### Original Article miR-224 suppresses HBV replication posttranscriptionally through inhibiting SIRT1-mediated autophagy

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**Abstract:** Hepatitis B virus (HBV) enters the host and successfully completes replication by using several mechanisms, including autophagy. However, previous studies revealed that microRNAs (miRNAs) widely participate in regulation of various cellular processes, such as autophagy and viral replication. Hence, the purpose of this study was to investigate the role of miR-224 in HBV infection and to determine whether its role depended on the miR-224/SIRT1/autophagy axis. Our results show that secretions of HBeAg and HBsAg, and HBV replication significantly declined in Huh7-1.3 cells, established by transfecting recombinant pcDNA 3.0-1.3 mer containing the 1.3 mer fragment of HBV genomic DNA, with miR-224 mimic transfection as compared to the Huh7-1.3 group. Moreover, it was discovered that HBV could induce autophagy, while miR-224 inhibited autophagy caused by HBV. Additionally, miR-224 could suppress SIRT1, LC3 expression, and facilitate p62 expression. SIRT1 was identified as the target gene of miR-224 and down-regulation of SIRT1 via miR-224 or si-SIRT1 transfected treatment in Huh7-1.3 cells repressed LC3 expression and enhanced p62 expression. In conclusion, these results suggest that miR-224 might hinder HBV replication through attenuating SIRT1-mediated autophagy, thereby these findings open a new avenue for the treatment of HBV infection.

**Keywords:** Hepatitis B virus (HBV) replication, microRNA-224 (miR-224), silent information regulator 1 (SIRT1), autophagy

#### Introduction

Hepatitis B virus (HBV), an enveloped, doublestranded DNA virus, chronically affects approximately 350~400 million humans across the world [1]. Patients with chronic HBV infection have a significantly increased risk from severe liver diseases, such as cirrhosis, and hepatocellular carcinoma, which remain major global public health problems [2]. At the current stage of knowledge and therapeutic options, patients with chronic HBV infection are commonly given drugs, including interferon- $\alpha$ , entecavir and tenofovir, that are effective in viremia suppression, decrease disease progression, and the eventual risk of hepatocellular carcinoma [3]. However, these treatments are rarely responsible for HBsAg clearance. The key obstacle against curing chronic hepatitis B is the inability to eradicate or inactivate covalently closed circular DNA (cccDNA) which is the transcriptional template of HBV [4]. Thus, it is urgent to understand the molecular mechanisms of HBV-host interactions and to identify new therapeutics for HBV infection.

Autophagy is a highly conserved multi-step selfdegradative physiological process where cellular components are sequestered in autophagosomes and subsequently fuse with lysosomes to degrade the sequestered contents [5]. Thereby, adequate autophagic responses protect cells from different kinds of stress and maintain cell survival mechanisms [6]. Currently, accumulating evidence has revealed that there is a close relationship between autophagy and pathogenesis of virus infection [7]. For example, human immunodeficiency virus type 1 (HIV-1) Nef-mediated inhibition of autophagy flux leads to cytotoxicity and death of cardio-

myocytes [8]; Tu elongation factor, mitochondrial (TUFM) acted as a host restriction factor that impeded avian-signature influenza A virus replication in human cells in a manner that correlated with autophagy [9]; hepatitis C virus (HCV) induced autophagy to modulate its RNA replication via homotypic fusion of phagophores in autophagosomes [10]. Silent information regulator 1 (SIRT1), a member of the conserved sirtuin family, exerts protective effects by inducing autophagy in a variety of diseases [11]. For instance, forced SIRT1 expression could maintain the physiological level of autophagy, which could further reverse hyperglycemia and repair damaged pancreatic β-cells in streptozotocin-induced diabetic mice [12]. However, the roles of autophagy and SIRT1 during HBV infection are still unclear.

microRNAs (miRNAs), a class of small, non-coding RNAs about 18~25 nucleotides in length, mainly function to negatively regulate gene expression by promoting mRNA degradation or inhibiting mRNA translation through interacting with perfect or imperfect complementary sequences between the miRNA seed and the 3'-untranslated regions (3'-UTR) of its target genes [13]. A growing number of studies have indicated that many miRNAs are involved in the infectious cycle of HBV [14]. For example, miR-101 suppressed HBV replication and expression by targeting forkhead box O1 (FOXO1) [15]; miR-449a facilitated HBV replication by targeting cAMP-responsive element binding protein 5 (CREB5) [16]; transcriptional repression of miR-224 directly targeted the HBV pgRNA and would inhibit HBV replication [17]. Therefore, miRNAs and autophagy are considered as important regulators in virus infection. Nevertheless, few studies have focused on whether aberrantly expressed miRNA could change HBV replication via regulating SIRT1-mediated autophagy. In the current study, we aimed to explore the function of miR-224/SIRT1/autophagy axis in HBV infection.

#### Materials and methods

### Cell culture and establishment of Huh7-1.3 cell lines

Hepatoma cell line Huh7 cells purchased from American Type Culture Collection (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, USA), and 100  $\mu$ g/ml streptomycin solution and 100 U/ml penicillin at 37°C in a humidified incubator with 5% CO<sub>2</sub>. Huh7-1.3 cells were established by transfection with recombinant pcDNA 3.0-1.3 mer which contained the 1.3 mer fragment of HBV genomic DNA with FuGENE® HD Transfection Reagent (Promega, USA) in accordance with the manufacturer's manuals.

#### Cell transfection and grouping

The experiment was divided into the following eight groups: Negative control (NC; i.e. Huh7 cells + NC plasmid), Control (i.e., Huh7-1.3 ce-IIs), Huh7-1.3 + Rampamycin, Huh7-1.3 + 3MA, Huh7-1.3 + SIRT1 siRNA, Huh7-1.3 + Rapamycin + SIRT1 siRNA, Huh7-1.3 + miR-224 and Huh7-1.3 + miR-224 inhibitor. Approximately 5×105 Huh7-1.3 cells per well were plated in 6-well plates and cells were grown to 80% confluence and subjected to the above treatments. Rapamycin is an autophagy activator, while 3MA is an autophagy inhibitor. The groups that included rapamycin or 3MA treatments were pre-incubated with the drug for 3 h at 37°C in the incubator. NC plasmid, SIRT1 siRNA, miR-224 mimic, and miR-224 inhibitor were obtained from Sangon Biotech, Shanghai, China and were transfected into the corresponding groups using Lipofectamine 2000 reagent (Invitrogen, USA) following the manufacturer's protocols. After 48 h transfection, the cellular supernatant and cells were harvested for subsequent analysis.

#### Enzyme-linked immunosorbent assay (ELISA)

The collected cellular supernatant was used to detect the level of HBV surface antigen (HBsAg) and antigen (HBeAg) with the commercially available ELISA kits (InTec, China) in line with the instructions of the supplier. A standard curve was drawn with the optical density (OD) values on the ordinate axis and standard concentrations on the abscissa axis. The corresponding concentrations were found on the standard curve with the OD values. Each experiment was repeated three times to obtain the mean values.

#### Extraction and quantification of HBV cccDNA

The samples were treated with 10  $\mu g$  DNase I for 16 h at 37°C. 100  $\mu I$  of lysis buffer (20 mM

Tris-HCl, 20 mM EDTA, 50 mM NaCl and 0.5% SDS) containing 50 µg proteinase K were added. After incubation at 65°C for 3 h, viral DNA was isolated by phenol/chloroform extraction and ethanol precipitation. The DNA pellet was rinsed with 70% ethanol and re-suspended in 10 µl of ddH<sub>2</sub>O. The cccDNA was later subjected to real-time-PCR using SYBR Green Realtime PCR Master Mix (Roche, Germany) and cccDNA-specific primers: 5'-TGCACTTCGCTTC-ACCT (forward) and 5'-AGGGGCATTTGGTGGTC (reverse). PCR was performed using an ABI PRISM® 7500 Sequence Detection System (Applied Biosystems, USA). cccDNA copy numbers were quantified according to a standard curve generated from an HBV plasmid in a concentration range of  $10^2 \sim 10^8$  copies.

#### Transmission electron microscopy

Transmission electron microscopy was used to identify the ultrastructure of autophagosomes/autolysosomes [18]. Briefly, Huh7 cells and Huh7-1.3 cells were washed with PBS twice and fixed in 2.5% glutaraldehyde overnight at 4°C. After washing with 0.1% sodium cacodylate buffer, the cells were post-fixed with 1% osmium acid for 3 h. Next, the cells were washed three times with PBS, dehydrated in ascending concentrations of ethanol and then transferred to absolute acetone for 20 min. The samples were placed in a 1:1 mixture of absolute acetone, followed by incubation in a Spurr resin mixture for 1 h at room temperature. Samples were incubated in a 1:3 mixture of absolute acetone, followed by the final resin mixture for 3 h and a final Spurr resin mixture overnight. Finally, the specimens were placed in capsules containing embedding medium and heated to 70°C for about 9 h. Ultra-thin (70 nm) sections were cut using an ultramicrotome (Leica, Germany). Specimen sections were stained with uranyl acetate and lead citrate for 10 min each and washed with water. The sections were observed by a transmission electron microscope (JEOL, Japan) operated at 60 kV.

#### Western blot (WB) analysis

Total protein from all samples was extracted by using ice-cold radioimmunoprecipitation (RIPA) assay buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% NP-40) supplemented with protease inhibitor cocktail for 30 min and the protein level was detected using the Bradford Protein Assay Kit (Beyotime, China). The proteins were then separated by 10~12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA) at 200 mA for 1.5 h by wet electrophoretic transfer. Following blocked with Tris-buffered saline-Tween-20 (TBST) containing 5% non-fat dry milk at room temperature for 1 h, the membranes were detected with the following specific primary antibodies: GAPDH (1:1000 dilution, as an internal reference; Abmart, USA), SIRT1 (1:1000 dilution; Abcam, USA), LC3 (1:1500 dilution; Millipore, USA) and p62 (1:1000 dilution; Abcam, USA). After incubation at 4°C overnight, the membranes were washed with TBST three times, followed by incubation with corresponding horseradish peroxidase-conjugated secondary antibody (1:12000 dilution; Abmart, USA) for 1 h at room temperature. The protein bands were visualized by using the ECL chemiluminescent reagent kit (Beyotime, China) and protein ratios were calculated following Image J densitomertric analysis.

## Cell staining for immunofluorescence (IF) microscopy

Cells, including NC, Huh7-1.3, Huh7-1.3 + 3 MA and Huh7-1.3 + miR-224 groups, cultured in sterile cover glasses in 24-well plates were fixed with 4% paraformaldehyde for 15 min and blocked with 3% goat serum for 20 min at room temperature, then incubated with LC3 antibody (1:200 dilution; Abcam, USA) for overnight at 4°C. The next day, cells were washed in 0.1 mM phosphate buffered saline (PBS) for 3 times and then incubated with the secondary antibody (1:200 dilution; Life Technologies, USA) for 1 hour at 37°C. After being washed with PBS twice, the LC3 dots in stained cells were observed under a fluorescence microscope (Leica, Germany). At least 20 cells were randomly selected for quantification of the LC3 puncta in triplicated samples.

### Construction of luciferase plasmids and reporter assay

The amplified human 3'-UTR segments of the SIRT1 gene (containing the predicted miR-224 binding site) were inserted into psiCHECK-2 vectors (Promega, USA) containing Renilla luciferase (Promega, USA) to generate the wild-type



**Figure 1.** miR-224 inhibits hepatitis B virus (HBV) antigen secretion and viral replication. Huh7-1.3 cells transfected with the 1.3 mer fragment of HBV genomic DNA were treated with miR-224 mimic. A. Culture medium from each group was collected and detected for levels of HBeAg by ELISA. B. Culture medium from each group was harvested and measured for levels of HBsAg by ELISA. C. The released levels of nuclear HBV cccDNA were determined by quantitative PCR in each group. \*indicates P < 0.05.

plasmid (SIRT1-WT) or mutant plasmid (SIRT1-Mutant) constructs. All constructs were further verified by sequencing. For luciferase assays, 293T cells were seeded in 96-well plates and co-transfected with the recombinant vectors along with control psiCHECK-2 plasmid, miR-224 mimics, miR-224 inhibitor, negative control (NC) plasmid, or NC inhibitor using Lipofectamine 2000 reagent. The cells were collected and analyzed by applying a Dual-Luciferase Reporter Assay System (Promega, USA) after 48 h. The luciferase activity values were normalized relative to that of the Renilla luciferase internal control. Each experiment was repeated three times in duplicate.

#### Statistical analysis

All data were carried out by SPSS 21.0 software (SPSS Inc., USA). Data are reported as the mean  $\pm$  standard deviation (SD) from at least three independent experiments. The differences between two groups were analyzed by two-tailed Student's test, and the differences between more than two groups were evaluated by one-way analysis of variance (ANOVA). A *p* value < 0.05 was considered to be statistically significant.

#### Results

### Effects of miR-224 on HBV antigen levels and HBV replication in Huh7-1.3 cells

HBeAg positivity and HBsAg positivity were both considered as evidence of HBV infection [19]. In this study, we first established a stably transfected Huh7-1.3 cell line which might have the features of HBV infection. It was found that the concentrations of HBeAg and HBsAg were increased in Huh7-1.3 cells as compared to Huh7 cells (i.e. NC group) (Figure 1A and 1B). Moreover, the released levels of nuclear HBV cccDNA were also elevated in Huh7-1.3 cells relative to the NC group (Figure 1C). Therefore, a highly transfection efficiency of the 1.3 mer fragment of HBV genomic DNA was achieved. Subsequently, the role of miR-224 on HBV antigen levels and HBV replication in Huh7-1.3 cells was further investigated and ELISA and PCR experiments revealed strong reductions in HBeAg (Figure 1A), HBsAg (Figure 1B) and cccDNA (Figure 1C) levels, indicating that miR-224 might inhibit HBV antigen secretions and viral replication.

### Influences of miR-224 on the autophagosome formation in Huh7-1.3 cells

Autophagy is a common phenomenon during virus infection [5, 7]. To investigate the influence of miR-224 on autophagosome formation in Huh7-1.3 cells, transmission electron microscopy was used to identify the ultrastructure of autophagosome. It was observed that Huh7 cells presented as basically mononuclear cells and possessed large numbers of oval or sausage-shaped mitochondria. Additionally, the cells had extensive endoplasmic reticulum in their overall cytoplasm, and their nuclear shape was as a regular ellipse and their chromatin was distributed uniformly (Figure 2A). However, intracellular autophagic vacuoles were seen in Huh7-1.3 cells, but after treatment with 3MA or miR-224, the number of intracellular autophagic vacuoles was significantly reduced (Figure 2A). Therefore, miR-224 might suppress autophagosome formation in Huh7-1.3 cells.

We further examined the expression of autophagy markers LC3 protein by WB and IF methods. During autophagosome formation, the



Figure 2. miR-224 suppresses autophagy induced by HBV. A. Autophagosomes were observed by transmission electron microscopy. Images were captured at  $\times$ 5,000 magnification. The representative features in each group were enlarged on the right. B. The expression of LC3 was examined by Western blotting in Huh7-1.3 cells with different treatments. C. The expression of LC3 in Huh7-1.3 cells was assayed using immunofluorescence. The representative images presented on the left panel, and the number of LC3 dots by GraphPad 5 was given in the histogram on the right panel. \*\*indicates P < 0.01.

cytosolic microtubule-associated protein 1 LC3-I is converted into LC3-II (a phosphatidylethanolamine-conjugated form) which then targets autophagic membranes [20]. Moreover, LC3-I was the upper band and LC3-II was the lower band in W.B. images. The results displayed a remarkably increase of LC3-II in the Huh7-1.3 group compared with the NC group, while the expression of LC3-II in Huh7-1.3 + 3MA and Huh7-1.3 + miR-224 groups was markedly lower than that in Huh7-1.3 group (**Figure 2B**). Furthermore, it was also discov-



**Figure 3.** Western-blot analysis for autophagic proteins in Huh7-1.3 cells treated with miR-224 mimic and miR-224 inhibitor. Representative graphs were presented (A) and the intensity quantifed by GraphPad 5 were calculated in the histogram (B).

#### A SIRT1 WT 5'-UUAAAAUUGUCAUUUGACUUU-3' miR-224 3'-UUGCCUUGGUGAUCACUGAAC-5' SIRT1 WT 5'-UUAAAAUUGUCAUUAUGCACU-3'



**Figure 4.** Direct targeting of the 3'-untranslated region (UTR) of SIRT1 mRNA by miR-224. A, B. Conserved miR-224 binding site in the 3'-UTR of SIRT1 mRNA. Predicted consequential pairing of the target regions and miR-224 (framed) was based on bioinformatic analysis. B. Analysis of luciferase activity in the 293T cells transfected with psiCHECK-2-SIRT1-WT and psiCHECK-2-SIRT1-mutant along with miR-224 mimic, miR-224 inhibitor, NC or NC inhibitor. \*\*P < 0.01.

ered that the number of LC3 dots determined by IF presented a similar expression trend with the W.B. results (**Figure 2C**). Hence, these results suggest that miR-224 might hinder autophagy induced by HBV.

#### miR-224 changed the expression of SIRT1 and autophagic proteins in Huh7-1.3 cells

SIRT1 has been demonstrated to play an important role in regulating the autophagy by deacetylation of autophagyrelated proteins [21]. Therefore, expression levels of SIRT1 and autophagic proteins were detected to further explore the role of miR-224. As illustrated in Figure 3A, our data show that the expression of SIRT1 was dramatically reduced when given 3MA or miR-224 mimic in Huh7-1.3 cells as compared to Huh7-1.3 and Huh7-1.3 + NC groups, whereas it was sharply enhanced in Huh7-1.3 cells transfected with miR-224 inhibitor. Meanwhile, LC3-II protein appeared to have a similar expression pattern as SIRT1 in each group. However, the expression of p62 was notably and slightly up-regulated in Huh7-1.3 cells treated with 3MA and miR-224, respectively, while it was clearly down-regulated in Huh7-1.3 cells treated with miR-224 inhibitor. The statistical graph of protein band density is presented in Figure 3B. Thereby, these data point out that miR-224 might impede the autophagy formation by repressing expression of SIR-T1 and autophagic proteins in Huh7-1.3 cells.

### SIRT1 might be the target of miR-224

Using the target prediction analysis, we found that a conserved sequence in the 3'-UTR of SIRT1 mRNA was a perfect match to the seed sequence

of miR-224 (**Figure 4A**). To investigate whether miR-224 regulates SIRT1 mRNA expression by directly targeting the predicted sequence, the SIRT1 3'-UTR fragments containing the normal or mutant binding site for miR-224 were cloned



**Figure 5.** Western blot was utilized to analyze the protein expression of SIRT1, LC3, and p62 in Huh7-1.3 cells with different treatments. A. Representative images of protein bands are shown. B. The ratios of SIRT1/GAPDH, LC3/GAPDH and p62/GAPDH are represented as mean  $\pm$  SD data from three independent experiments. \*\*, P < 0.01.

down-stream of the firefly luciferase coding region in the psiCHECK-2 to replace the luciferase 3'-UTR. Our results demonstrate that mimics of miR-224 could significantly attenuate the luciferase activity of the wild-type SIRT1 3'-UTR reporter but not that with mutated miR-224 binding site (**Figure 4B**). In contrast, inhibition of endogenous miR-224 by miR-224 inhibitor significantly increased the luciferase activity of the wild-type reporter but not that of the mutant (**Figure 4B**). Thus, this result hinted that miR-224 might directly target to SIRT1 3'-UTR.

# SIRT1 treatments in Huh7-1.3 cells altered the expression of SIRT1 and autophagic proteins in Huh7-1.3 cells

W.B. analysis were performed in Huh7-1.3 cells after transfection with NC plasmid, SITR1 siRNA and miR-224 mimic, or treating with rapamycin, 3MA, or rapamycin + SIRT1 siRNA to address the role of SIRT1. It was uncovered that expression of SIRT1 and LC3 was signifi-

cantly decreased in Huh7-1.3 cells treated with 3MA, SIRT1 siRNA, or rapamycin + SIRT1 siRNA as well as miR-224 mimic when compared to Huh7-1.3 and Huh7-1.3 + NC groups, while they were obviously increased in Huh7-1.3 cells treated with rapamycin. Furthermore, the decreased degrees of SIRT1 and LC3 in Huh7-1.3 + rapamycin + SIRT1 siRNA group were apparently lower than those in Huh7-1.3 + SIRT1 siRNA group compared with Huh7-1.3 and Huh7-1.3 + NC groups. Additionally, p62 expression in Huh7-1.3 cells with 3MA, SIRT1 siRNA, rapamycin + SIRT1 siRNA as well as miR-224 mimic treatments was remarkably upregulated as compared with Huh7-1.3 and Huh7-1.3 + NC groups, whereas it was markedly down-regulated in Huh7-1.3 cells with rapamycin treatment (Figure 5A). Moreover, the band intensities of each protein were calculated and shown in a histogram (Figure 5B). Hence, these data show that SIRT1 might be involved in the autophagy process in Huh7-1.3 cells via being regulated by miR-224.

#### Discussion

HBV is the one of etiological factors for liver damage [1]. It has been estimated that nearly 5% of world population is chronically infected with HBV, which could develop a necroinflammatory liver disease with different patterns of severity and course, ranging from persistent injury to cirrhosis, hepatic failure and hepatocellular carcinoma; thereby it remains a major public health concern worldwide [2]. It is wellknown that miRNAs act as key factors in several biological processes, such as cell proliferation, differentiation and apoptosis, etc [22]. Moreover, evidence is emerging that miRNAs are key mediators in the replication and propagation of viruses [23]. For example, miR-23b detected in Enterovirus 71(EV71)-infected cells could inhibit virus replication by targeting the EV71 VP1 RNA coding region [24]; miR-548g-3p suppressed the recruitment of the viral RNA-dependent RNA polymerase (NS5) to the viral genome which ultimately resulted in a blockade of viral replication via targeting the stem loop A promoter element of Dengue virus (DENV) 5'-UTR [25]. In the present study, we found that miR-224 overexpression by transfection of a miR-224 mimic into Huh7-1.3 cells inhibited HBV antigen secretions, including HBeAg and HBsAg, and viral cccDNA replication. The released levels of HBeAg and HBsAg are commonly used to analyze the conditions of HBV replication [19]. Therefore, our results suggest that miR-224 might repress HBV replication.

Autophagy, a catabolic process that is important for maintaining cellular homeostasis and can act as an important regulator of the cell immune response in viral infection [26]. For example, HCV use autophagy to augment replication by providing membrane sources for the assembly of HCV RNA replication complexes [27, 28]. Autophagy induced by porcine epidemic diarrhea virus (PEDV) infection accelerates expression of inflammatory cytokines and has a positive feedback loop with the NF-kB signaling pathway during PEDV infection, which could further benefit PEDV replication [29]. In addition, a growing set of studies have demonstrated that deregulated miRNAs induced by virus infection could regulate autophagy which can further promote development of virus infection [5]. For instance, miR-30a-5p facilitated replication of porcine circovirus type 2 (PCV2)

through enhancing autophagy by targeting 14-3-3 gene [30]; EV71 infection resulted in the reduction of cellular miR-30a, which led to the inhibition of autophagosome formation and eventually enhanced virus replication [31]. Hence, in our study, we also investigated the interacting roles of autophagy and miR-224 in HBV infection and it was discovered that autophagosome was affected, LC3-II expression was increased, and the number of LC3 dots was elevated in Huh7-1.3 cells. In contrast, after transfecting with miR-224 these phenomena were reversed. Thus, these findings indicate that miR-224 might hinder autophagosome formation caused by HBV replication.

SIRT1, functions as both metabolic sensor and transcriptional regulator, has broad cellular functions, such as metabolic homeostasis, stress response, tumorigenesis, and autophagy [32]. The role of SIRT1 in the life cycle of viruses has gradually been revealed in past years [33-35]. For example, SIRT1 inhibits EV71 genome replication and RNA translation by interfering with the viral polymerase and 5'-UTR RNA [36]; SIRT1 also regulates HIV-1 transcription through deacetylating Tat protein [37]. Additionally, several recent in vitro studies demonstrate that deacetvlation of LC3 by SIRT1 allows LC3 to bind with Atg7 and other elements that are involved in autophagy, suggesting that SIRT1 could regulate autophagy induction [38]. Hence, we speculated that miR-224 might reduce HBV replication through attenuating SIRT1-mediated autophagy. LC3-II and p62 both are essential markers of autophagy [39]. LC3-II converted from LC3-I via proteolytic cleavage is regarded as a hallmark of mammalian autophagy, whereas p62 mainly mediates the recruitment of cargo in the autophagosomes [39, 40]. We next examined the two proteins and W.B. results displayed that miR-224 dramatically down-regulated the expression of SIRT1 and LC3-II and up-regulated the expression of p62.

Subsequently, based on the inverse expression of miR-224 and SIRT1, dual-luciferase reporter assay was used to identify the interaction between miR-224 and SIRT1 and the data revealed that miR-224 could directly target 3'-UTR of SITR1. Finally, down-regulation of SIRT1 with siRNA and miR-224 mimic transfection in Huh7-1.3 cells further verified that diminished SIRT1 sharply descended LC3-II level and ascended p62 level, concluding that miR-224 might regulate autophagy via targeting SIRT1.

In summary, we uncovered that miR-224 could impede HBV replication by declining the secretions of HBV surface antigen and cccDNA replication in Huh7-1.3 cells. Furthermore, the formation of autophagosome induced by HBV and the expression of LC3-II were suppressed in Huh7-1.3 cells transfected with miR-224 mimic. Additionally, miR-224 also inhibited SIRT1 expression and promoted p62 expression. The target interaction between miR-224 and SIRT1 was demonstrated. Ultimately, it was further found that when Huh7-1.3 cells were treated with si-SIRT1, LC3-II protein was down-regulated and p62 protein was up-regulated. Taken together, these data suggest that the miR-224/ SIRT1/autophagy axis is closely associated with HBV replication. Furthermore, the data also offer the possibility of targeting the miR-224/SIRT1/autophagy pathway for the treatment of HBV patients.

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#### Disclosure of conflict of interest

None.

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