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Original Article

Decreased miR-34a promotes growth by regulating MAP4K4 in hepatitis B virus related hepatocellular carcinoma

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Abstract: Many results suggest that microRNAs participate in malignant progression in tumor by regulating gene expression at post-transcriptional level. In hepatocellular carcinoma (HCC), miRNAs are also dysregulated and involved in tumorigenesis. In the present study, we tried to investigate the molecular mechanism of miR-34a in hepatitis B virus (HBV)-related HCC. Quantitative real-time PCR (qRT-PCR) was used to detect miR-34a expression in 17 cases of HBV-related HCC tissues and their adjacent non-cancerous tissues. Hepatitis B virus X protein (HBx) expression vector was transferred into HepG2 and Hep3B and the biologic effects on miR-34a and MAP4K4 were observed. By overexpression of miR-34a, CCK-8 and colony formation assay to evaluate HepG2.2.15 cell growth, flow cytometry to evaluate cell apoptosis. Western Blot and immunohistochemistry to test protein expression. And potential mechanisms were analyzed with luciferase activity assay. The results showed that miR-34a was significantly downregulated in HBV-related HCC and cells. MAP4K4 mRNA was a target of miR-34a and overexpression miR-34a reduced MAP4K4 protein levels. HBx suppressed miR-34a expression while upregulated MAP4K4 in HCC cells in vitro. MiR-34a overexpressed in HepG2.2.15 cells significantly inhibit cell proliferation and induced cell apoptosis. Our results demonstrated that induced MAP4K4 expression by HBx involved in decreasing of miR-34a expression in HBV-related HCC. This revealed a new pathophysiology mechanism of HBV-related HCC.

Keywords: Hepatocellular carcinoma, hepatitis B virus, miR-34a, MAP4K4

Introduction

Hepatocellular carcinoma (HCC) is a worldly malignant tumor and the incidence is still increasing [1]. Hepatitis B virus (HBV) is a DNA virus that has participated in HCC tumorigenesis. Through integrating into the host DNA, HBV could adjust host gene expression and thus promote HCC progression [2]. High replication status, persistent inflammation, viral mutations of HBV virus intimately correlated with the HBV-related HCC [3, 4]. Recent research results have revealed that chronic inflammation and regeneration of the damaged liver involved in the malignant progression of HBV-related HCC [5]. However, the detailed molecular mechanism has not been well illuminated yet. It is still urgent to investigate molecular mechanisms of the HBV-related HCC.

MicroRNAs (miRNAs) are a class of small noncoding RNAs, which have been identified to function important roles in the cancer malignant development [6, 7]. The expression of miRNAs was frequently dysregulated in HBV-related HCC and this obviously enhanced HBV-related HCC progression [8]. For example, miR-145 was dramatically downregulated in HBV-related HCC and might act as tumor suppressor [9]. However, higher miR-331-3p expression involved in HBV-related HCC progression [10]. HBx has been shown to play a critical role in the molecular pathogenesis of HBV-related HCC. And it was also found that HBx could adjust miRNAs expression to promote HBV-related HCC progression [11]. These results strongly provided new theories for elaborating the mechanism of HBV-related HCC and possibly supplied an effective way for the HBV-related HCC diagnosis and treatment.

MiR-34a was frequently downregulated in multiply cancer such as breast cancer [12], esoph-

ageal squamous cancer [13], and colon cancer [14]. However, the expression situation and functions of miR-34a in HBV-related HCC have not been investigated. In the present study, the expression level of miR-34a in HBV-related HCC tissues and HepG2.2.15 cell line was evaluated, and the effects of miR-34a overexpression on HepG2.2.15 cell proliferation and apoptosis were observed. We also identified MAP4K4 was a directly target of miR-34a.

Materials and methods

Human tissues

17 pieces of HBV-related HCC tissues and adjacent non-tumor liver tissues were obtained at Taihe Hospital Affiliated to Hubei Medical University from June of 2015 to October of 2015. This study was officially approved by the medical ethics society of Hubei Medical University. And the informed consent forms for use of tumor tissues and paraffin slides were got from all the patients. All the tissues were frozen in liquid nitrogen.

Cell culture

The HepG2.2.15 cells were purchased from National Platform of Experimental Cell Resources (Beijing, China) and were cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone) supplemented with 10% fetal bovine serum (FBS, Gibco) and 380 mg/L antibiotic G-418 sulfate (Promega), at 37°C in an atmosphere of 5% CO_2. Human normal liver cell line LO2, HepG2 and Hep3B were available in the laboratory and cultured in DMEM supplemented with 10% FBS and antibiotics (penicillin and streptomycin) at 37°C in an atmosphere of 5% CO_2.

Detection of miR-34a expression level

Total RNAs of tissues and cells were extracted by TRIzol reagent (Invitrogen), according to the manufacturer's instructions. An amount of 1 mg total RNA was reverse-transcribed to cDNA using All-in-OneTM miRNA First-Strand cDNA Synthesis Kit (Gene-Copoeia). Then, All-in-OneTM miRNA qRT-PCR Detection Kit (Gene-Copoeia) was used to detect the expression level of miR-34a. U6 RNA was used to normalize the relative abundance of miR-34a. After denaturation at 95°C for 10 min, 40 amplification cycles were followed: denaturation at 95°C for 10 s, annealing at 60°C for 20 s, and extension at 72°C for 30 s. Fluorescence was collected automatically during amplification, and the melting curve for the product was obtained. Relative miR-34a expression was calculated with $2^{-\Delta\Delta CT}$ method.

CCK-8

Hep2.2.15 was cultured at 1,000 cells per well in 96-wells. The next day, the cells were transfected with miR-34a and negative control as above. Then, 10 mL of CCK-8 reagents were separately added into cells each well at before transfection and 24 h, 48 h or 72 h after transfection. The OD values were shown at 450 nm via a microplate reader (Bio-Rad).

Colony formation assay

After transfection of mi-R34a for 48 hrs in Hep2.2.15 cells, 500 cells were plated into 6-well plates and continually cultured at 37°C and at an atmosphere of 5% CO_2 for 10 d. The supernatants were then discarded and cells were rinsed in PBS for twice, and fixed with 75% ethanol for 15 mins. The cells were stained with 0.1% crystal violet for 10 mins. The plates were dried at room temperature and the colony numbers containing more than 50 cells were microscopically counted. The experiments were triplicated.

Transfection of miRNA, and HBx cDNA

miR-34a and negative control were purchased from RiboBio (Guang Zhou, China). The HBx open reading frame (Adr subtype, AB299858) was cloned into the PCDNA3.1 (+) plasmid (Invitrogen) to create PCDNA3.1-hbx in GenePharma (Shanghai, China). The transfection was performed in a final concentration of 100 nM using Lipofectamine TM 2000 (Invitrogen, Carlsbad, CA, USA) according to the instructions provided by the manufacturer. RNA transfection efficiency is approximately 70%-80% and the overexpression of miRNA for at least 48 hours. For the HBx cDNA plasmid and miR-34a mimics combination experiment, HepG2 and/or Hep3B cells were co-transfected with HBx cDNA plasmid (2 μg) and miR-34a mimics (100 nM) for 72 h.

Apoptosis analysis

Flow cytometry was performed using an annexin V-fluorescein-5-isothiocyanate Apoptosis

Detection Kit (Nanjing KeyGEN Bio-tech. CO., LTD, China). Hep2.2.15 cells were transfected with miR-34a and negative control as above. Forty-eight hours after transfection, the cells were trypsinized, washed twice with cold PBS, and resuspended in binding buffer. The cell suspension (100 μl) was incubated with 5 μl of annexin-V and 1 μl of propidium iodide at room temperature for 15 min. The stained cells were analysed with fluorescent-activated cell sorting (FACS) using BD LSR II flow cytometry (BD Biosciences, San Diego, CA, USA). The results were calculated as percentages of apoptotic cells.

Luciferase activity assay

The 3'-UTR of MAP4K4 containing one putative miR-34a binding sites was amplified and cloned into pGL3 vector separately (Life technologies, Carlsbad, CA, USA). The complementary sites with the sequence in MAP4K4 3'-UTR were mutated, so that not complement to miR-34a any more. HepG2 cells were cultured in 24-well plates and co-transfected with 100 ng of wild-type or mutated MMAP4K4 3'-UTR constructs, or 100 nM of negative control or miR-34a mimics. Luciferase activity was measured with the dual luciferase reporter assay system (Promega).

Immunohistochemical analysis

HBV-related HCC paraffin embedded blocks were cut into 3-μm thick slides. The slides were first dewaxed in xylol, rehydrated in graded alcohol series, and antigen repaired in 98°C for 15 minutes in 10 mM sodium citrate (pH 6.0). Endogenous peroxidase activity was blocked with 3% H_2O_2-methanol and non-specific binding sites were closed by normal goat serum. An anti-human MAP4K4 rabbit monoclonal antibodies (1:50, Abcam Inc., Cambridge, CA) incubated in 4°C for overnight. The next morning, the slides were washed three times with PBS and then incubated with biotin labeled second antibody for 15 minutes. DAB color-substrate solution for half minute. The slides were counterstained with hematoxylin for 1 minute, dried in 60°C overnight, dehydrated in gradient ethanol, sealed with neutral neutral gum. The first antibody was omitted in the negative controls and breast cancer tissues as the positive controls that had been confirmed overexpression for the MAP4K4 protein.

Immunohistochemical results evaluation

According to the methods reported previously [15], high MAP4K4 expression was defined as ≥10% of the tumor cells were positive in the cytoplasmic staining, and low MAP4K4 expression (MAP4K4-L) was defined as <10% of the tumor cells were positive staining or no cytoplasmic staining.

Immunohistochemical scores were also assessed with a semi-quantitative method which combining staining intensity and the percentage of positive cells. The scores of staining intensity were defined as 0, negative; 1, weak; 2, moderate; and 3, strong. The scores of positive cells were defined as (0, <21%; 1, 21% to 40%; 2, 41% to 60%; 3, 61% to 80%; and 4, 81% to 100%). The final staining score was calculated by multiplying the intensity staining scores and percentage of positive cells scores. All the sections were independently reviewed by two independent pathologists, and the final consistent immunohistochemical diagnosis for conflicting results was reached by discussing.

Western blot analysis

Seventy-two hours after transfection with miR-34a and negative control, cells were collected and extracted protein for assessing MAP4K4 expression with western blot. Total proteins were separated by 10% SDS-PAGE gel and then transfected to PVDF membranes. The member was blocked 2 h at room temperature with 5% milk. After washing with TBST three times, the membrane was incubated with primary antibodies against human MAP4K4 proteins (Abcam, USA) at 4°C overnight. After washing three times, the second antibody with HRP-labeled goat anti-rabbit IgG (BZSGB Technology, China) was incubated at room temperature for 2 h. Finally, the membranes were incubated with ECL reagent (Millipore, USA) and quickly wrap the membrane in plastic wrap and expose. The GAPDH (Cell Signaling technology) was used as the control.

Statistical analysis

The statistical analysis was carried out with SPSS 17.0 software. Student's t-test was adopted to evaluate statistical differences between experimental groups. A P value of less than 0.05 was considered to be statistically significant. All the experiments were performed in triplicate.

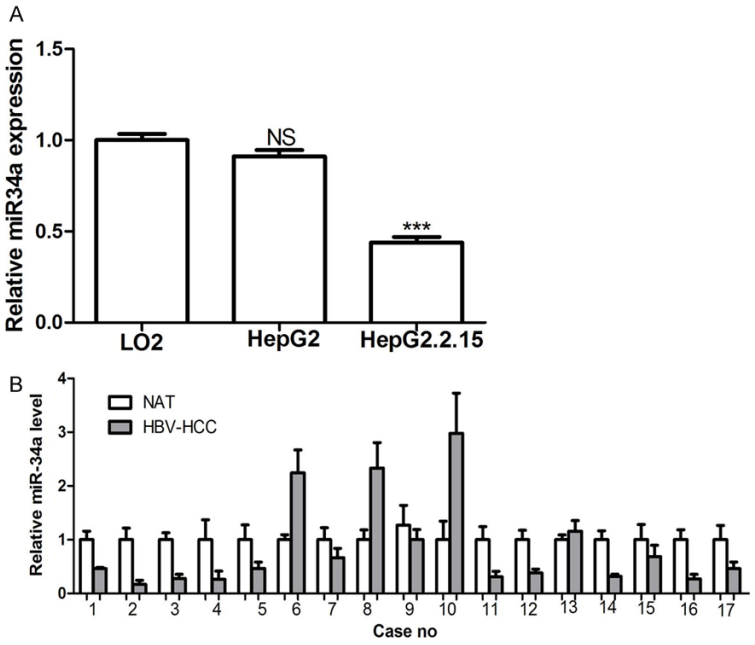

Figure 1. Decrease miR-34a expression in HBV-related HCC cells and tissues. A. Expression of miR-34a in normal liver cell line LO2, HepG2 and HepG2.2.15 HCC cell lines detected by stem-loop real-time PCR. B. The relative expression mature of miR-34a was further down-regulated in HBV-related HCC tissues compared with matched noncancerous liver tissues. U6 RNA was used as an internal control. The results were analyzed with Student's t-test (***P<0.001).

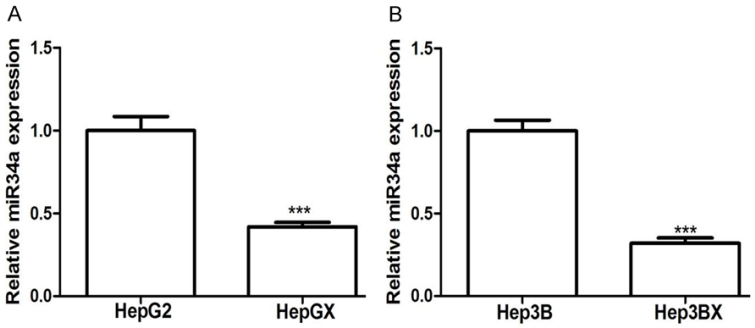

Figure 2. HBx expression vector was transfected into HepG2 and/or Hep3B cells. miR-34a expression was significantly down-regulated in HepG2x (A) and/or Hep3Bx (B) cells compared to the parental cells. These results were analyzed with Student's t-test (***P<0.001).

Results

miR-34a was significantly downregulated in HBV-related HCC

To investigate the potential roles of miR-34a in HBV-HCC, we first detected the expression level of miR-34a in HepG2.2.15, HepG2 and LO2 by qRT-PCR. The result showed that miR-34a was obviously lower in HepG2.2.15 than in HepG2 and LO2 (P<0.01) (**Figure 1A**). We then qualitified miR-34a expression level in 17 HBV-HCC tissues and accompanying non-tumor liver tissues with qRT-PCR. Surprisingly, about 82.4% miR-34a was significantly decreased in HBV-HCC issues compared to normal liver tissues (**Figure 1B**). These results confirmed the decreased tendency of miR-34a in HBV related HCC cell lines and tissues. And this suggested that decreased miR-34a expression might participate in HBV-related HCC progression.

HBx suppresses miR-34a expression in HCC cells

To further identify whether HBX could regulated miR-34a in hepatocarcinoma cells, we tested miR-34a expression in cells that were stably or transiently transfected with HBx expression vector in HepG2 and/or Hep3B cells. The relative expression levels were determined with qRT-PCR. miR-34a expression was significantly down-regulated in HepG2X and/or Hep3BX cells compared to the parental cells (**Figure 2**). This result strongly supported the idea that HBx could inhibit miR-34a expression in HCC cells.

MAP4K4 was a biologic target of miR-34a

To further investigate the molecular mechanism of miR-34a mediated pathological functions in HBV-related HCC, we used bioinformatics algorithms to analyze the miR-34a targeted genes. The miRNA: mRNA alignment analysis showed the 3'-UTR of MAP4K4 mRNA sequence contained one putative binding site for miR-34a located at 840-846 nt (**Figure 3A**). Then we cloned the putative binding site of miR-34a into the pGL3 vector to validate direct targeting of MAP4K4 by miR-34a in HepG2.2.15 cells. As showed

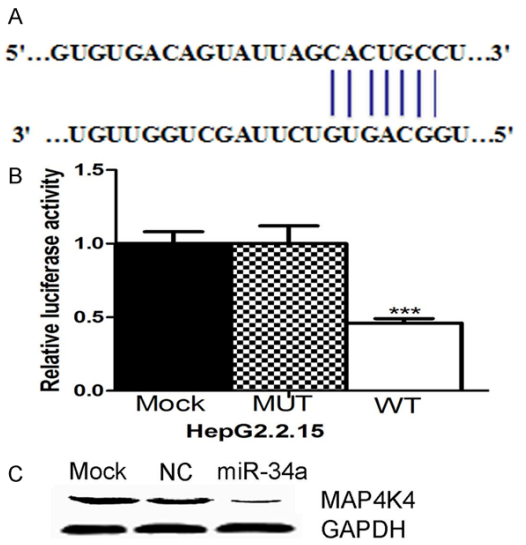

Figure 3. MAP4K4 is a direct target of miR-34a. A. Sequence alignment of miR-34a with 3'-UTR of MAP4K4. There are one direct recognition sites of MAP4K4 3'-UTR by miR-34a. B. 512 bp sequence from the MAP4K4 3'-UTR containing the miR-34a target sequence (position of 334-846 for UTR-wt, or identically insert with a mutated MAP4K4 3'-UTR constructs (UTR-mut) were cloned into the pGL3 vector separately (Life technologies, Carlsbad, CA, USA). Luciferase activity was measured with the dual luciferase reporter assay system (Promega). The result was analyzed with Student's t-test (***P<0.001). C. Western lot demonstrated that miR34a could inhibit MAP4K4 protein in HepG2.2.15 cells.

in **Figure 3B**, apparently, co-transfection of HepG2.2.15 with miR-34a mimics and the pGL3-wt 3'-UTR vector showed a significantly decreased luciferase activity compared to those cells transfected with the negative control. But co-transfection of HepG2.2.15 with miR-34a mimics and the pGL3-MU 3'-UTR vector which with several nucleotide substitutions in the core binding sites had no apparent affection on fluorescence. This suggested that miR-34a could regulate MAP4K4 expression by targeting MAP4K4 mRNA. We then test MAP4K4 expression in HepG2.2.15 after miR-34a re-expression with Western blot. And the result demonstrated that miR-34a could significantly inhibit MAP4K4 protein expression (**Figure 3C**).

Upregulation of MAP4K4 by HBx in HCC cells in vitro

First, IHC was used to examine MAP4K4 expression in 17 HBV-associated HCC, and results showed that MAP4K4 was positive expression in 13 tumor tissues (76.5%). And the relation between MAP4K4 IHC scores and relative miR-34a expression was analyzed, the result showed that there was a reverse correlation between MAP4K4 and miR-34a in HBV-related HCC (**Figure 4B**).

Then HBx expression vector was transfected into to HepG2 and/or Hep3B cells, after 48 hours, no obviously differences of MAP4K4 mRNA were observed between the HBx-expressing plasmid (HepG2-hbx) or empty vector (HepG2-vc) (**Figure 4C**). However, higher MAP4K4 protein was observed in HBx transfected HepG2 and/or Hep3B cells compared to HepG2-vc groups (**Figure 4D**). These results strongly indicated that miR34a might regulate MAP4K4 expression in HBV-related HCC in a post-transcription model.

Increased MAP4K4 expression by HBx involved in decreasing miR-34a expression in HBV-related HCC

Since HBx protein could suppress miR-34a expression and up-regulate MAP4K4 expression in the same time. We further investigated whether decreased miR-34a expression involved in increasing MAP4K4 expression by HBx in HCC. We then test MAP4K4 expression accompanying over-expression of miR-34a in HBx transferred HepG2 and/or Hep3B cells. And results revealed that miR-34a re-expression in HBx transferred HepG2 and/or Hep3B cells apparently inhibit MAP4K4 upregulation (**Figure 4D**). These suggested that HBx could up-regulate MAP4K4 expression through decreasing miR-34a expression in HBV-related HCC.

Re-expression of miR-34a suppressed HepG2.2.15 growth and induced apoptosis

To understand whether re-expression of miR-34a inhibits HepG2.2.15 cells viability, CCK-8 assay was used to test the viability effect of re-expression of miR-34a in HepG2.2.15 cells. As shown in **Figure 5A**, re-expression of miR-34a significantly inhibited HepG2.2.15 cell viability at 48, 72 and 96 h compared to negative control group. A colony formation assay also showed that re-expression of miR-34a could significantly inhibit HepG2.2.15 colony formation compared to the negative control group (**Figure 5B**).

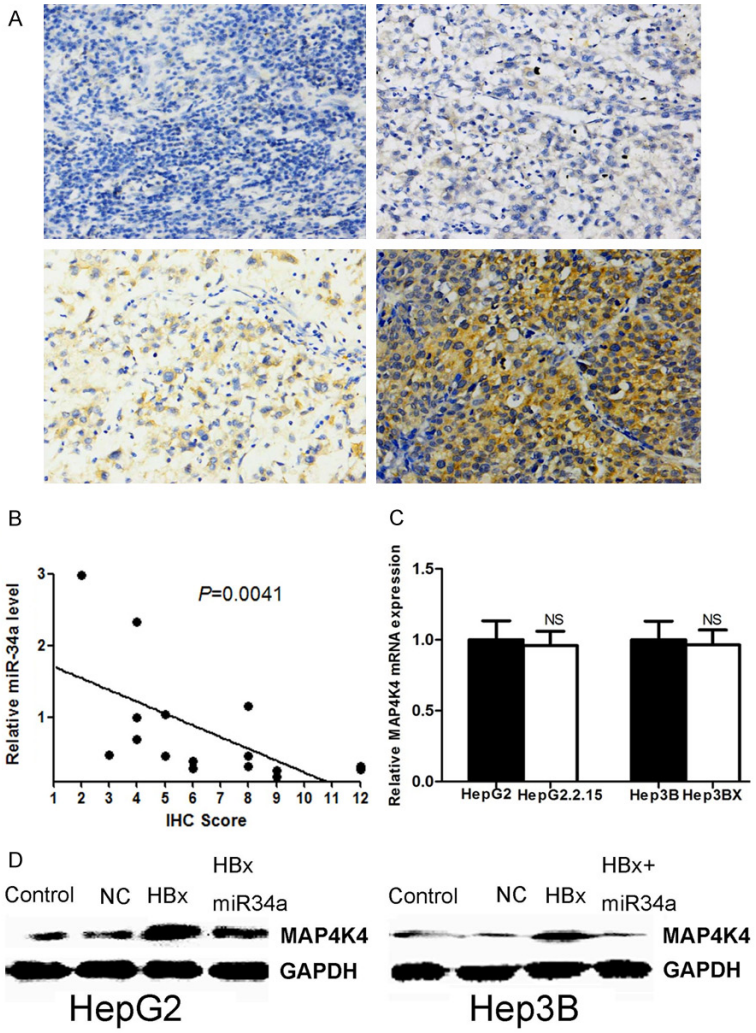

Figure 4. Reverse relationship between MAP4K4 and miR-34a in HBV-related HCC tissues. A. Representative MAP4K4 microphotographs of immunohistochemistry. B. Reverse relationship of MAP4K4 immunohistochemistry scores and relative miR-34a expression in HBV-related HCC tissues and matched noncancerous liver tissues. C. Western blot to test overexpression of miR-34a on HepG2 and/or Hep3B MAP4K4 mRNA and protein expression. D. And miR-34a re-expression in HBx transferred HepG2 and/or Hep3B cells apparently inhibit MAP4K4 upregulation.

Cell apoptosis is an important cause of viability suppression, so we also performed a cell apoptosis assay with a flow cytometer. The percentage of apoptosis in HepG2.2.15 cells was greatly increased in the miR-34a re-expression group (**Figure 5C**). Our results showed that decreased miR-34a had a tumor growth-promoting effect in HBV-related HCC.

Discussion

HBV infection is regarded as the chief cause of HCC in China [16]. However, the intricate mechanisms that promote HBV-related HCC development have not been completely clarified. In addition, there are a small number of therapeutic strategies for HBV-related HCC at present [17]. Recent research results demonstrated that some miRNAs participated in HBV-related HCC progression [18]. For example, miR-122 was downregulated after HBV infection [19]. On the contrary, miR-331-3p [14], miR-181a [20], were upregulated after HBV infection. Moreover, some miRNAs were differently expressed in HBV-related HCC development process and intimately correlated with worse prognosis [21]. In the present study, we found that the expression of miR-34a was obviously decreased in HBV-related HCC tissues and HepG2.2.15 cell line compared with their relative normal controls. This result revealed that HBV infection could inhibit miR-34a expression and miR-34a might supply an effective molecular therapy target for HBV-related HCC.

The interacts between miRNAs and HBV infection in HBV-related HCC are complicate and many questions have not been well clarified, yet [22]. It has been proved that miRNAs could affect viral replication either by directly interacting with the HBV genome or indirectly regulating transcription factors. For example, miR-26b could repress HBV replication by targeting the host factor cysteine- and histidine-rich domain containing 1 (CHORDC1) which increased HBV enhancer/promoter activities and promoted viral transcription, gene expression, and replication [23]. MiRNAs could also indirectly regulate HBV replication. MiR-130a was found to reduce HBV replication by targeting two major metabolic regulators PGC1α and PPARγ, both of which could stimulate HBV replication [24]. On the contrary, HBV infection also adjusted miRNAs

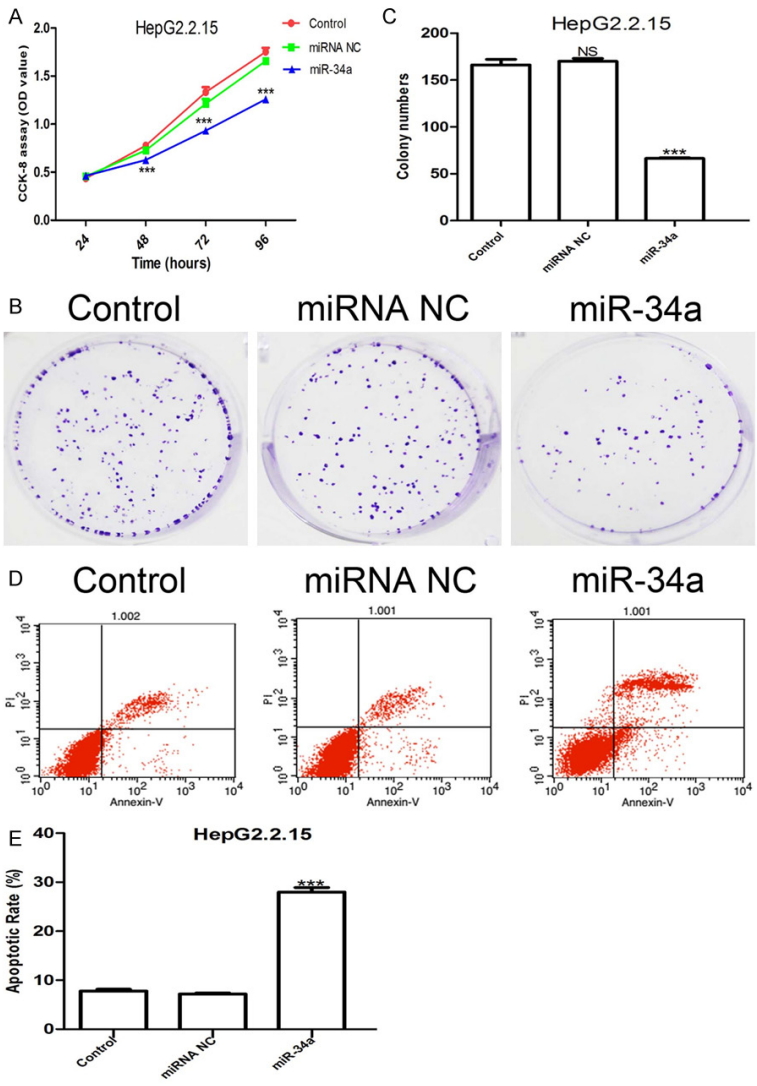

Figure 5. Restored expression of miR-34a in HepG2.2.15. (A) CCK8 to test pre-miRNA transfected or 100 nM miR-34a mimics transfected HepG2.2.15 proliferation. (B) Colony formation assay for proliferation. (C) And it showed that miR-34a could inhibit HepG2.2.15 colony formation. (D) Annexin V-FITC/PI assay for apoptosis determination. (E) Overexpression of miR-34a had the apoptosis induction effect on HepG2.2.15. The results were analyzed with Student's t-test (***P<0.001).

expression and thus promoted HBV-related HCC progression [25, 26].

HBx has been reported to play a key role in HBV-related HCC progression [27]. HBx could induce multiple miRNAs upregulation such as miR-21 [28]. MiR-21 was critical to promote early carcinogenesis of hepatocytes upon HBV infection [29]. However, HBx could suppress miR-205 expression through inducing hypermethylation of miR-205 promoter [30]. It is already demonstrated that human papillomaviruse (HPV) oncogene E6 could effectively decrease miR-34a level by inhibiting p53 expression and that miR-34a is transcriptionally regulated by tumor suppressor p53. This resulted in the increased expression of multiple genes which were targeted by miR-34a. The upregulation of these genes correlatd with tumor cell proliferation, survival and migration [31]. In the present study, we found that HBx could suppress miR-34a expression in HCC cells. And this might explain the downregulation reason of miR34a expression in HBV-related HCC tissues. However, the molecular mechanisms that HBx inhibit miR-34a expression in HCC cells had not been revealed.

MAP4K4 plays an important role in malignant cell proliferation and apoptosis processes [32]. For example, silencing MAP4K4 induces gastric cancer cell apoptosis by increasing the ratio of Bax/Bcl-2 [33]. In HCC, it was also reported that MAP4K4 expression was higher compared to normal liver tissues. And knockdown MAP4K4 expression repressed multiple tumor progression-related signaling pathways including JNK, NF-kB, and toll-like receptors [34]. In the present study, MAP4K4 was identified as one of miR-34a targets by luciferase assay. By overexpression of miR-34a in HepG2.2.15 cells, MAP4K4 protein level was obviously inhibit. This suggest that miR-34a might inhibit cell proliferation and induce apoptosis in HepG2.2.15 by suppressing MAP4K4 expression.

In the whole, this research firstly found that miR-34a was downregulated in HBV-related HCC. MAP4K4 was a directly target of miR-34a. And miR-34a effectively inhibit MAP4K4 expression in HepG2.2.15 cells. MiR-34a overexpression in HepG2.2.15 cells also decreased cell

proliferation and induced apoptosis. However, MAP4K4 surely is not the only molecular target of miR-34a, and more studies are required to deeply elucidate the mechanisms of miR-34a in HBV-related tumorigenesis.

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Disclosure of conflict of interest

None.

Authors' contribution

Conceived and designed the experiments: Qin Ou. Performed the experiments: Qin Ou; Bei Li. Analyzed the data: Wen-Fang Li; Geng Wang. Wrote the paper: Qin Ou. Revisions for the paper: Wen-Fang Li.

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