslated region (UTR) of the target gene messenger RNA, usually inducing their target gene translational repression, deadenylation, and degradation [8,9]. Abundances of studies demonstrated that miRNAs were involved in almost all biological processes such as cell proliferation, differentiation, apoptosis, neuroprocesses, carcinogenesis, immune response and many physiological activities and disease procedures [10,11].

Several studies demonstrated that HBx-mediated miRNA regulation serves a crucial function in hepatocarcinogenesis. For example: HBx suppresses the p53-mediated activation of miR-148a, thus induces the activation of mTOR to promote cancer growth and metastasis of HCC. HBx suppresses the expression of miR-15b to enhance the proliferation of HCC. HBx abrogates the effect of miR-205 on tumor suppression in carcinogenesis. HBx can induce the high expression of miR-224, thus promotes hepatoma cell migration and tumor formation by silencing its target gene Smad4 [12–16].

In the present study, we first explored the different expression profiles of microRNAs between $HBV^+$ and $HBV^-$ liver tissues.

* Corresponding authors.
*E-mail addresses:* j_pzhang@suda.edu.cn (J. Gan), ganjianhe@aliyun.com (J. Zhang).
[1] These authors contributed equally to this work.

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Microarray results suggested that HBV infection significantly changed the expression patterns of microRNAs. Among them, miR-203a was one of most significantly increased in $HBV^+$ liver tissues compared to that in $HBV^-$ liver tissues. Over-expression of HBx in HepG2 cells could induce high expression of miR-203a. Further experiments demonstrated that miR-203a increased the apoptosis and proliferation of HepG2 cells and production of some proinflammation cytokines such as IL-6 and IL-8, but down-regulated the production of TGFβ and IFNγ. Extensive studies suggested that miR-203a targeted Rap1a, thus affected the PI3K/ERK/p38/NFκB pathways. All together, the present studies have suggested that HBx induced high expression of miR-203a and then exerted pivotal role in the inflammation formation of HBV infection.

## 2. Materials and methods

### 2.1. Cell culture and transfection

Hepatoma cell line HepG2 was cultured in Dulbecco's modified Eagle's medium (HyClone, Logan, Utah) with 10% FCS (Gbico, HongKong) and 1% Penicillin-Streptomycin Solution. Liver cell lines LO2 was cultured in RPMI-1640 medium (HyClone, Logan, Utah) with 10% FCS (Gbico, HongKong) and 1% Penicillin-Streptomycin Solution. HBx encoded plasmid pCDNA-HBx and pCDNA3.1 vector were transiently transfected into HepG2 cells by using Lipofectamine 2000 (Invitrogen, MA, USA) according to the manufacturer's instruction.

### 2.2. Plasmid construction

The fragment containing precursor of miR-203a was amplified from mouse genomic DNA by PCR with the following primers: F: 5′ CCGCTCGAGGCGTCTAAGGCGTCCGGTACGGC 3′ R: 5′ CCGGAATTCCTGGGAATTCCACGGAGTTTCGAGG 3′. The fragment was digested with restriction enzymes *Xho*I and *Eco*RI and inserted into the vector of pMDH1-PGK-GFP, and confirmed by sequencing. Rap1a coding plasmid (Ubi-MCS-3FLAG-SV40-cherry-Rap1a) and its vector Ubi-MCS-3FLAG-SV40-cherry were purchased from Shanghai Genechem Co., Ltd (shanghai, China).

### 2.3. Apoptosis assay

Single cell suspensions were stained with APC- Annexin V (Biolegend, San Diego, CA, USA) in Annexin V binding buffer. After staining, Propidium Iodide Solution (Biolegend, San Diego, CA, USA) was added to the cell solution to final concentration 10 μg/ml. Cells were analyzed by using BD FACS CantoII. FACS data were analyzed using FlowJo software (Tree Star, Inc).

### 2.4. BrdU staining

APC-BrdU Flow Kit was purchased from BD Bioscience For BrdU assay, cells were treated with BrdU for 4 h. Then cells were harvested and processed according to the manufacturer's protocol using APC-anti-BrdU and 7-AAD. After staining, cells were analyzed by using BD FACS CantoII. FACS data were analyzed using FlowJo software (Tree Star, Inc).

### 2.5. Real-time PCR assay

Total RNAs were all extracted using RNAiso Plus reagent (Takara Biotechnology Co. LTD). To detect the expression of miR-203a and miR-203b, RNA was first reversed using the M-MLV reverse transcriptase with the following miR-203 specific reverse primer: miR-203a 5′ CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCTAGTGGT 3′, quantitative real-time PCR were performed in triplicate by using FastStart Universal SYBR Green Master (Roche Diagnostics GmbH, Mannheim, Germany) on an eppendorf Real-Time Detection System. To detect the expression of other genes, total RNAs were first reversed using M-MLV reverse transcriptase with Oligo(dT). Transcripts were quantified by real-time PCR and normalized to the amount of GAPDH mRNA expression. The PCR primers are listed in Supplementary Table 1.

### 2.6. Luciferase assay

The 3′UTR of Rap1a containing miR-203a binding sites predicted by targetscan software was amplified by PCR (the primers were shown in Supplementary Table 2) and fragments were cloned into pMIR-report vector to construct luciferase plasmid for evaluating the binding activity between miR-203a and 3′UTR of Rap1a. Approximately $10^5$ 293 T cells seeded in a 24-well plate were transiently transfected with 0.5 μg of firefly luciferase reporter plasmid pMIR-report-Rap1a, 0.1 μg of Renilla luciferase TK vector, and 1.0 μg of pMDH1-PGK-GFP-miR-203a. pMDH1-PGK-GFP-miR-184 was used as a negative control. TK vector was used to normalize transfection efficiency. Both firefly and Renilla luciferase activity were detected after 24 h. Normalized relative units represent firefly luciferase activity/Renilla luciferase activity.

### 2.7. Western blot

The relative cells were lysed with RIPA lysis buffer containing 1 mM PMSF for 10 min. The 6×SDS loading buffer was added to the supernatants of lysis. The mixture was boiled for 10 min and subjected to 12% SDS–PAGE gel with the same amount of total proteins. After running for 1 h about, the gel was transferred to polyvinylidene fluoride membrane by electro-blotting. Proteins were detected with a 1:1000 dilution of mouse anti-Rap1a antibody (Abcam, Cambridge, MA, USA), a 1:500 dilution of anti-MAPK/pMAPK antibody (Cell Signaling Techology, Danvers, MA, USA), a 1:1000 dilution of anti-Erk/pErk antibody (Cell Signaling Techology, Danvers, MA, USA), a 1:1000 dilution of anti-Akt/pAkt antibody (Cell Signaling Techology, Danvers, MA, USA) and anti-PI3K/pPI3K antibody (Cell Signaling Techology, Danvers, MA, USA), a 1:5000 dilution of HRP-linked anti-rabbit antibody (Cell Signaling Techology, Danvers, MA, USA) and HRP-conjugated donkey anti-mouse antibody (Proteintech, Chicago, IL, USA). GAPDH was used as loading reference.

### 2.8. Microarray

Total RNAs were extracted from relative tissues using TRIzol reagent (TaKaRa) according to the manufacturer's description, microRNAs were purified using the miRVana miRNA Isolation Kit (Ambion, Austin, TX, USA), tailed with polyadenylation polymerase, ligated with biotinylated DNA dendrimers, and hybridized to Affymetrix GeneChip miRNA arrays using the FlashTag Biotin RNA Labeling Kit (Genisphere, Hatfield, PA, USA) according to the manufacturer's instructions. Slides were scanned with the Affymetrix GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA, USA), and miRNA data were analyzed using the miRNA QC Tool (Affymetrix).

### 2.9. Protein mass spectrum assay

Proteins from HepG2 cells transfected with pMDH1-PGK-GFP-miR-203a and pMDH1-PGK-GFP were extracted with RIPA buffer in the presence of fresh protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) and quantified with BCA protein assay (Thermo Fisher, shanghai, China). Same amount of proteins from two samples were individually precipitated using cold acetone and solubilized with 8 M urea in 50 mM HEPES (pH =8.0). Proteins (100 μg each) were reduced with dithiothreitol, alkylated with iodoacetamide, digested with endoproteinase Lys-C (Wako, Japan) for 3 h at room temperature and again with sequencing grade modified trypsin