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Regulation of HBV replication and gene expression by miR-501-3p via targeting ZEB2 in hepatocellular carcinoma

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Hepatitis B virus (HBV) infection is a major public health issue with serious medical consequences. The roles of microRNAs (miRNAs) in HBV replication and expression have been generally recognized, and the abnormal expression of miR-501 has been reported in patients with HBV infection. However, the function and mechanism in HBV replication remain elusive. The expression patterns of miR-501-3p and ZEB2 in HBV-related hepatocellular carcinoma (HCC) tissues and HBV-expressing HCC cells were determined by qRT-PCR and western blot assays. HBV replication and expression were evaluated through detecting the copies of HBV DNA and the secretion of HBV surface antigens HBsAg and HBeAg by real-time PCR and ELISA analyses. Bioinformatics and dual-luciferase reporter analyses were employed to validate the functional interaction between miR-501-3p and ZEB2. miR-501-3p was significantly upregulated, while ZEB2 was downregulated in HBV-related HCC tissues and cells compared with relative controls without HBV infection. Knockdown of miR-501-3p hampered HBV replication and gene expression in HepG2.2.15 and HepAD38 cells. ZEB2 was identified as a functional target of miR-501-3p. The absence of ZEB2 abolished the inhibitory effects of anti-miR-501-3p on HBV replication and gene expression. Our data indicated that miR-501-3p participated in the regulation of HBV replication and gene expression partially via repressing ZEB2 in HepG2.2.15 and HepAD38 cells, providing a promising antiviral avenue for HBV infection.

Key words: Hepatitis B virus, miR-501-3p, ZEB2, replication, expression, hepatocellular carcinoma

Hepatitis B virus (HBV) infection is a global health problem with more than 240 million chronically infected patients, leading to substantial human morbidity and mortality [1]. HBV, a member of the hepadnavirus family, has been identified to replicate by reverse transcription in human hepatocytes [2]. The virus genome is a circular double-stranded DNA, which can be transcribed into 4 virus transcripts of different lengths (3.5 kb, 2.4 kb, 2.1 kb, and 0.7 kb). As a pregenomic RNA (pgRNA), the longest RNA plays a vital role in the modulation of HBV DNA replication [3]. Despite the great progress in antiviral therapy, chronic HBV infection remains a major problem for hepatocellular carcinoma (HCC) patients [4]. Therefore, it is urgent to investigate the pathogenesis of HBV infection to identify promising targets for HBV prevention and therapy.

MicroRNAs (miRNAs) are defined as a group of single-stranded noncoding RNAs with 19–25 nucleotides (nt) in length [5]. Accumulating evidences indicate that miRNAs serve as crucial regulators of gene expression through imperfect base pairing with the 3'UTR of target mRNAs [6]. Recently, various miRNAs have been confirmed to be associated with HBV infection, and the discovery of miRNAs may be helpful for the development of available strategies for HBV prevention and therapy [7]. For instance, miR-101 exerts a vital role by interacting with FOXO1 in the regulation of HBV replication and expression [8]. miR-29b is reported to be distinctly triggered HBV replication by binding to SMARCE1 in HCC [9].

miR-501, a cancer-related miRNA, is frequently dysregulated in several types of human cancers, including HCC [10], cervical cancer [11], and prostate cancer [12]. Emerging evidences confirm that miR-501-3p exerts elevated expression in patients with chronic HBV infection, highlighting its potential diagnostic and therapeutic values in HBV infec-

tion [13]. Also, miR-501-3p expression showed a 7.01-fold increase in HBV-related HCC tumor versus non-tumor liver tissue, suggesting a poor overall survival in HBV-related HCC tumor [14]. Although the relationship between miR-501-3p expression and HBV-related HCC has been elucidated, its biological function and specific mechanism in HBV replication and gene expression remain unclear. In this study, we initially detected the expression of miR-501-3p in HBV-related HCC tissues and HBV-expressing HCC cell lines. Next, the functional roles of miR-501-3p in HBV replication and expression as well as its underlying mechanism were further investigated, so as to provide a novel biological target for HBV prevention and therapy.

Patients and methods

Clinical samples. Human HCC tissues and paired non-cancerous tissues were collected from 18 patients who were diagnosed with HBV-related HCC in the Fifth Affiliated Hospital of Sun Yat-sen University between March 2016 and June 2017. After surgical resection, fresh samples were immediately frozen in liquid nitrogen and stored at –80 °C before used. The experiment was performed following the approval of the Institutional Ethics Committee of the Fifth Affiliated Hospital of Sun Yat-sen University with written informed consent from all participants.

Cell culture. 293T cells, human HCC cell line HepG2 and HepG2.2.15 cells (stably HBV-transfected HepG2 cells) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). HCC cell line HepAD38 (in which HBV replication can be modulated by tetracycline) were obtained from BeNa Culture Collection (Beijing, China). 293T cells were cultured in DMEM medium containing 10% fetal calf serum (FBS) (Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin (Solarbio, Beijing, China). HCC cells were grown in Eagle's Minimal Essential Medium (EMEM, ATCC) in the presence of 10% fetal calf serum (FBS) and 1% penicillin/streptomycin. All cells were maintained in a humidified incubator of 5% CO_2 at 37 °C. To block HBV replication, HepAD38 cells were cultured in a complete medium with 0.3 μg/ml of tetracycline.

Plasmid construction and cell transfection. HBV-expressing plasmid pCH9/3091 (HBV1.1) (containing 1.1-unit-length HBV genome and CMV promoter) was obtained from Lin Lan (The Third Military Medical University, China) and transfected into HepG2 cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). miR-501-3p inhibitor (anti-miR-501-3p) and inhibitor control (anti-NC), miR-501-3p mimics (miR-501-3p) and mimics control (NC), small interference RNA targeting ZEB2 (si-ZEB2) and its scramble control (siRNA) were all synthesized by (Thermo Fisher Scientific, Rockford, IL, USA). ZEB2-expressing plasmid (ZEB2) was constructed by GenePharma Co. Ltd. (Shanghai, China) through inserting the full-length of ZEB2 sequences into pcDNA3.1 vector. HepG2.2.15 cells and HepAD38 cells (removal of tetracycline) were transfected with oligonucleotides or plasmid using Lipofectamine 2000 reagent for 48 h according to the manufacturer's protocol.

Quantitative real-time PCR (qRT-PCR). Total RNA was extracted from clinical tissues and cells using TRIzol Reagent (Invitrogen) following the instructions of the manufacturer. After purity analysis of RNA, MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) or M-MLV reverse transcriptase (Thermo Fisher Scientific) was used for cDNA synthesis. The relative expression of miR-501-3p was measured by using TaqMan miRNA assay (Thermo Fisher Scientific) and normalized to U6 snRNA. ZEB2 expression was detected by Fast SYBR Green Master Mix (Thermo Fisher Scientific) and normalized to β-actin. The primer sequences were 5'-CATAGCACGCGGGCTAG-3' (forward) and 5'-GACGTGGGACGGAGGT-3' (reverse) for miR-501-3p, 5'-CTCGCTTCGGCAGCACA-3' (forward) and 5'-AACGCTTCACGAATTTGCGT-3' (reverse) for U6 snRNA, 5'-GAAGATGAAATAAGGGAGGG-3' (forward) and 5'-CTGGGTAAATAATGGCTGTG-3' (reverse) for ZEB2, 5'-ACAGAGCCTCGCCTTTGCCG-3' (forward) and 5'-ACATGCCGGAGCCGTTGTCG-3' (reverse) for β-actin. The results from three repetitions were calculated by using $2^{-\Delta\Delta Ct}$ method.

For HBV DNA replication assay, DNA from the cell culture supernatant was isolated using MiniBEST Viral DNA Extraction kit (Takara, Dalian, China) in line with the manufacturer's instructions, and quantified by real-time PCR using an ABI 7500 Real-time PCR Systems (Applied Biosystems, Foster, CA, USA).

ELISA assay for HBsAg and HBeAg detection. The culture supernatant of HepG2.2.15 and HepAD38 cells were harvested for the detection of HBsAg and HBeAg concentration using ELISA kits (BioLegend, San Diego, CA, USA) according to the manufacturer's instruction. Absorbance was determined in a microplate reader with dual-wavelength measurement (450/630 nm).

Luciferase reporter assay. For luciferase activity assay, partial sequences of ZEB2 3'UTR containing the putative binding sites of wild-type miR-501-3p were obtained from PCR amplification and inserted into psiCHECK-2 luciferase vector (Promega, Madison, WI, USA), generating wild-type ZEB2 (ZEB2-WT). ZEB2 mutant (ZEB2-MUT) was produced by mutating the binding site of miR-501-3p in the 3'UTR of ZEB2 using TaKaRa MutanBEST Kit (Dalian, China). ZEB2-WT or ZEB2-MUT reporter was transfected into 293T cells along with miR-501-3p, anti-miR-501-3p, or relative control for 48 h. Afterward, the luciferase activity in cell lysates was examined using a Dual-Luciferase Reporter Assay System (Promega).

Western blot assay. The protein expression of ZEB2 was determined by western blot assay. Briefly, total proteins in HepG2.2.15 and HepAD38 cells were extracted by using RIPA reagents (Thermo Fisher Scientific) in the presence

of protease inhibitors (Solarbio), followed by the quantification of protein concentration using a BCA protein assay kit (Thermo Fisher Scientific). Equal amounts of proteins denatured at 95°C for 5 min were separated on the SDS-PAGE gel, transferred onto PVDF membrane (Millipore, Bedford, MA, USA), and incubated with rabbit anti-ZEB2 (1:1000, ab223688, Abcam, Cambridge, UK) and rabbit anti-β-actin (1:5000, ab8227, Abcam) antibodies. After further incubation with goat anti-rabbit IgG antibody (HRP) (1:5000, ab6721, Abcam), protein signals were detected by Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific) and the gray values were quantified using ImageJ software.

Results

miR-501-3p expression was upregulated in HBV-related HCC tissues and HBV-expressing cells. In the present study, the expression patterns of miR-501-3p were initially investigated in 18 HBV-related HCC tissues and adjacent normal tissues by qRT-PCR. The results showed that the miR-501-3p expression was strikingly elevated in HBV-related HCC tissues relative to the paired normal tissues (Figure 1A). Moreover, the abundance of miR-501-3p was markedly increased in HBV-expressing HepG2.2.15 and HBV1.1-transfected HepG2 cells compared with relative controls without HBV infection (Figures 2B, 2D). Give that tetracycline can inhibit HBV replication in HepAD38 cells, we further tested the expression of miR-501-3p in HepAD38 cell line, and found that miR-501-3p level was markedly higher in HepAD38 cells than that in tetracycline-treated HepAD38 cells (Figure 2C). These findings indicated the potential interaction between miR-501-3p expression and HBV replication.

Knockdown of miR-501-3p inhibited HBV replication and expression. In the present study, loss-of-function assays were conducted to investigate the effect of miR-501-3p on HBV replication. As a result, the introduction of anti-miR-501-3p caused a decreased level of miR-501-3p in Hep2.2.15 and HepAD38 cells compared with that in the anti-NC group (Figure 2A), indicating the desirable transfection efficiency of miR-501-3p inhibitor. Next, the results of the real-time PCR assay revealed that knockdown of miR-501-3p significantly decreased the copies of HBV in Hep2.2.15 and HepAD38 cells (Figure 2B). Moreover, the ELISA assay showed that miR-501-3p absence by the miR-501-3p inhibitor led to a distinct decrease in the secretion of HBsAg and HBeAg in Hep2.2.15 and HepAD38 cells (Figures 2C, 2D).

ZEB2 was identified as an authentic target of miR-501-3p. Given that miRNAs exert their functions mainly by repressing the expression of target genes, thereby we further explore the molecular mechanism of miR-501-3p in HBV replication via searching for the target mRNA. The prediction of TargetScan software validated that the 3'UTR of ZEB2 possessing the putative binding sequences of miR-501-3p (Figure 3A). Next, the luciferase reporter assay was performed to determine the precise interaction between miR-501-3p and ZEB2. As shown in Figure 3B, the luciferase activity of ZEB2-WT reporter was markedly suppressed by the overexpression of miR-501-3p, and notably induced in the presence of miR-501-3p knockdown in 293T cells, but no significant changes were observed in ZEB2-MUT reporter. Moreover, the regulatory effect of miR-501-3p on ZEB2 expression was also demonstrated by transfecting miR-501-3p, anti-miR-501-3p, or relative control into HepG2.2.15 cells. The results of western blot assay

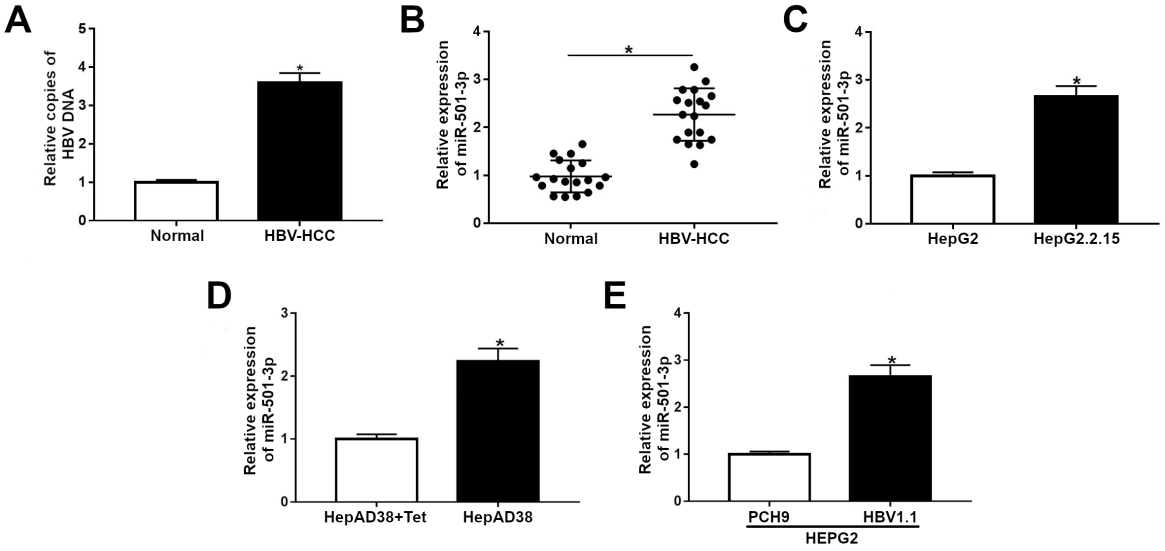

Figure 1. miR-501-3p expression was upregulated in HBV-related HCC tissues and HBV-expressing cells. A) The abundance of miR-501-3p in HBV-related HCC tissues (n=18) and paired normal tissues (n=18) was determined by qRT-PCR analysis. B–C) The expression of miR-501-3p in HBV-expressing HCC cells (including HBV stably expressing cell line HepG2.2.15, HepGAD38 cells, and HBV1.1-transfected HepG2 cells) and corresponding HCC cells without HBV infection were detected by qRT-PCR analysis. *p<0.05

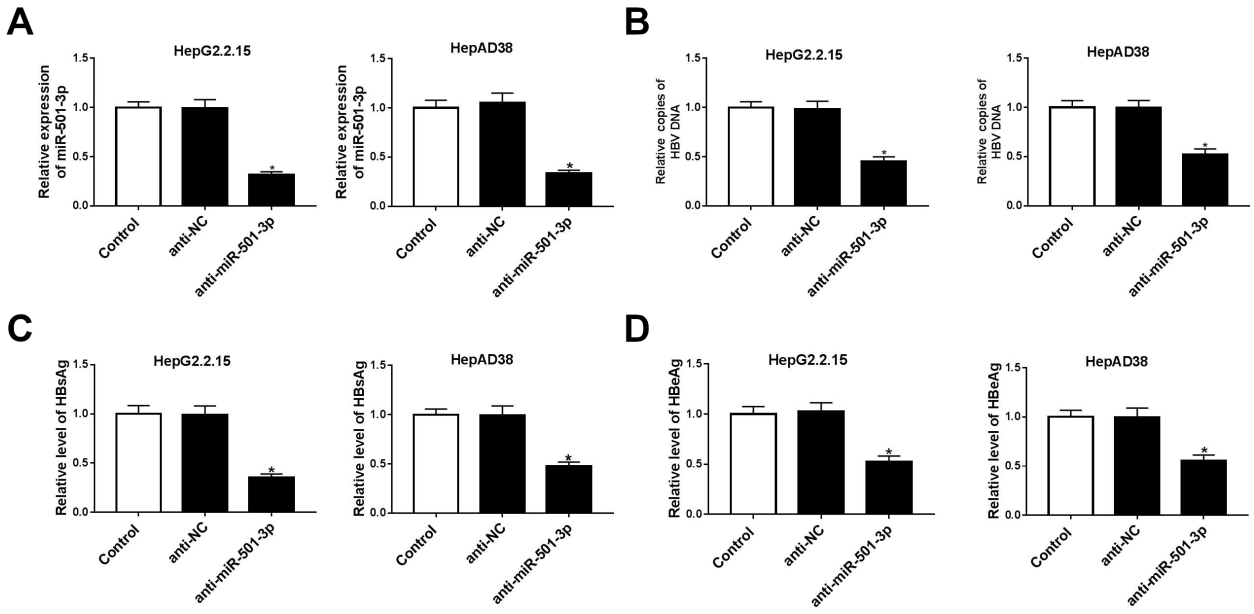

Figure 2. miR-501-3p depletion hampered HBV replication and expression in Hep2.2.15 and HepAD38 cells. A–D) Hep2.2.15 and HepAD38 cells were transfected with anti-NC or anti-miR-501-3p with the non-transfected group as a blank control. About 48 h after transfection, miR-501-3p expression was tested by qRT-PCR assay (A). Relative copies of HBV DNA were measured by real-time PCR analysis (B). The secretion of HBsAg and HBeAg was examined by the ELISA assay (C, D). *p<0.05

indicated that the absence of miR-501-3p led to a striking elevation of ZEB2 expression, but miR-501-3p supplement caused a marked reduction of ZEB2 level (Figure 3C). These data suggested that miR-501-3p negatively regulated ZEB2 expression via direct interaction.

ZEB2 exhibited an inhibitory effect on HBV replication and expression. In the present study, we first determined the abundance of ZEB2 in HBV-related HCC tissues and paired normal tissues, and results displayed that ZEB2 expression at mRNA level was obviously downregulated in HBV-related HCC tissues relative to nearby normal tissues (Figure 4A). Following correlation analysis disclosed the negative relationship between miR-501-3p and ZEB2 expression in clinical samples (Figure 4B). Likewise, the protein levels of ZEB2 were distinctly decreased in HBV-expressing HCC cells (Hep2.215, HepAD38 without tetracycline, and pCH9/3091-transfected HepG2 cells) compared with corresponding controls (HepG2, HepAD38 with tetracycline, HBV1.1-transfected HepG2 cells) (Figures 4C–E). To further explore the influence of ZEB2 on HBV replication and expression, ZEB2 was overexpressed by the transfection of ZEB2-expressing plasmid into HepG2.2.15 and HepAD38 cells (Figure 4F). Following gain-of-function analysis revealed that the high expression of ZEB2 remarkably hindered HBV replication and expression, showing as the reduction of HBV DNA copies, and HBsAg and HBeAg secretion in Hep2.2.15 and HepAD38 cells (Figures 4G–I). These results illuminated that ZEB2 served as a suppressor in HBV replication and expression.

The knockdown of ZEB2 reversed the inhibitory effects of anti-miR-501-3p on HBV replication and expression. To elucidate whether the role of miR-501-3p in HBV replication and expression was modulated by ZEB2, rescue experiments were conducted by transfecting anti-miR-501-3p along with or without si-ZEB2 into Hep2.2.15 and HepAD38 cells. The outcomes indicated that the miR-501-3p knockdown resulted in an enhanced abundance of ZEB2 in Hep2.2.15 and HepAD38 cells, whereas si-ZEB2 introduction reversed the effect of miR-501-3p downregulation on ZEB2 expression (Figure 5A). Functionally, the knockdown of miR-501-3p blocked HBV replication and expression, presenting as the decrease of virus DNA copies, and the production of HBsAg and HBeAg. However, the inhibitory effects of miR-501-3p knockdown on HBV replication and expression were abolished following si-ZEB2 introduction (Figures 5B–D). Taken together, these data indicated that miR-501-3p inhibited HBV replication and expression through repressing ZEB2.

Discussion

HBV-related liver disease is a serious problem for numerous patients due to its high morbidity and mortality. Although great progress has been achieved in the prevention and treatment of HBV infection, the ultimate cure for HBV-related liver diseases is still difficult [15]. Thereby, it is urgently necessary to develop a promising therapeutic strategy for improving the consequence of patients with HBV infection.

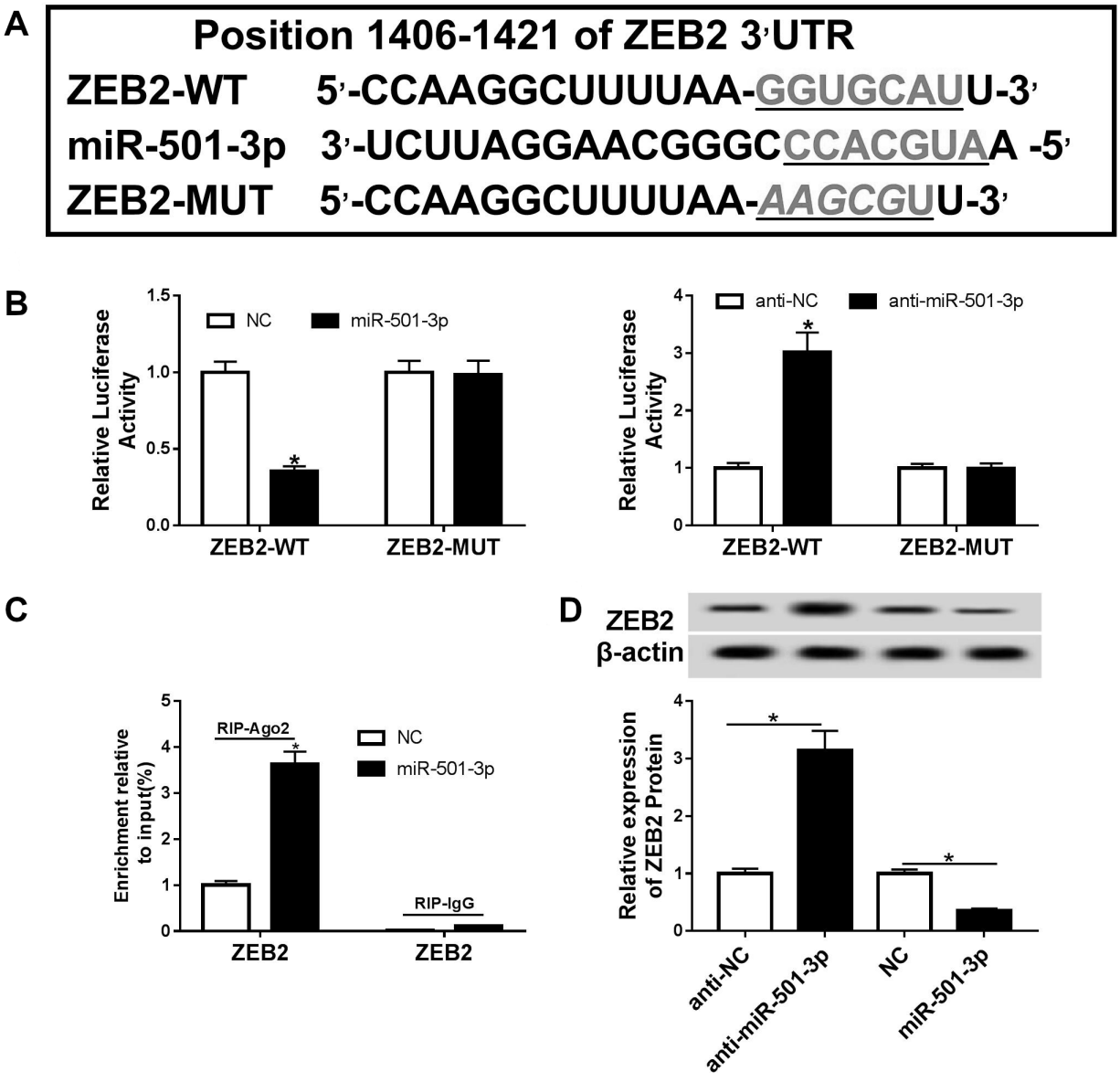

Figure 3. ZEB2 was a functional target of miR-501-3p. A) The predicted binding sites of miR-501-3p in the 3'UTR of ZEB2, as well as the mutant in ZEB2-MUT reporter. B) The luciferase activity of ZEB2-WT and ZEB2-MUT constructs was determined by luciferase reporter assay in 293T cells transfected with miR-501-3p, anti-miR-501-3p, or relative controls. C) ZEB2 expression at the post-transcriptional level was detected in miR-501-3p or anti-miR-501-3p-transfected Hep2.2.15 cells by western blot analysis. *p<0.05

Accumulated evidences have demonstrated that miR-501 can act as an oncogene or tumor suppressor in several types of malignancies. As reported by Yu et al., it facilitated the epithelial-mesenchymal transition by repressing JDP2 in HCC [16]. Zhang et al. stated that miRNA-501-3p impeded prostate cancer growth and induced cell cycle arrest through suppressing CREPT-mediated cyclin D1 expression [12]. Ling et al. proposed that miR-501-3p played a major regulator in the progression of pancreatic cancer progression by negatively regulating E-cadherin [17]. Interestingly, previous studies also indicated the dysregulation of miR-501-3p has been linked to the HBV-infected patients and HBV-related HCC [13, 14].

Consistent with the previous studies, we observed that miR-501-3p expression was significantly upregulated in HBV-related HCC tissues relative to nearby normal tissues. Moreover, miR-501-3p expression was increased in HBV-expressing HepG2.2.15 and HepAD38 cells compared with the controls without HBV expression. Next, loss-of-function assays were carried out to investigate the regula-

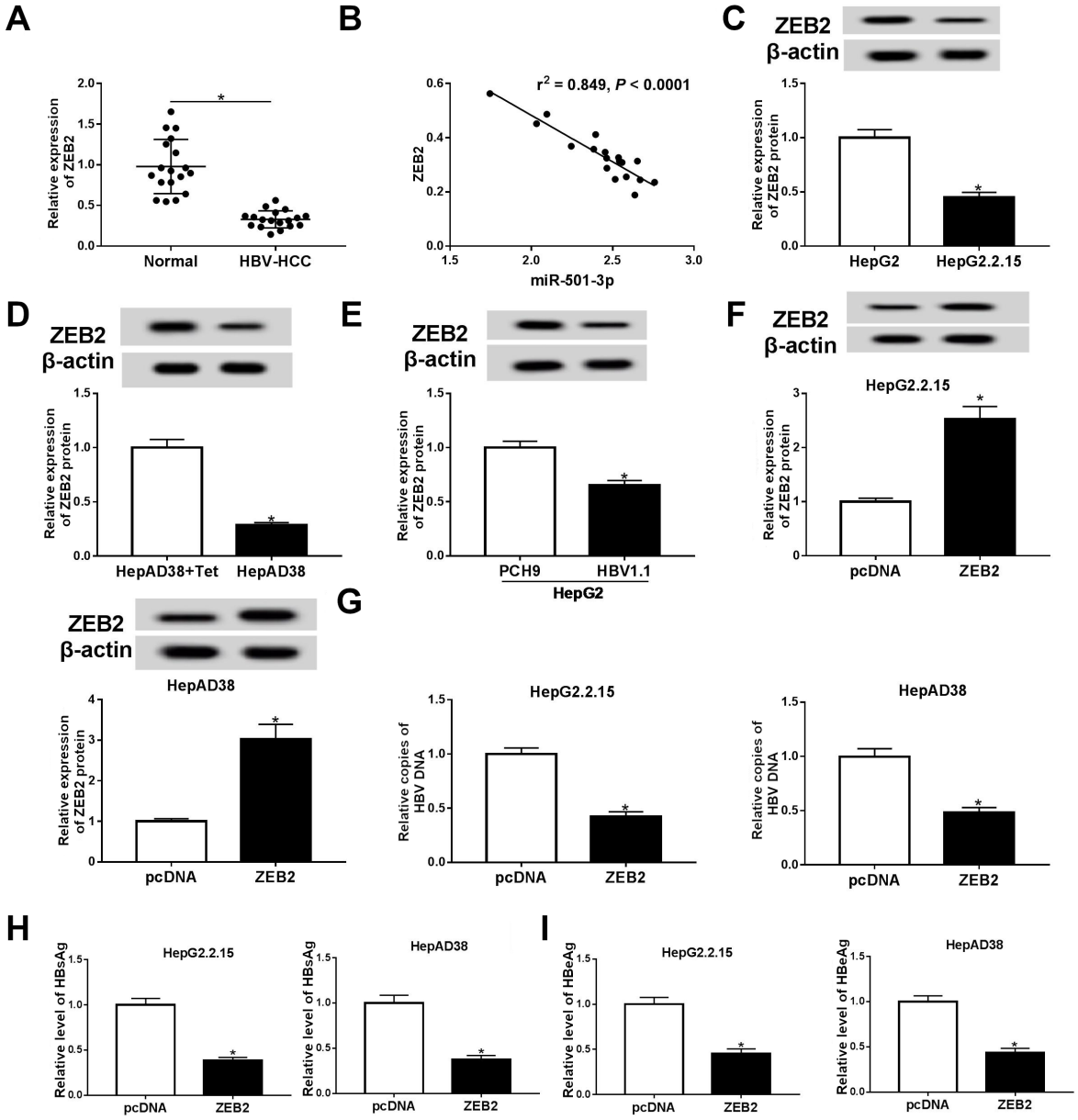

Figure 4. ZEB2 suppressed HBV replication and expression in Hep2.2.15 and HepAD38 cells. A) The expression level of ZEB2 in HBV-related HCC tissues (n=18) and adjacent normal tissues (n=18) was measured by qRT-PCR analysis. B) The correlation between miR-501-3p and ZEB2 expression. C–E) The abundance of ZEB2 in HBV-expressing HCC cells and corresponding HCC cells without HBV expression were detected by western blot analysis. F–I) Hep2.2.15 and HepAD38 cells were transfected with pcDNA or ZEB2 expressing plasmid (ZEB2). About 48 h thereafter, ZEB2 expression was tested by western blot assay (F). Relative copies of HBV DNA were detected by real-time PCR analysis (G). The production of HBsAg and HBeAg was tested by ELISA assay (H, I). *p<0.05

tory roles of miR-501-3p in HBV replication and expression in HCC. As a result, knockdown of miR-501-3p hindered HBV replication and gene expression in Hep2.2.15 and HepAD28 cells, showing as the decrease of virus DNA copies, and the production of the surface antigens HBsAg and HBeAg. Collectively, we put forward an original point that miR-501-3p might function as an effective inducer in HBV infection.

miRNAs have been verified to affect host antiviral activity by indirectly regulating the expression of host genes required for virus replication or by directly targeting gene transcripts. For example, knockdown of miR-26b leads to a reduction in its target gene CHORDC1, which triggers the activity of HBV enhancer/promoter, and facilitates viral replication and gene expression [18]. Decreased expression of miR-200c-mediated nuclear factor IA (NFIA) attenuates the activity of HBV

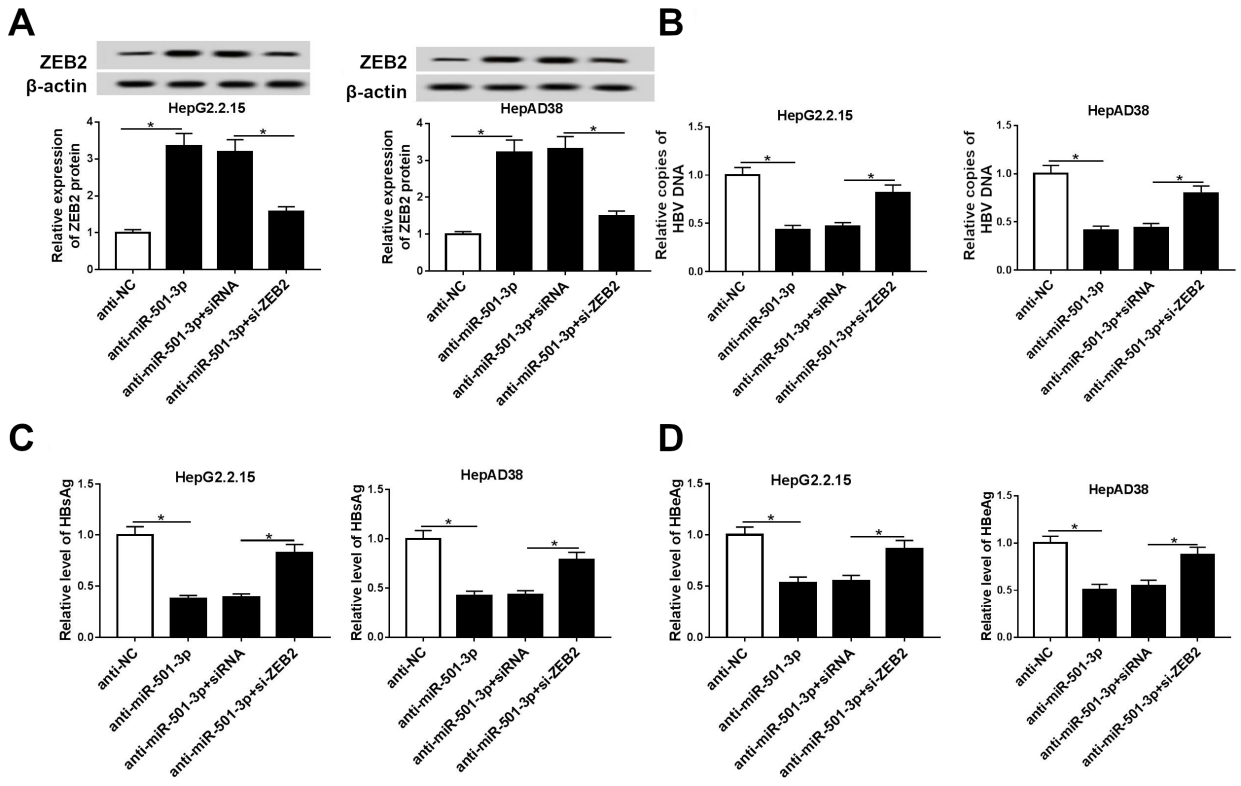

Figure 5. The inhibitory effect of miR-501-3p depletion on HBV replication and expression was abrogated by ZEB2 knockdown in Hep2.2.15 and HepAD38 cells. A–D) Hep2.2.15 and HepAD38 cells were transfected with anti-NC or anti-miR-501-3p, or co-transfected with anti-miR-501-3p+siRNA or anti-miR-501-3p+ZEB2. About 48 h thereafter, ZEB2 expression at the protein level was tested by western blot assay (A). Relative copies of HBV DNA were evaluated by qRT-PCR analysis (B). The secretion of HBsAg and HBeAg was measured by ELISA assay (C, D). *p<0.05

Enhancer I, leading to the repression of HBV replication and gene expression [19]. In the following study, we aimed to determine whether miR-501-3p affected HBV replication and expression by targeting the host gene. By bioinformatics and luciferase reporter assays, ZEB2 was identified to be an authentic target of miR-501-3p.

ZEB2 (also known as SMADIP1 or SIP1), a member of the Zinc finger E-box binding homeobox 2 (ZEB) family, is originally identified as a transcription factor that plays a crucial role in the TGF-β signaling pathway [20]. Reliable evidences have confirmed that ZEB2 exerts a functional role in the regulation of epithelial-mesenchymal transition (EMT) by repressing the genes of epithelial cell-cell junctions, including E-cadherin [21]. Moreover, ZEB2 can functions as an oncogene by inducing cell proliferation, migration, and invasion in glioma [22], nasopharyngeal carcinoma [23], lung cancer [24], and HCC [25]. A recent study discloses that ZEB2 suppresses HBV transcription and replication via targeting virus core promoter [26]. Besides, similar research also revealed the inhibitory effect of ZEB2 on HBV replication and expression in HCC [27]. In the present study, we observed that overexpression of ZEB2 suppressed HBV replication and expression in HCC, which completely consistent with previous reports. Subsequent rescue experiments uncovered that the ZEB2 knockdown abolished the inhibitory effect of anti-miR-501-3p on virus replication.

In conclusion, miR-501-3p expression was upregulated in HBV-related HCC tissues and cells with HBV infection, and inhibition of miR-501-3p blocked HBV replication and expression by directly targeting ZEB2. This work not only highlights the roles of miR-501-3p in HBV replication and expression *in vitro* but also implicates the potential application of miR-501-3p in antiviral therapy by acting as a novel molecular target.

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