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miRNA-548ah promotes the replication and expression of hepatitis B virus by targeting histone deacetylase 4

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ABSTRACT

Aim: Many studies have shown that some microRNAs (miRNAs) play an important role in the pathogenesis of chronic hepatitis B (CHB) infection. In this study, we aimed to explore the role and molecular mechanism of miRNA-548ah in the replication and expression of the hepatitis B virus (HBV).

Main methods: Overexpression and knockdown of miRNA-548ah were performed in three hepatoma cell lines with HBV replication and in a murine HBV model injected with adenovirus HBV vector. The effect of miRNA-548ah on its target gene, histone deacetylase (HDAC) 4, were confirmed in in vitro studies and further investigated in liver tissues from CHB patients.

Key findings: miRNA-548ah significantly increased the expression of HBV in hepatoma cell lines and in a HBV mouse model. The expression level of covalently closed circular DNA (cccDNA) in the miRNA-548ah mimics group was significantly higher than the negative control group and significantly lower in the miRNA-548ah inhibitor group. The HBV core antigen promotes the expression of miRNA-548ah in hepatocytes. Finally, we observed a negative correlation between the expression of miRNA-548ah and HDAC4 in the liver tissue of patients with CHB.

Significance: miRNA-548ah promoted the replication and expression of HBV through the regulation of the target gene, HDAC4. Inhibition of HDAC4 by miRNA-548ah might influence the deacetylation state of histones binding to cccDNA, thereby enhancing the replication of cccDNA. The HBV core antigen might increase the expression of miRNA-548ah. These results may provide new potential molecular targets for the prevention and treatment of CHB.

1. Introduction

Chronic hepatitis B (CHB) viral infection is a serious disease with a substantial impact on human health. According to the latest data published by the WHO, there are about 240 million people infected with CHB worldwide, of which 20–30% will progress to liver cirrhosis and hepatocellular carcinoma [1]. Antiviral therapy is an effective method for the treatment of CHB. However, current treatments are limited, due to factors, such as viral relapse after drug withdrawal and other adverse reactions [2]. Thus, there is an urgent need to elucidate the pathogenesis of CHB infection and explore new methods of diagnosis and treatment.

MicroRNAs (miRNAs) are small RNA molecules that regulate gene expression at the post-transcriptional level and play important roles in many physiological and pathological mechanisms [3]. Recent evidence has suggested that miRNAs play a role in CHB infection [4,5]. During CHB infection, the products of viral replication and expression lead to abnormal expression of endogenous miRNAs and contribute to persistent infection [6,7]. However, host cell miRNAs, such as miRNA-122, miRNA-1231, and miRNA-1, affect the replication and expression of hepatitis B virus (HBV) by direct or indirect mechanisms [8,9].

Previous studies have shown that miRNA-548 is a poorly conserved mammalian-specific gene family. There are 69 miRNA-548 species in humans, which are distributed on almost all of the human chromosomes [10]. Recent studies have shown that miRNA-548 family members may play an important role in regulating the immune response, especially the interferon signal response [11,12].

We previously determined that miRNA-548ah is highly expressed in

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the PBMCs of CHB patients [6]. However, whether miRNA-548ah is expressed in liver cells, and how it might affect the replication and expression of HBV, has not yet been reported. In this study, the effects of miRNA-548ah on HBV replication and expression and its mechanism of action were investigated. The findings presented herein provide new potential molecular targets for the prevention and treatment of CHB.

2. Materials and methods

2.1. Subjects

Liver tissue samples from 18 patients with CHB infection were collected from October 2015 to December 2016 in Taizhou People's Hospital, Taizhou, China. Diagnostic criteria were based on the 2010 Chronic Hepatitis B Prevention Guide of China [13]. Patients were excluded for any of the following criteria: (1) receiving therapy with nucleos(t)ide analogues, interferon-α, or steroids in the preceding six months; (2) comorbidity with other causes of liver disease, such as hepatitis A, C, D, or E virus, pregnancy, alcoholism, or drug abuser; or (3) comorbidity with HIV infection, or severe active disease (e.g., heart, brain, kidney, hypertension, and diabetes). This experimental protocol was approved by the ethical commission of Taizhou People's Hospital. Written informed consent was obtained from all subjects.

2.2. Materials

HepG2 cells, HepG2.2.15 cells, Huh7 cells, and 293 T cells were purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China). miRNA-548ah mimics and inhibitors, agomiRNA-548ah, and the negative control were purchased from Genepharma Co., Ltd. (Shanghai, China). Lipofectamine™ and the RNA extraction kit were purchased from Invitrogen (Hercules, CA, USA). SYBR Green I was purchased from TaKaRa Bio Inc. (Beijing, China). Hepatitis B surface antigen (HBsAg) and hepatitis B e-antigen (HBeAg) chemiluminescent microparticle immunoassay kits were purchased from American Abbott (Chicago, IL, USA). A HBV DNA real-time PCR kit was purchased from Acon Biotech Co., Ltd. (Hangzhou, China). The psiCHECK-2 plasmid vector was purchased from Promega Corporation (Madison, WI, USA). Mouse anti-human histone deacetylase (HDAC) 4 antibody was purchased from Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA). The mouse anti-hepatitis B c-antigen (HBcAg) was purchased from Abcam (Cambridge, MA, USA). The acetylated H3 histone specific antibody (AcH3) and the total H3 histone specific antibody were purchased from Millipore (Burlington, MA, USA). The rAAV8-1.3HBV virus was purchased from Beijing FivePlus Molecular Medicine Institute Co. Ltd. (Beijing, China). The pHBV1.3 plasmid and HDAC4 expression plasmid were kindly provided by Professor Zhang Xiaoyong (Southern Medical University, Guangzhou, China).

2.3. Cell culture and transfection

HepG2 cells, HepG2.2.15 cells, and Huh7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS)(Gbico, Grand Island, USA) at 37 °C in a 5% CO_2 incubator. miRNA-548ah mimics and inhibitors were transfected into HepG2.2.15 cells using Lipofectamine reagent. HepG2 cells and Huh7 cells were co-transfected with pHBV1.3 plasmid and miRNA-548ah mimics or inhibitors using Lipofectamine, respectively. Cell culture supernatants and cells were collected at 48 h or 72 h post-transfection, respectively.

HepG2.2.15 cells were transfected with siRNA, HDAC4-over-expressing plasmid, miRNA-548ah mimics, or miRNA-548ah inhibitors using Lipofectamine reagent. The cells were collected at 48 h post-transfection and detected by fluorescence quantitative PCR and western blot.

2.4. Quantitative detection of miRNA-548ah and HDAC4

The total RNA of cells was extracted using TRIzol(Invitrogen, Carlsbad, CA, USA). The primers of *miRNA-548ah*, *HDAC4*, *U6*, and *GAPDH* were designed and synthesized by Shanghai Bioengineering Co., Ltd. (Supplemental Table 1). cDNA was synthesized through reverse transcription. The PCR reaction was carried out on the real-time fluorescent quantitative PCR detector (ABI 7300; Thermo Fisher Scientific, Waltham, MA, USA). The reaction conditions of the *miRNA-548ah* PCR were as follows: 1 cycle at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 40 s. The small nuclear RNA *U6* gene was used as an internal reference. *HDAC4* and *GAPDH* reaction conditions were as follows: 1 cycle at 95 °C for 30 s, followed by 40 cycles at 95 °C for 15 s and 60 °C for 30 s. For HBV pregenomic RNA (pgRNA) amplification, the primers were designed and synthesized by Shanghai Bioengineering Engineering Co., Ltd. (Supplemental Table 1). PCR reaction conditions were as follows: 1 cycle at 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s and 60 °C for 60 s. *GAPDH* was used as internal reference. The ΔΔCt method was used to calculate the ratio of gene expression relative to the control gene in the experiments.

2.5. Western blotting

Cells were harvested using 1 × RIPA buffer and incubated on ice for 10 min. The supernatant was immediately transferred into a new centrifuge tube after centrifugation. The protein concentration was determined by using a bicinchoninic acid (BCA) assay. The total protein of each sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride membranes. The membranes were blocked with 5% BSA solution and incubated with anti-human HDAC4 and HBcAg antibodies. Secondary antibodies labeled with horseradish peroxidase were added to the membranes and incubated. GAPDH was used as the loading control. Enhanced chemiluminescence was used to visualize the membranes prior to imaging. The gray value of each specific strip was digitized by ImageJ analysis software (NIH, Bethesda, MD, USA)(https://imagej.en.softonic.com/).

2.6. Construction of reporter gene vectors and detection of luciferase activity

The miRNA-548ah binding sites at the 3′ untranslated region (3′ UTR) of the *HDAC4* gene and HBV sequence were synthesized. Restriction enzyme sites for *Xho* I and *Not* I were added at both ends of the fragment, respectively. The synthetic gene fragments were cloned into the PUC57 vector and confirmed by sequencing. PUC57 vectors were then subcloned into psiCHECK-2 plasmid vectors, identified by restriction enzyme digestion, and confirmed by sequencing. Site-directed mutagenesis was performed at the 3′ UTR seed area of the *HDAC4* and *HBV* sequence (1091–1097) by using a site-directed mutagenesis kit(NEB, Ipswich, MA, USA). Both the psiCHECK-HDAC4-mut group and psiCHECK-HBV-mut group were verified by sequencing. Next, 293 T cells were co-transfected with the plasmids and miRNA-548ah mimics by using Lipofectamine 2000. The cells were cultured for 4 h, and then the media was replaced with complete solution and incubated for 48 h. Luciferase assays were performed using the Dual-luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA). Firefly luciferase was used as the internal reference. The ratios of luciferase activity were calculated between Renilla and firefly luciferase.

2.7. Construction and transfection of pcDNA3.1 plasmid vector expressing HBV S antigen, C antigen, and X antigen

The pcDNA3.1 plasmid vectors expressing HBV S antigen, C antigen, or X antigen were constructed. HepG2 cells were transfected with the

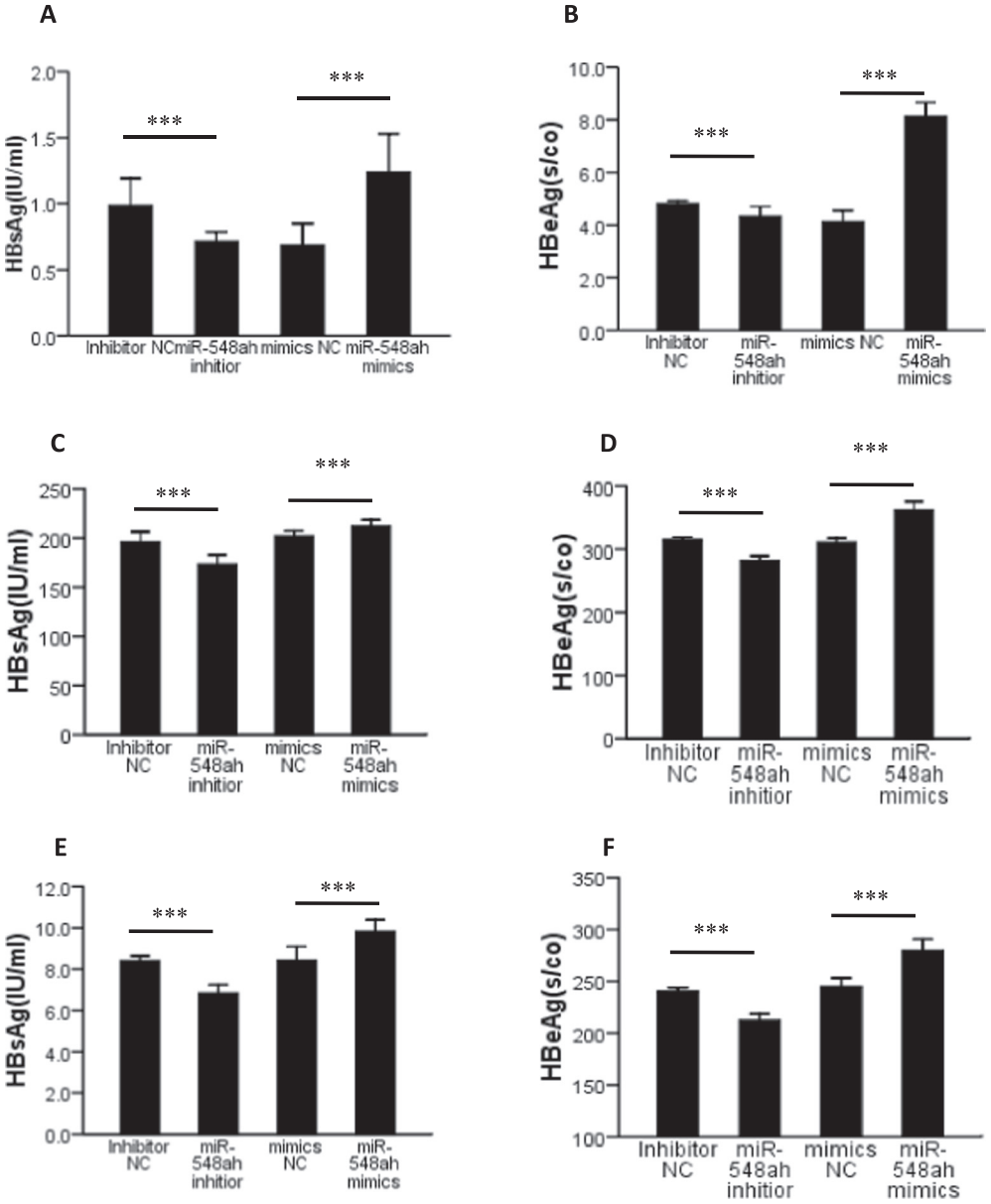

Fig. 1. Effect of miRNA-548ah on expression of HBV in three hepatoma cell lines. A and B are the expression of HBsAg and HBeAg in HepG2 cell transfected with pHBV1.3. C and D are the expression of HBsAg and HBeAg in Huh7 cell transfected with pHBV1.3. E and F are the expression of HBsAg and HBeAg in HepG2,2,15 cell. *** $P < 0.001$.

three plasmids and negative control space carrier (NC) using Lipofectamine reagent. The transfection medium was replaced after 6 h of transfection with DMEM culture medium containing 10% FBS. The cultured cells were collected at 72 h. The levels of *miRNA-548ah* cDNA were detected by Real time-PCR.

2.8. Detection of HBV DNA, HBsAg, and HBeAg

The levels of HBsAg and HBeAg in the cell culture supernatant were detected by using a chemiluminescent microparticle immunoassay, according to the manufacturer's instructions. The level of HBV DNA in the cell culture supernatant was detected by fluorescence quantitative PCR. The lower limit of HBV DNA quantitative detection was 20 IU/mL.

2.9. CCK-8 cell proliferation assay

HepG2.2.15 cells were routinely cultured and transfected with miRNA-548ah mimics or inhibitors by Lipofectamine, according to the manufacturer's instructions. The cells were harvested into single cell suspensions 48 h post-transfection. The cells were inoculated into cell culture plates and treated with CCK-8 at 0, 24, 48, 72, and 96 h. The OD values were detected at 450 nm using a microplate reader.

2.10. HBV mouse model

Recombinant adeno-associated (rAAV)8–1.3HBV virus (1×10^{12} vg/100 μL/mouse) was injected into C57BL/6 mice (6-week-old males) via the tail vein. Serum was collected at four weeks post-injection and stored at −80 °C. All mice were divided into two groups ($n = 6$/group). The agomiRNA-548ah (1 OD/100 μL/mouse) and negative control (1 OD/100 μL/mouse) were injected into the tail vein on days 0, 4, 7, and 10. Serum was collected on days 5, 9, 14, and 21 post-injection and stored at −80 °C. Two mice were euthanized on day 7, and the remaining mice were euthanized at day 21. The protocol was approved by the Committee on the Ethics of Animal Experiments of Affiliated Fifth Hospital of NanTong University.

2.11. Chromosome immunoprecipitation assays

HepG2.2.15 cells were collected and resuspended in 1 mL of ChIP lysis buffer. The nuclei were collected after centrifugation and fixed with 1% formalin (final concentration) and incubated for 10 min at 37 °C. The nuclei were washed two times with phosphate buffer saline, and then SDS lysis buffer containing protease inhibitors was added. The sample was then incubated for 30 min on the ice. The resulting chromatin solution was ultrasonicated for 10 min using a Bioruptor (Diagenode, Liege, Belgian). The sample was then diluted with buffer, added to magnetic beads coupled to anti-rabbit IgG, and mixed for 30 min at 4 °C. The beads were absorbed for 2 min at 4 °C with Magnetic Separation Rack(BioMag Scientific Inc., Wuxi, China). The supernatants were divided into two groups and then to immunoprecipitated overnight at 4 °C using antibodies specific to AcH3 and H3. The corresponding non-specific IgGs were used as negative controls. The ChIP elution buffer was added and incubated for 2 h at 62 °C. RNase (final concentration 1 μg/mL) were added and incubated for 20 min at 37 °C. The beads were absorbed for 2 min at 4 °C with Magnetic Separation Rack. The target DNA fragments were eluted and purified. The products were amplified by PCR. The reaction conditions were as follows: 1 cycle at 95 °C 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s.

2.12. Statistical analysis

Data analysis was performed using SPSS17.0 statistical software (SPSS, Inc., Chicago, IL, USA). Data were expressed as mean ± standard deviation. Student's *t*-test was used for comparison between two groups, and one-way ANOVA and SNK-q tests were used for multiple comparisons. The X^2 test was used for count data. Pearson's correlation was used to determine associations between variables. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of miRNA-548ah on the replication of HBV in three hepatocellular carcinoma cell lines

HepG2 cells and Huh7 cells were co-transfected with the pHBV1.3 plasmid and miRNA-548ah mimics or inhibitors. Our results showed that the expression levels of HBsAg, HBeAg, and HBV DNA were significantly increased in the miRNA-548ah mimics group as compared with the mimics control group. The levels of HBsAg, HBeAg, and HBV DNA were significantly decreased when comparing the miRNA-548ah inhibitors group with the control group ($F = 20.61$, 276.87, 110.03, $P < 0.001$) (Fig. 1A, B, and Fig. 2A) and ($F = 51.45$, 197.65, 275.09, $P < 0.001$) (Fig. 1C, D, and Fig. 2B). Compared with control group, the levels of HBsAg, HBeAg, and HBV DNA in HepG2.2.15 cells transfected with miRNA-548ah mimics were significantly increased; however, they were decreased in HepG2.2.15 cells transfected with miRNA-548ah inhibitors ($F = 73.09$, 163.48, 3079.39, $P < 0.001$) (Fig. 1E, F, and Fig. 2C). Furthermore, the levels of HBcAg expression were detected in HepG2, Huh7, and HepG2.2.15 cells by western blot. The results showed that compared to the negative control group, HBcAg expression was significantly increased in the miRNA-548ah mimics group and significantly decreased in the miRNA-548ah inhibitor group (Supplemental Fig. 1).

3.2. Effect of miRNA-548ah on the proliferation of HepG2.2.15 cells

The effect of miRNA-548ah on the proliferation of hepatoma cell lines was detected by CCK-8 analysis. The results showed that the cellular proliferation of the miRNA-548ah mimics group was slightly increased relative to the control group at 24, 48, and 72 h ($P = 0.078$). However, there was no significant difference in the cellular proliferation of the miRNA-548ah inhibitors group compared to the control group at 24, 48, and 72 h ($P = 0.399$) (Supplemental Fig. 2).

3.3. Effect of miRNA-548ah on the replication of HBV in a murine HBV infection model

AgomiRNA-548ah is specially labeled and chemically modified microRNA. An in vivo HBV infection model was established, and agomiRNA-548ah was injected into the tail vein of mice in order to assess the impact of miRNA-548ah on HBV replication over 21 days. There was no significant difference in the expression level of HBV DNA between agomiRNA-548ah-treated mice and agomiRNA-negative control mice at day 5 post-injection ($t = 1.43$, $P = 0.204$). However, the expression level of HBV DNA in the serum of the agomiRNA-548ah group was significantly increased compared to the the agomiRNA-negative control group at days 9, 14 and 21 post-injection ($t = 3.35$, 6.52, 3.42, $P = 0.015$, 0.001, 0.014, respectively) (Fig. 3A).

3.4. Prediction of target genes and analysis of the miRNA-548ah binding sites

Target genes of miRNA-548ah were predicted by using the Targetscan, Mirdb, and MiRanda databases. From the three databases, 195 common candidate target genes of miRNA-548ah were identified. HDAC4 was one of the candidate genes. The binding sites of miRNA-548ah and HDAC4 were analyzed by using Targetscan. We identified three binding sites of miRNA-548ah in the *HDAC4* gene sequence (Supplemental Fig. 3).

3.5. Expression of miRNA-548ah and HDAC4 in human hepatocellular carcinoma cell lines and HBV mouse liver tissue

The mRNA expression of *miRNA-548ah* and *HDAC4* molecules were detected in three different hepatocellular carcinoma cell lines by fluorescence qPCR. Our results showed that the mRNA expression of *miRNA-548ah* in Huh7, HepG2, and HepG2.2.15 cells was 0.70 ± 0.26, 1.72 ± 0.55, and 2.37 ± 0.82, respectively ($F = 6.14$, $P = 0.035$). The expression of *HDAC4* in Huh7, HepG2, and HepG2.2.15 cells was 0.97 ± 0.03, 14.31 ± 1.71, and 7.65 ± 0.05, respectively ($F = 136.96$, $P < 0.001$) (Fig. 5A). Moreover, the expression level of *miRNA-548ah* and *Hdac4* in the liver tissue of HBV-infected mice was detected by fluorescence qPCR. The results showed that the expression level of *miRNA-548ah* in the experimental group was significantly increased after miRNA-548ah treatment compared with the negative control group (2.73 ± 1.36 vs. 0.12 ± 0.09, $t = 3.84$, $P = 0.009$). The expression level of *Hdac4* was decreased compared with the negative control group (1.09 ± 0.22 vs. 1.39 ± 0.10, $t = 2.54$, $P = 0.040$) (Fig. 3B).

3.6. Effect of miRNA-548ah on the expression of HDAC4 mRNA and protein in HepG2.2.15 cells

The effect of miRNA-548ah on the expression of *HDAC4* mRNA was detected in HepG2.2.15 cells by fluorescence qPCR. The results showed that the level of *HDAC4* significantly decreased upon transfection of miRNA-548ah mimics and significantly increased upon the transfection of miRNA-548ah inhibitors compared to the control group ($F = 9.34$, $P < 0.01$) (Fig. 4A). Western blot results showed that the expression of HDAC4 protein was significantly inhibited in HepG2.2.15 cells transfected with miRNA-548ah mimics compared with the control group ($t = 3.58$, $P = 0.02$). Furthermore, the expression of HDAC4 protein was slightly increased in HepG2.2.15 cells transfected with miRNA-548ah inhibitor compared with the control group; however, the difference was not statistically significant ($t = 0.99$, $P = 0.38$) (Fig. 4C and D).

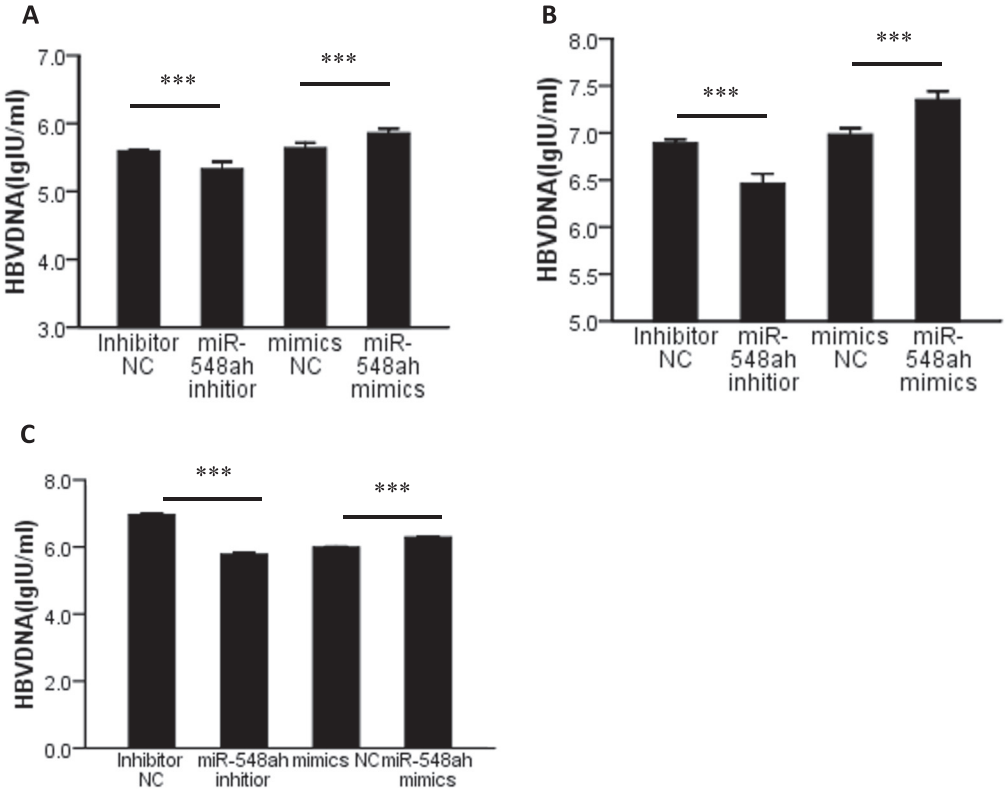

Fig. 2. Effect of miRNA-548ah on replication of HBV in three hepatoma cell lines. A is the levels of HBV DNA in HepG2 cell transfected with pHBV1.3. B is the levels of HBV DNA in Huh7 cell transfected with pHBV1.3. C is the levels of HBV DNA in HepG2,2,15 cell. GAPDH were used as internal reference. *** $P < 0.001$.

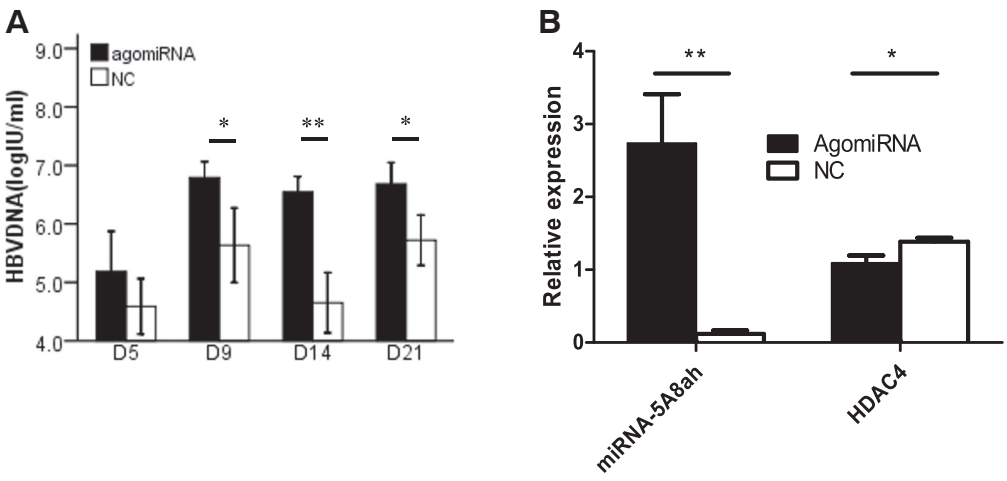

Fig. 3. Effect of miRNA-548ah on replication of HBV in HBV mouse model. (A) The expression of HBVDNA increased in HBV mouse injected with agomiRNA-548ah than that of negative controls at day 9, day 14, and day 21. (B) The expression of miRNA-548ah are higher than that of negative controls and HDAC4 are lower than that of negative controls in mouse liver tissue. U6 and GAPDH were used as internal reference. * $P < 0.05$ and ** $P < 0.01$.

3.7. Effect of miRNA-548ah on the luciferase activity of a double luciferase expression vector of HDAC4

The double luciferase expression vectors containing the HDAC4 wild type or mutant type (3170–3176 location of the mutated sequence) sequence were successfully constructed, identified by restriction enzyme digestion, and confirmed by sequencing. The sequencing analyses are depicted in Supplemental Fig. 4. The luciferase activity assay showed that the luciferase activity of psiCHECK-HDAC4 was significantly inhibited by miRNA-548ah mimics compared to the control group ($t = 3.18$, $P = 0.03$). No significant inhibition of luciferase activity of the psiCHECK-HDAC4-mut in the miRNA-548ah mimics group was observed ($t = 1.66$, $P = 0.17$) (Fig. 4B).

3.8. Expression and correlation of miRNA-548ah and HDAC4 in liver tissues of patients with CHB

The mRNA levels of *miRNA-548ah* and *HDAC4* in the liver tissue of 18 patients were detected by fluorescence qPCR. The baseline data is presented in Supplemental Table 2. The mean expression levels of *miRNA-548ah* and *HDAC4* were 1.26 ± 0.73 and 1.04 ± 0.44, respectively, in all 18 patients. Pearson's correlation analysis showed no significant correlation between the expression of *miRNA-548ah* and *HDAC4* ($r = 0.175$, $P = 0.488$). Of the 18 cases, 9 were CHB cases and 9 were HBV-related liver cirrhosis cases. The expression of *miRNA-548ah* in the liver tissue of patients with CHB (0.90 ± 0.56) was significantly lower than the expression of *miRNA-548ah* in the liver tissue of patients with HBV-related liver cirrhosis (1.62 ± 0.71) ($t = 2.41$, $P = 0.028$). There was no statistically significant difference found regarding the

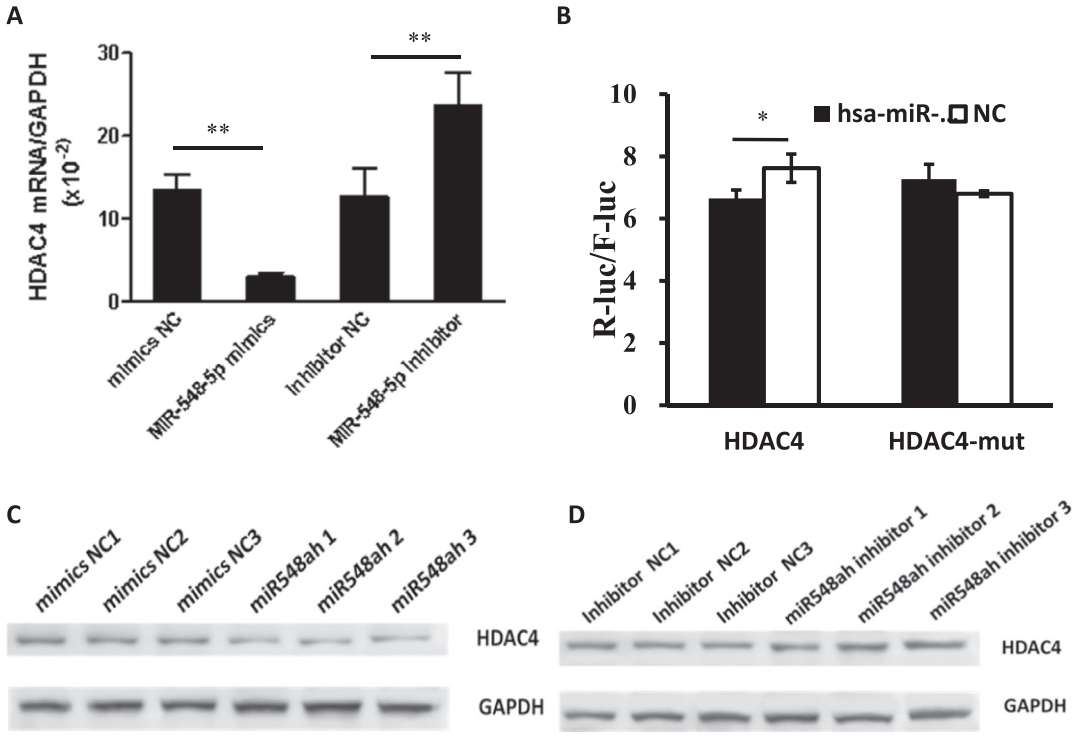

Fig. 4. Effect of miRNA-548ah on expression of HDAC4 in HepG2.2.15 cell lines. (A) The expression of HDAC4 increased and decreased in miRNA-548ah mimics group and miRNA-548ah inhibitors group than that of negative controls, respectively. (B) Effect of miRNA-548ah on luciferase activity of double luciferase recombinant vector containing HDAC4. C and D are the effect of miRNA-548ah on expression of HDAC4 protein. GAPDH was used as internal reference. * $P < 0.05$ and ** $P < 0.01$.

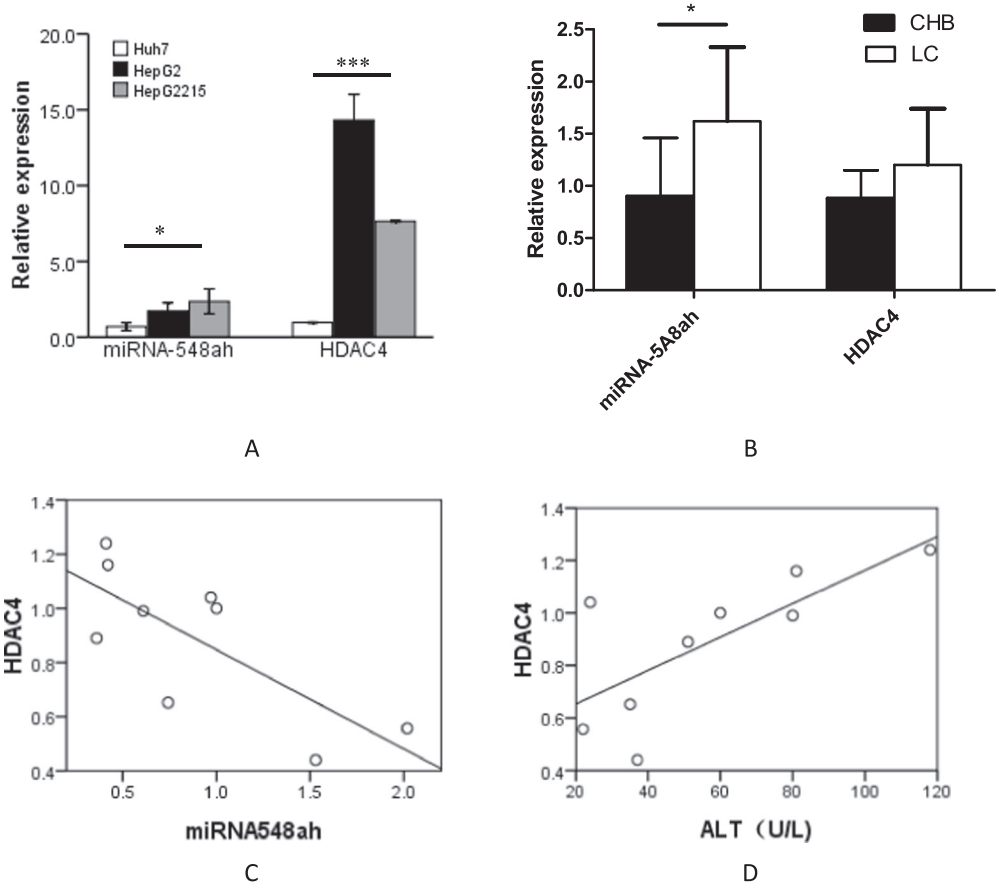

Fig. 5. Expression and correlation of miRNA-548ah and HDAC4 in human liver tissues and hepatoma cell lines. (A) Expression of miRNA-548ah and HDAC4 in hepatoma cell lines. (B) Expression of miRNA548ah and HDAC4 in patients with chronic hepatitis B(CHB) and liver cirrhosis(LC). (C) Correlation between HDAC4 and miRNA548ah in patients with chronic hepatitis B. (D) Correlation between HDAC4 and ALT in patients with chronic hepatitis B. U6 and GAPDH were used as internal reference. * $P < 0.05$ and *** $P < 0.001$.

expression of *HDAC4* in the liver tissue between the two groups of patients (CHB: 0.88 ± 0.27; cirrhosis: 1.20 ± 0.54) ($t = 1.52$, $P = 0.146$) (Fig. 5B).

3.9. Correlation between miRNA-548ah and HDAC4 in liver tissues and alanine aminotransferase (ALT) levels in CHB patients

Pearson's correlation analysis showed that the expression of miRNA-548ah was inversely correlated with HDAC4 in the liver tissue of patients with CHB ($r = 0.746$, $P = 0.021$) (Fig. 5C). No significant correlation was found between the expression of miRNA-548ah and HDAC4 in the liver tissue of patients with HBV-related cirrhosis ($r = -0.341$, $P = 0.369$). Furthermore, there was no significant correlation between the levels of miRNA-548ah in the liver tissue and HBV DNA or ALT levels in the serum ($r = 0.192$, $P = 0.621$; $R = 0.666$, $P = 0.050$). There was no significant correlation between the expression of HDAC4 in liver tissue and HBV DNA levels in the serum ($r = 0.129$, $P = 0.741$). However, the expression level of HDAC4 in liver tissue was positively correlated with serum ALT levels ($r = 0.733$, $P = 0.025$) (Fig. 5D).

3.10. Direct effect of miRNA-548ah on the replication of HBV

Bioinformatic prediction revealed that there was a miRNA-548ah binding site in the genome of HBV C and D genotype (1091–1097) (Fig. 6A). Luciferase expression vectors containing HBV wild type and mutant (1091–1097 position of mutated sequence) were successfully constructed, identified by restriction enzyme digestion, and confirmed by sequencing. The luciferase activity was detected by a dual luciferase reporter kit. Our results showed that there was no significant effect of hsa-miR-548ah on the luciferase activity of psiCHECK-HBV vector and psiCHECK-HBV-mut vector ($t = 0.28$, 0.05, $P > 0.05$) (Fig. 6B).

3.11. Effect of different HBV antigens on miRNA-548ah expression in HepG2 cells

The expression of *miRNA-548ah* in HepG2 cells transfected with HBV S, C, or X antigen plasmids was detected by fluorescence qPCR. Our results showed that the expression level of *miRNA-548ah* in HBV S antigen and X antigen plasmid groups was not significantly different. However, the expression of *miRNA-548ah* was significantly increased in the HBV C antigen plasmid group compared with the control group ($F = 13.29$, $P = 0.001$) (Fig. 6C).

3.12. Effect of co-transfection of miRNA-548ah and HDAC4 on HBV pregenomic RNA (pgRNA) and HBcAg

miRNA-548ah mimics and HDAC4-overexpression plasmid or miRNA-548ah inhibitor and HDAC4-interference fragment (si2981) were co-transfected into HepG2.2.15 cells. We then analyzed the expression level of HBV pgRNA by fluorescence qPCR and the expression of HBcAg by western blot. There was no statistically significant difference in the expression level of HBV pgRNA between miRNA-548ah mimics and the HDAC4-overexpressed plasmids group and negative control group (1.05 ± 0.12 vs. 0.90 ± 0.11) ($t = 1.59$, $P = 0.19$). The expression level of HBV pgRNA in miRNA-548ah inhibitor and HDAC4-interference fragment co-transfection group and the negative control groups was 0.71 ± 0.02 and 0.67 ± 0.07, respectively; this difference was not statistically significant ($t = 0.83$, $P = 0.45$). The expression of HBcAg in the miRNA-548ah mimics and the HDAC4-overexpression plasmid co-transfection group and the negative group was 0.260 and 0.295, respectively. The expression of HBcAg in the miRNA-548ah inhibitor group and HDAC4-interference fragment co-transfection group and the negative group were 0.256 and 0.175, respectively.

3.13. Effect of miRNA-548ah and HDAC4 on the expression of covalently closed circular DNA (cccDNA)

We performed a ChIP assay using HepG2.2.15 cells transfected with miRNA-548ah mimics, miRNA-548ah inhibitors, or HDAC4 siRNA vector. We detected the expression of cccDNA by fluorescence qPCR. The results showed that the expression level of cccDNA in the AcH3 group was significantly increased relative to the negative control group (1.22 ± 0.11 vs. 0.29 ± 0.05) ($t = 14.02$, $P < 0.001$). However, the

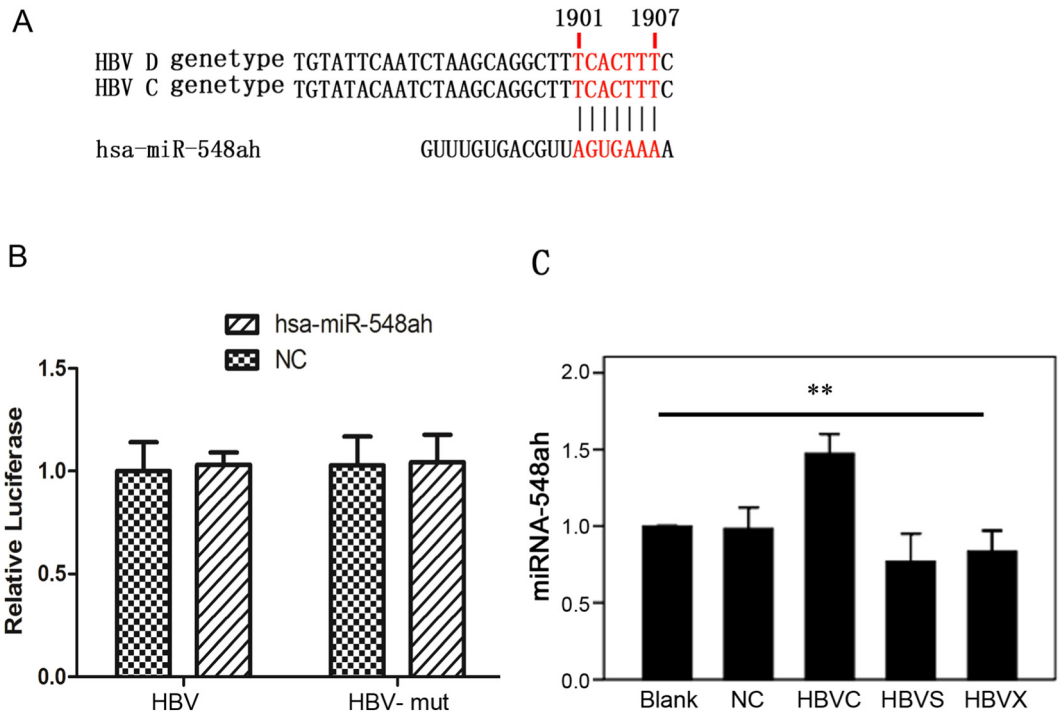

Fig. 6. Interaction of miRNA-548ah and HBV. (A) Binding sites of miRNA-548ah and HBV C gene type sequence. (B) Direct effect of miRNA-548ah on replication of HBV. (C) Effect of different HBV antigen on miRNA-548ah expression in HepG2 cells. U6 was used as internal reference. ** $P < 0.01$.

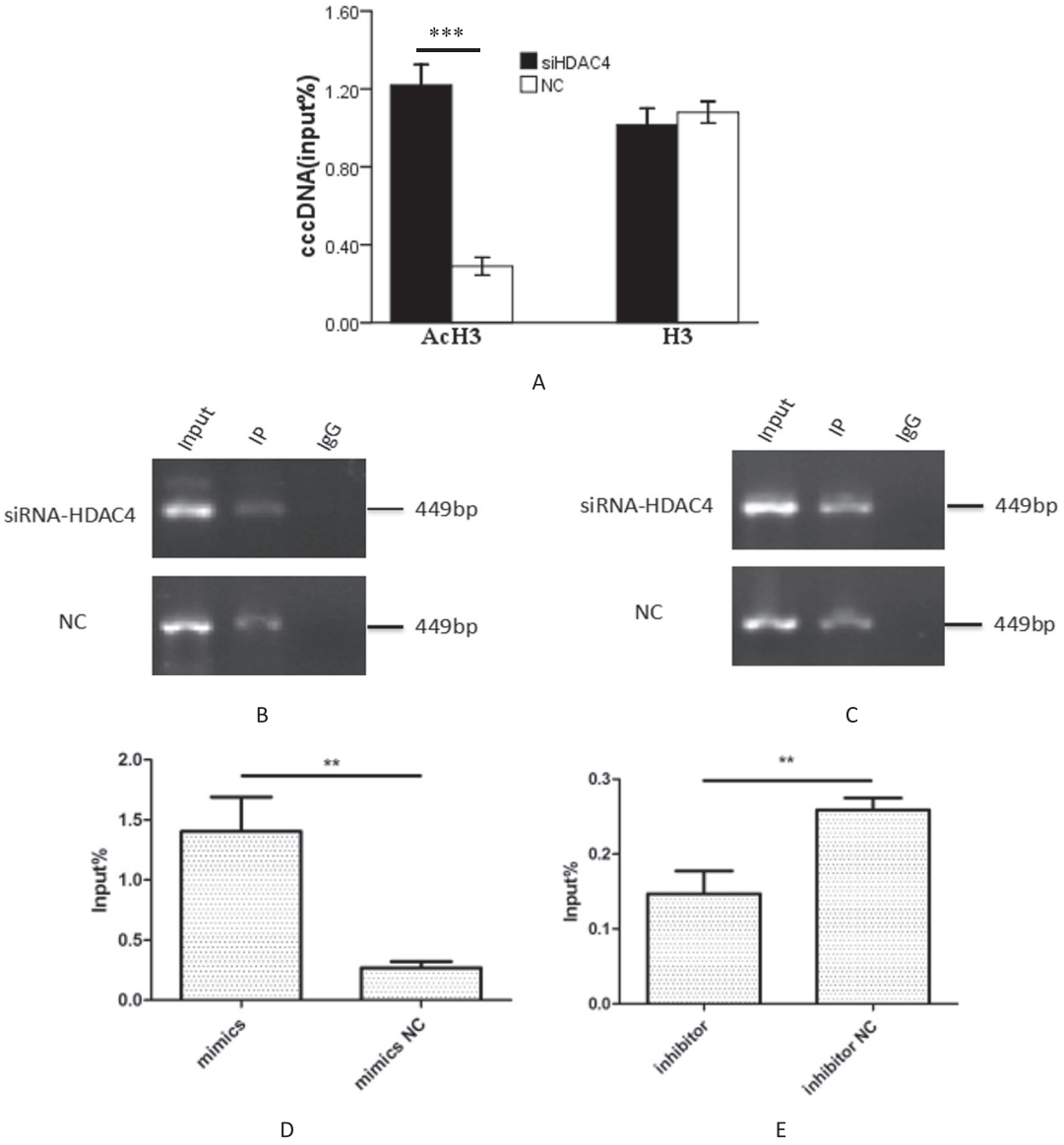

Fig. 7. cccDNA expression after HDAC4 interference and miRNA-548ah in HepG2.2.15 cell lines. (A) cccDNA expression after HDAC4 interference in HepG2.2.15 cell lines. (B and C) electrophoretogram of cccDNA expression in Chip of two group (B:AcH3;C:H3). (D) Effect of cccDNA expression of miRNA-548ah mimics in HepG2.2.15 cell lines (AcH3). (E) Effect of cccDNA expression of miRNA-548ah inhibitors in HepG2.2.15 cell lines (AcH3). ** $P < 0.01$ and ***$P < 0.001$.

expression level of cccDNA in the H3 group was not significantly different than the negative control group (1.01 ± 0.08 vs. 1.08 ± 0.05) ($t = 1.09$, $P = 0.337$) (Fig. 7A–C). Compared with the negative control group, the expression level of cccDNA in the miRNA-548ah mimics group significantly increased (1.40 ± 0.29 vs. 0.27 ± 0.06) ($t = 6.70$, $P = 0.003$) and significantly decreased in the miRNA-548ah inhibitor group (0.15 ± 0.03 vs. 0.26 ± 0.02) ($t = 5.19$, $P = 0.007$) (Fig. 7D, E).

4. Discussion

Previous studies have shown that there are many abnormal expression patterns of miRNA molecules, such as miRNA-122, miRNA-199, and miRNA-20, in CHB infection, which may play an important role in regulating the replication and expression of HBV and disease progression [14–16]. miRNA-548ah was previously identified to be highly expressed in the PBMCs of CHB patients [17]. In this study, the expression and function of miRNA-548ah in hepatocytes were explored. Our results showed that miRNA-548ah was expressed in hepatoma cell lines, and specifically, the expression of miRNA-548ah was lower HepG2 cells than in HepG2.2.15 cells. The levels of HBV DNA, HBsAg, and HBeAg in cell culture supernatants were significantly increased in HepG2.2.15 cells transfected with miRNA-548ah mimics and decreased upon transfection with miRNA-548ah inhibitors. One miRNA-548ah binding site was found in the genome sequence of HBV C and D genotypes; however, miRNA-548ah displayed no direct effect on the replication of HBV through the detection of luciferase activity. Moreover, no significant effect on the proliferation of hepatoma cell lines was found upon transfection with miRNA-548ah mimics or inhibitors. miRNA-548ah promoted the replication and expression of HBV in mice infected with recombinant HBV adenovirus vector. These results suggest that miRNA-548ah can promote the replication and expression of HBV, and its mechanism is worthy of further research.

The acetylation and deacetylation of histones is an important mechanism by which gene expression is regulated. This process is primarily modulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs). The acetylation of histones promotes transcription, while the deacetylation of histones inhibits transcription.

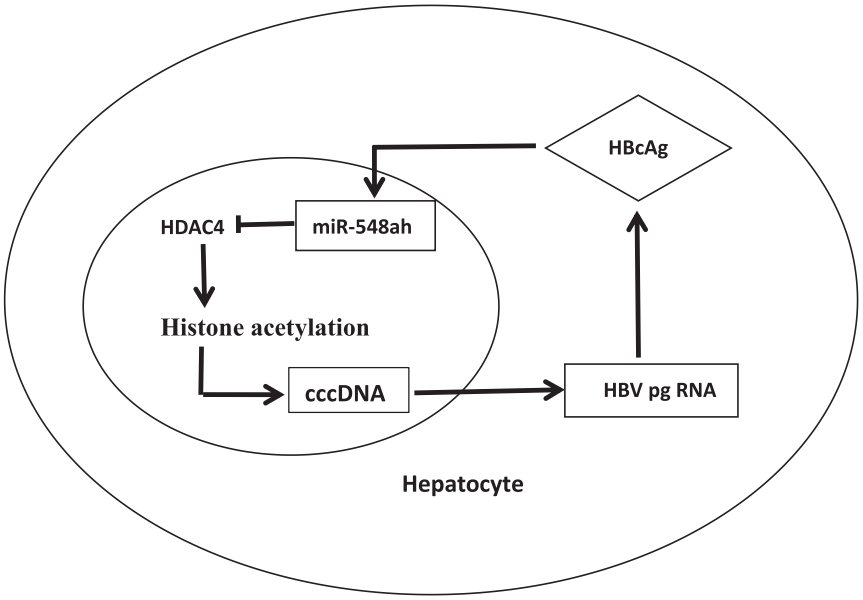

Fig. 8. Feedback loop of interaction between miRNA-548ah and HBV in liver cells. miRNA-548ah might promote the replication and expression of HBV through the regulation of its target gene, HDAC4. Consequently, HDAC4 might increase the replication of cccDNA through inhibiting the deacetylation of histone combining with cccDNA. HBV C antigen might enhance the expression of miRNA-548ah in hepatocyte. Interactions between miRNA-548ah and HBV forms a feedback loop, which plays an important role in the mechanism of chronic HBV persistent infection.

Bioinformatic analysis showed that HDAC4 is one of the candidate gene targets of miRNA-548ah. Consequently, we found three miRNA-548ah binding sites within the *HDAC4* gene sequence. Studies have previously shown that HDAC4 can regulate the expression of genes in multiple cell types, and HDAC4 possessed various regulatory functions involved in gene transcription, cell growth, and development [18]. Furthermore, HDAC4 plays an important role in the pathogenesis of some diseases, such as breast cancer and diabetic nephropathy [19,20]. Our results showed that HDAC4 was expressed in HepG2 cells and HepG2.2.15 cells, which is consistent with the report by Yuan et al. [21]. The level of HDAC4 expression was significantly lower in HepG2.2.15 cells than in HepG2 cells, which was the opposite of miRNA-548ah expression in these two cells lines. The inhibition of miRNA-548ah expression increased the expression of HDAC4 in HepG2.2.15 cells. The expression of miRNA-548ah was negatively correlated with the expression of HDAC4. Western blot analysis showed that the expression of HDAC4 protein was significantly inhibited in HepG2.2.15 cells transfected with miRNA-548ah mimics. The luciferase activity of psiCHECK-HDAC4 vector was significantly inhibited after transfection of miRNA-548ah mimics. Importantly, the expression of miRNA-548ah was inversely correlated with HDAC4 in liver tissues of patients with CHB. These studies suggest that HDAC4 is likely a target gene of miRNA-548ah.

Some reports have shown that the activity of cccDNA is regulated by the change in the acetylation state of histones H3 and H4, which in turn affects the transcription and expression of HBV in vivo and in vitro [22,23]. Interferon alpha (IFN-α) is one of the most effective drugs for the treatment of CHB. Belloni et al. [24] found that IFN-α can lead to a decrease in the degree of histone acetylation combined with cccDNA, thereby inhibiting cccDNA transcription. Palumbo et al. [25] suggested that IL-6 could inhibit the replication of HBV by targeting the epigenetic control of cccDNA. Some miRNAs have been found to regulate the function of HBV minichromosomes. Zhang et al. [26] showed that miRNA-1 affected the replication and expression of HBV by acting on multiple target genes, such as *HDAC4*. Chen et al. [27] found that the class I and II HDAC inhibitor, Trichostatin A (TSA), enhanced the replication of HBV. In this study, the expression level of cccDNA in the miRNA-548ah mimics group was significantly increased, while it was decreased in the miRNA-548ah inhibitor group compared with the negative control group. Furthermore, the level of cccDNA expression significantly increased after inhibiting the expression of HDAC4 through miRNA-548ah mimics and knockdown of HDAC4. These results suggest that miRNA-548ah can promote the replication and expression of HBV by targeting HDAC4 and regulating the deacetylation of histone H3 binding with cccDNA. Recent studies reported that the transcription of the cccDNA microchromosome is regulated by histone acetylation in natural HBV infection [28,29]. The mechanism of miRNA-548ah in natural HBV infection will need to be explored further.

The expression level of miRNA-548ah in HepG2.2.15 cells was significantly increased compared to that in HepG2 cells. This suggests that HBV infection may enhance the expression of miRNA-548ah. HBV can express several antigens, including surface antigen X, core antigen, and e-antigen. Some studies have reported that the HBV X-antigen inhibited the expression of miRNA-15b and miRNA-125a and promoted the expression of miRNA-146a [30–32]. In this study, we show that the expression level of miRNA-548ah was significantly increased in the HBV core antigen expression plasmid group relative to the control group. However, there was no significant difference in the expression of miRNA-548ah in the HBV S antigen and X antigen expression plasmid groups. These results indicate that the HBV core antigen promotes the expression of miRNA-548ah. Our results show that miRNA-548ah may function by inhibiting the deacetylation of the histone binding with the cccDNA, thereby enhancing the replication of cccDNA through the inhibition of the target gene HDAC4. Ultimately, miRNA-548ah promotes the replication and expression of HBV. This interaction between miRNA-548ah and HBV forms a positive feedback loop, which might play an important role in the mechanism of persistent CHB infection (Fig. 8).

5. Conclusions

Our data suggest that miRNA-548ah promotes the replication and expression of HBV through the regulation of its target gene, HDAC4. Consequently, HDAC4 appears to increase the replication of cccDNA by inhibiting the deacetylation of the histone combining with cccDNA. Moreover, the HBV C antigen might enhance the expression of miRNA-548ah in hepatocytes (Fig. 8). These findings not only provide a new mechanism for the molecular pathogenesis of CHB, but also offer potential new molecular targets for the prevention and treatment of CHB.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2018.12.057.

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