

Alteration of microRNA profiles by a novel inhibitor of human La protein in HBV-transformed human hepatoma cells

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A pyrazolopyridine HBSC11 was previously identified as a novel inhibitor of human La protein with anti-hepatitis B virus (HBV) activity. However, the underlying mechanism(s) of HBV inhibition by HBSC11 remains unclear. This study aimed to examine the regulation of microRNA (miRNA) by HBSC11 in HBV-transformed human hepatoma HepG2.2.15 cells using microarray and quantitative real-time PCR. Target genes of the differentially expressed miRNAs were predicted and subjected to bioinformatics analysis. Results showed that HBSC11 significantly upregulated the expression of miR-3912-5p, miR-6793-5p, and miR-7159-5p in HepG2.2.15 cells. Target genes of the three miRNAs were mainly involved in the regulation of nucleic acid-templated transcription, negative regulation of gene expression, nucleic acid binding transcription factor activity and regulation of phosphorylation. In addition, target genes were enriched in certain regulatory pathways related to HBV infection and HBV-associated disease progression, such as the transforming growth factor (TGF)- β , Wnt, and p53 signaling. Our study demonstrates the involvement of miR-3912-5p, miR-6793-5p, and miR-7159-5p and the potential modulation of specific pathways (TGF- β , Wnt, and p53 signaling) in HBSC11-mediated inhibition of HBV replication. This study provides insight into the molecular mechanism of the action of HBSC11 against HBV infection and will support the development of antiviral drugs targeting La protein.

KEYWORDS

HBSC11, hepatitis B virus, La protein, miRNA microarray

1 | INTRODUCTION

Despite the development of effective antivirals and vaccines, hepatitis B virus (HBV) infection remains a severe global health problem. It is estimated that more than 350 million people worldwide are chronically infected with HBV.¹ HBV infection can cause chronic liver inflammation and progressive fibrosis, which lead to hepatic decompensation and cirrhosis, and ultimately hepatocellular carcinoma.²

Human La protein, also known as Sjogren syndrome antigen B (SS-B), is a 47-kDa conserved multifunctional RNA-binding phosphoprotein. La protein was initially considered as the autologous antigen produced in patients with systemic lupus erythematosus (SLE) and Sjogren syndrome.³ There are three domains of La proteins: the highly conserved 60-amino acids N-terminal domain that contains the La motif, the moderately conserved RNA recognition motif (RRM, also known as RNP), and the non-conserved C-terminal domain that contains an

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atypical RRM and nuclear localization sequence (NLS).⁴ Mammalian La protein has been shown to specifically bind to a predicted stem-loop in HBV RNA (1275-1291 nt) and thus stabilize the viral RNA.⁵ In addition, the RRM-2 and RRM-3 are separately required for the binding, indicating a cooperative function of these two RRMs.⁶ More recently, it has been reported that inhibition of human La protein by RNA interference downregulates the expression of HBV mRNA and protein.⁷ Therefore, La protein is an important target for inhibiting HBV infection. In our previous studies, a novel inhibitor of human La protein, a pyrazolopyridine HBSC11, with anti-HBV activity, was discovered by structure-based virtual screening and in vitro evaluation.^{8,9}

microRNAs (miRNAs) are an abundant class of small non-coding RNA molecules, containing about 21-23 nucleotides. miRNA duplex was generated by Dicer cleavage of the double-stranded region of a 60-70-nt RNA hairpin precursor. The regulatory functions of miRNAs are accomplished through binding to the consensus sequences of target gene mRNA and activation of the RNA-induced silencing complex (RISC),^{10,11} which degrades mRNA or inhibits mRNA translation.^{12,13} It has been demonstrated that miRNAs play an significant role in cell proliferation,¹⁴ growth,¹⁵ and apoptosis,¹⁶ as well as viral replication.¹⁷ Notably, La protein has been reported to act as a pre-miRNA-binding protein that regulates miRNA processing in vitro.¹⁸

To further investigate the potential mechanism and key regulatory factor of HBSC11-mediated inhibition of HBV, miRNA microarray analysis was performed in the stably HBV-expressing human hepatoblastoma HepG2.2.15 cells with or without HBSC11 treatment to identify the differentially expressed miRNAs. The differentially expressed miRNAs were validated by quantitative real-time PCR, and bioinformatics analysis was performed to explore the possible target genes involved in certain regulatory pathways related to HBV infection and HBV-associated disease progression.

2 | MATERIALS AND METHODS

2.1 | HepG2.2.15 cell culture and HBSC11 treatment

HepG2.2.15 cells stably transformed with HBV were kindly donated by the Shanghai Public Health Clinical Center Affiliated to Fudan University. HepG2.2.15 cells were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 380 µg/mL G418 (Gibco) at 37°C in a humidified atmosphere with 5% CO₂. HBSC11 was synthesized by the School of Pharmacy, Shanghai Jiao Tong University, and the purity was 99.71%. For HBSC11 treatment, a 1 M stock solution of HBSC11 dissolved in DMSO was diluted in complete DMEM to a concentration of 50 µM and filtered through a 0.22-µm membrane. Cells were treated with this medium containing 50 µM HBSC11 for 24 h. Cells treated with DMSO served as a vehicle control. The concentration of HBSC11 and time of treatment were based on our published studies.⁸ The final DMSO concentration in the growth media was 0.005%.

2.2 | RNA extraction

After treatment, cellular RNA was extracted using the mirVana™ miRNA Isolation Kit without phenol (Ambion, Austin, TX) according to the manufacturer's instructions. RNA quality and concentration were confirmed using the NanoDrop ND-2000 (Thermo Fisher Scientific, Waltham, MA). RNA purity and integrity (RIN >7.0) were also verified using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) before miRNA array analysis.

2.3 | miRNA array

Qualified RNA from HBSC11-treated and vehicle-treated HepG2.2.15 cultures were subjected to analysis with the miRNA Complete Labeling and Hyb Kit (Agilent Technologies) and then the Agilent Human miRNA (8*60 K) array. The array was derived from the Sanger microRNA miRBase (V21.0) that contains 2549 human miRNAs. Briefly, miRNAs were fluorescently labeled and hybridized in a hybridization oven at 55°C and 20 rpm for 20 h. After washes in staining dishes (Thermo Fisher Scientific) with Gene Expression Wash Buffer Kit (Agilent Technologies), the array was scanned using the Agilent Microarray Scanner. Data were recorded using the Feature Extraction software 10.7.1.1, and raw data were normalized by the Quantile algorithm, GeneSpring Software 11.0 (Agilent Technologies). miRNAs that were differentially expressed with statistical significance were identified through Scatter Plot, Volcano Plot filtering and hierarchical clustering. *P* values were calculated using Student's *t*-test. The significance thresholds set for the differentially expressed miRNAs were fold changes ≥2.0 or ≤0.5 and *P* ≤ 0.05.

2.4 | Validation of differentially expressed miRNAs by quantitative real-time PCR

According to the miRNA microarray results, several differentially expressed miRNAs were identified in HepG2.2.15 cells after HBSC11 treatment. Quantitative real-time PCR (qRT-PCR) was then performed to validate the array results. RNA was reverse transcribed into cDNA using the miScript II RT Kit (Qiagen, Valencia, CA) and subjected to qRT-PCR using the miRcute miRNA qPCR Detection Kit (SYBR Green, Tiangen, Shanghai, China) on a 7900 HT Sequence Detection System (ABI, Foster City, CA). The PCR program was: 95°C, 2 min, ×1; 94°C, 15 s, 60°C, 1 min, ×40. RNU6B (U6) was used for data normalization. Primers used for miRNA qRT-PCR were synthesized by Sangon Biotech (Shanghai, China) and are listed in Supplementary Table S1. Data were analyzed using the 2^{-ΔΔCt} method.

2.5 | miRNA target prediction

miRNA target prediction was performed using five online software programs including TargetScan (http://www.targetscan.org/vert_71/), PicTar (<http://pictar.mdc-berlin.de/>), miRanda (<http://www.microrna>

org/microma/home.do), miRDB (<http://mirdb.org/miRDB/>), and probability of interaction by target accessibility (PITA) (https://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html) with human selected as the species.

2.6 | Bioinformatic analyses of predicted target genes

After target prediction of the differentially expressed miRNAs, Gene Ontology (GO) and Kyoto Encyclopedia of genes and genomes (KEGG) pathway analyses was performed. The above predicted miRNA target genes were input into the online DAVID database. These bioinformatics resources are able for utilizing the GO database to provide functional information about gene products and to describe functions through the adoption of domain-specific ontologies, particularly suitable for functional analysis of miRNAs. Further, Fisher's exact test and chi-square test were applied to the GO annotations to calculate the *P* values. Finally, the false discovery rate (FDR) was assessed by conducting multiple comparisons of the null hypothesis tests. The smaller the *P* value, the more significant the function of target genes in the GO enrichment analysis. In addition, the target genes input into the online DAVID bioinformatics resources were linked to the KEGG pathway database to create the KEGG pathway annotations. The coded genes in the module were used as target genes and compared to reference genes in the KEGG pathways. Statistical analyses were similar to the analyses in GO annotation analysis.

2.7 | Statistical analysis

The data are expressed as mean ± standard deviation of three independent experiments with three biological replicates. Statistical differences were identified by Student's *t*-test using SPSS 17.0 software (SPSS, Inc., Chicago, IL), and a significance difference was defined as *P* < 0.05 with miRNA expression showing ≥2.0-fold upregulation or ≤0.5-fold downregulation. Additionally, the Benjamini-Hochberg FDR was used for multiple-testing correction (cutoff was 0.05). *P*-values < 0.05 (two-tailed) were considered statistically significant.

3 | RESULTS

3.1 | miRNAs differentially expressed after HBSC11 treatment

Using the human miRNA (8 × 60 K) array, three miRNAs (hsa-miR-3912-5p, hsa-miR-6793-5p, and hsa-miR-7159-5p) were identified

significantly upregulated by HBSC11 treatment in HepG2.2.15 cells. The fold changes in expression were 21.2329 (*P* = 0.0078), 16.3439 (*P* = 0.0029), and 45.3816 (*P* = 0.0026), respectively (Table 1). To directly view the differentially expressed miRNAs, a scatter diagram of miRNA expression was plotted (Figure 1A). The scatter plot showed the genes that were 2-fold upregulated and downregulated (red and green dots, respectively) and filtered the genes with a fold-change value between 0.5- and 2-fold (black dots). However, considering that *P* values were not considered in the scatter plot, the further statistical analysis of miRNA expression was performed by volcano scatter plot analysis (Figure 1B). The red point in the plot represents the genes that were differentially expressed with statistical significance. As shown in Figure 1B, there were three miRNAs with fold changes >2 and *P* values < 0.05. The heat map (Figure 1C) shows distinctive expression patterns for specific miRNAs associated with the HBSC11-treated group and DMSO control group. Specifically, hsa-miR-3912-5p, hsa-miR-6793-5p, and hsa-miR-7159-5p were up-regulated in the HBSC11-treated group.

3.2 | Validation of miRNA expression by qRT-PCR

To confirm the miRNA array data, qRT-PCR was used to measure the expression of the three miRNAs. As shown in Figure 2, the three miRNAs were validated to be upregulated by HBSC11 treatment in HepG2.2.15 cells. The differences were significant, consistent with the microarray results.

3.3 | Prediction of miRNA target genes

miRNAs play important roles in biological processes through inhibition of target gene expression, and online software were used to predict the target genes of the three differentially expressed miRNAs. A total of 3184 target genes were identified, some of which are involved in HBV replication and HBV-induced hepatocarcinogenesis, for example, CCND2, HNF-4A, CUL4A, PTEN, CCND1, SPP1, TP53, and BCL2. Supplementary Table S2 lists some of the predicted targets.

3.4 | Bioinformatics analyses of candidate target genes

3.4.1 | GO annotation of target genes

GO annotation of the target genes was performed to identify the possible regulatory functions of the miRNAs. Figure 3 shows that the candidate target genes were mainly involved in regulation of

TABLE 1 miRNAs differentially expressed after HBSC11 treatment

miRNA name	<i>P</i> value	Fold change	Up/down	Mirbase accession no.	Chr
hsa-miR-3912-5p	0.0078	21.2329	Up	MIMAT0027036	Chr5
hsa-miR-6793-5p	0.0029	16.3439	Up	MIMAT0027486	Chr19
hsa-miR-7159-5p	0.0026	45.3816	Up	MIMAT0028228	Chr6

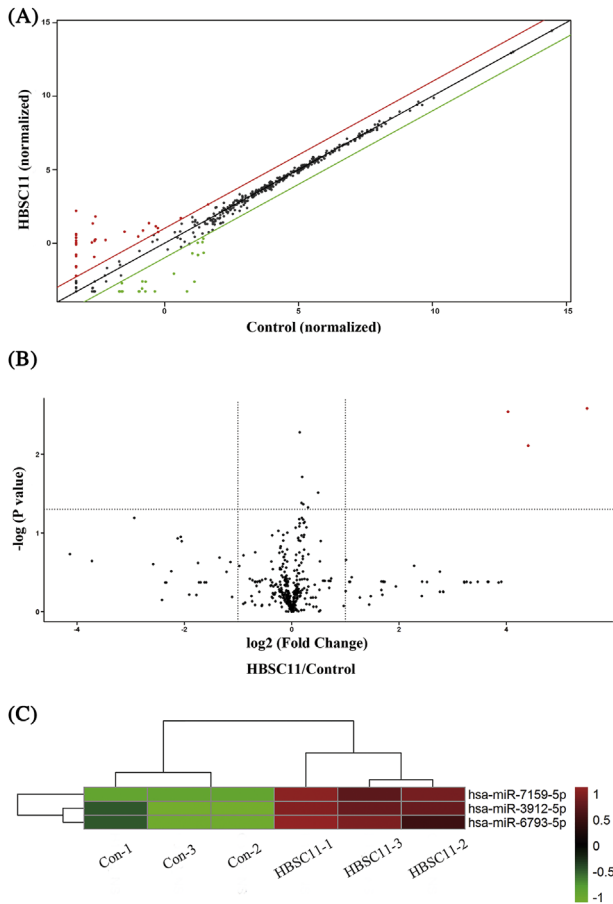


FIGURE 1 Scatter plot (A) and volcano plot (B) and heat map (C) of miRNAs differentially expressed in HepG2.2.15 cells after HBSC11 treatment. A, The X-axis of the scatter plot is the normalized signal values of the DMSO control group, and the Y-axis represents the normalized signal value in the HBSC11-treated group (the ratio scale). B, The plots were constructed using fold-change values and *P* values. The vertical lines correspond to 2.0-fold upregulated and downregulated, and the horizontal line represents *P* value = 0.05. C, Red indicates high relative expression and green indicates low relative expression

transcription, DNA-templating, regulation of nucleic acid-templated transcription, transcription from RNA polymerase II promoter, regulatory region DNA binding, nucleic acid-binding transcription factor activity, and negative regulation of gene expression as well as regulation of phosphorylation, etc. These data suggest that the inhibition of La protein by HBSC11 might regulate the transcription of HBV RNA via these target genes of the three upregulated miRNAs.

3.4.2 | KEGG pathways of target genes involved

KEGG is a pathway-based analysis to explore the biological functions of genes. Further KEGG analysis was carried out to explore the pathways of candidate target genes with which the three miRNAs may be associated. As shown in Figure 4, the target genes were primarily

enriched in the KEGG pathways of ubiquitin-mediated proteolysis, transforming growth factor (TGF)- β signaling, Wnt signaling, and p53 signaling.

4 | DISCUSSION

HBSC11 is a pyrazolopyridine that has been identified as a novel inhibitor of human La protein. Our earlier study using structure-based virtual screening and in vitro evaluation showed that HBSC11 exerted great inhibitory effects on HBV replication.⁸ This study revealed that HBSC11 upregulated the expression of three miRNAs, hsa-miR-3912-5p, hsa-miR-6793-5p, and hsa-miR-7159-5p in HepG2.2.15 cells. These miRNAs target a pattern of genes that are widely enriched in several pathways involved in regulating HBV replication and associated disease progression.

As the target of HBSC11, the La protein is a multifunctional RNA-binding protein. La can interact with a variety forms of RNA, including pre-tRNAs and 5S rRNAs, via targeting the oligouridylylate stretch (UUU_{OH}) at the 3' end of the newly RNA polymerase III-transcribed RNA,⁴ exhibiting RNA chaperone-like activity. Later, La protein was reported to be involved in mRNA translation of a variety of viruses, including HCV and poliovirus.¹⁹ In view of the interaction of La protein with HBV RNA, La specifically recognize a predicted stem-loop located between nt 1275 and 1291 in the viral RNA structure⁵ and modulate the spatial configuration.²⁰ This change conceals the nuclear RNase site near the La protein-binding motif on HBV RNA and thus protects the viral RNA from enzymatic degradation.²¹ The binding of human La protein with HBV RNA contributes to the stabilization of the viral RNA structure and translation of viral proteins. Indeed, it has been shown that phosphorylated La protein was present at much higher levels in HBV-positive liver tissues than in HBV-negative liver tissues, with

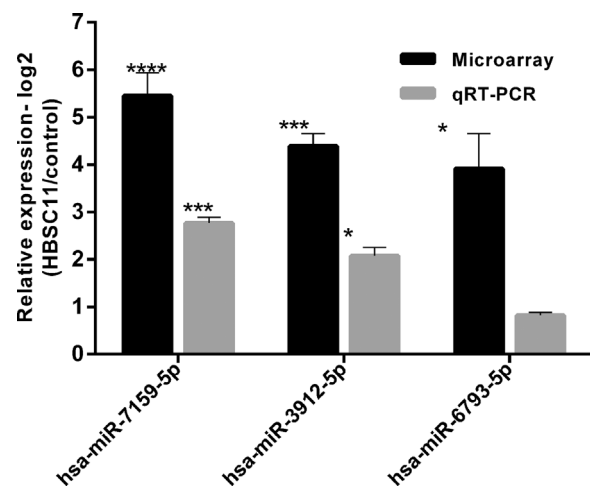


FIGURE 2 Validation of differentially expressed miRNA by qRT-PCR. Comparison of fold change ($\log_2[\text{HBSC11}/\text{control}]$) in miRNA expression between microarray and quantitative RT-PCR results (**P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001)

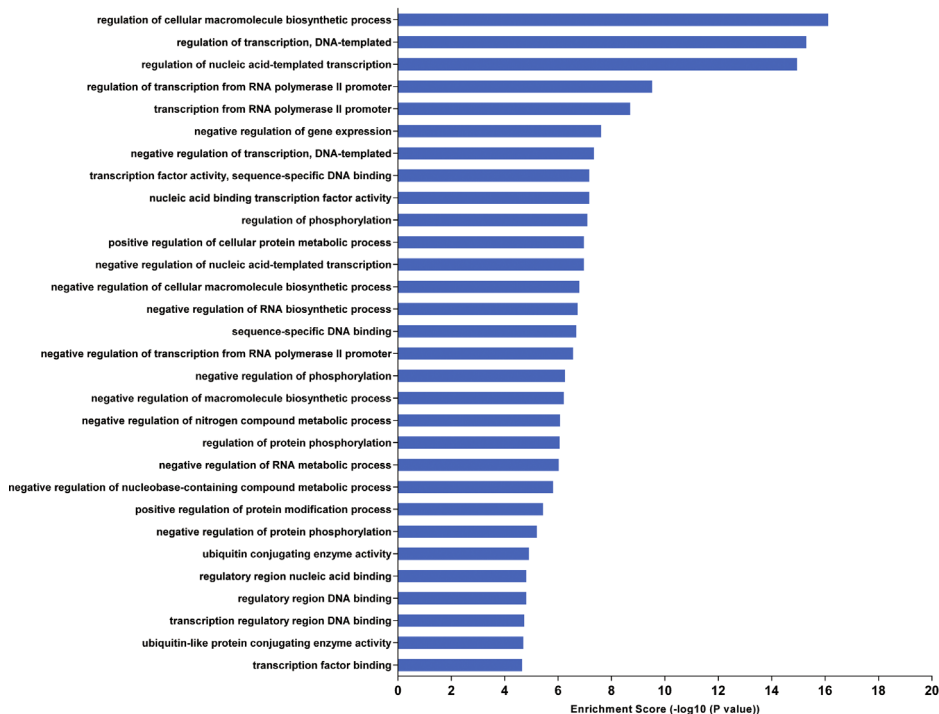


FIGURE 3 GO analysis based on miRNA target genes. The vertical axis is the GO category, and the horizontal axis is the enrichment score, which is equal to $-\log_{10}(P \text{ value})$ that represents the significant level of GOs. GO: Gene ontology. $P < 0.05$ was considered statistically significant

the phosphorylation of the serine 366 residue of La protein by casein kinase II (CK 2) being later demonstrated to promote HBV replication and expression.²² Therefore, novel antiviral strategies were suggested to disrupt the human La-HBV RNA interaction to lead to HBV RNA degradation. We previously screened a number of compounds with the ability to enter a pocket composed of Gln20, Tyr23, Arg57, Leu124, Asn139, and Ile140 on La protein. Among those compounds, HBSC11 was found to form hydrogen bonds with the nitrogen atom of main chain of Asn139 and Ile140 of La protein as analyzed by docking simulation. HBSC11 has the most obvious inhibitory effect on La transcription and expression and was tested to have anti-HBV activity in Hep2.2.15 cells.

MiRNAs are a class of small RNAs that control numerous physiological and pathological processes. A number of studies have demonstrated their involvement in HBV infection and the development of HBV-related diseases. For example, miR-122 was reported to inhibit HBV replication via NDRG3.²³ Loss of miR-122 expression in patients with HBV enhanced viral replication through cyclin G (1)-modulated P53 activity.²⁴ Later, HBV mRNA was reported to inhibit miR-122 expression and thus upregulated PTTG1-binding protein, which promotes hepatocellular carcinoma tumor growth and cell invasion.²⁵ MiR-15a/miR-16-1 were also identified to be down-regulated by HBV RNA in hepatocytes.²⁶ In addition, liver levels of hsa-miR-125a-5p have been correlated with liver HBV DNA level

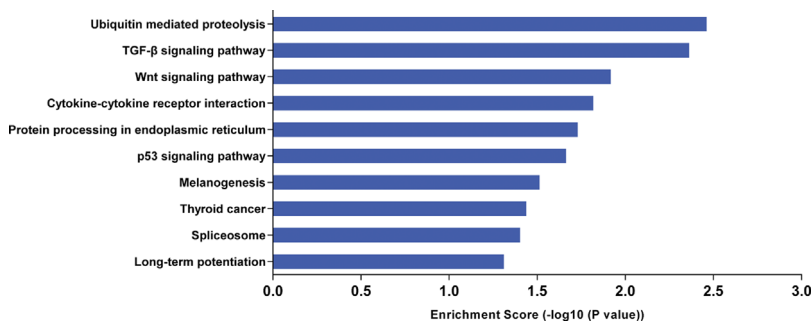


FIGURE 4 KEGG analysis based on miRNA target genes. The vertical axis is the pathway category, and the horizontal axis is the enrichment of pathways, which is equal to $-\log_{10}(P \text{ value})$ that represents the significant level of GOs. KEGG: Kyoto Encyclopedia of Genes and Genomes; TGF-β: transforming growth factor beta; p53: tumor protein p53. $P < 0.05$ was considered statistically significant

and fibrosis score, implicating this miRNA as an independent predictor of disease progression.²⁷ In contrast, miR-125b inhibits HBV expression in vitro through targeting of the SCNN1A gene.²⁸ As for miRNA biogenesis, typically, the primary miRNA (pri-miRNA) transcripts are processed by the nuclear Drosha complex into ~70-nt stem-loop pre-miRNAs. These pre-miRNAs are further cleaved by the cytoplasmic Dicer complex into mature miRNAs. Recently, La protein was further shown to recognize the characteristic stem-loop structure of pre-miRNAs, of which the majority lack a 3' UUU terminus.¹⁸ As such, La protein may act as a global regulator of miRNA expression. Considering the binding sites of HBSC11 to La locate within this region, it is reasonable to assume that HBSC11 will affect processing of certain miRNAs.

The present study showed that the La-specific inhibitor HBSC11 triggered the expression of three miRNAs in Hep2.2.15 cells, including hsa-miR-3912-5p, hsa-miR-6793-5p, and hsa-miR-7159-5p. To date, these miRNAs have not been reported except for a recent computational analysis showing that hsa-miR-3912-5p has the ability to target an unknown genetic motif of molluscum contagiosum virus subtype 1 (MCV).²⁹ They may act as new regulator miRNAs controlling HBV replication.

Prediction of the possible target genes of these three miRNAs revealed a total of 3184 target genes, including some important factors that have been implicated in the regulation of HBV replication, such as CCND2, HNF4A, TNFSF10, SUZ12. CCND2 regulated HBV replication via enhancing the activity of HBV core promoters.³⁰ TNFSF10 has been reported to participate in HBV evasion through HBX-induced autophagy.³¹ SUZ12 was activated during both HBV replication and liver carcinogenesis.^{32,33} Therefore, the La inhibitor HBSC11 may downregulate these genes and thus suppress HBV replication. In addition, target genes also included those associated with HBV-related hepatocellular carcinoma, such as PTEN, IGF1, IGF2, Bcl2, and CCND1. These factors are related to certain cellular biological processes, such as cell proliferation, migration, apoptosis, and angiogenesis. For example, PTEN is well known as a key factor in the PI3K/Akt signaling and acts as a suppressor gene of hepatocellular carcinoma.³⁴ To our surprise, La and La-related protein are closely connected to liver cancer.^{35,36} Therefore, it is of clinical significance to study whether La protein plays a role in HBV-related liver cancer via regulation of HBV expression. In our previous systematic analysis, TNFSF10, CCND1, and Bcl2 were also included in the overlapping target genes of La, CK2, and HBV.³⁷ Therefore, La inhibitor HBSC11 may regulate these genes and thus suppress HBV replication.

Further bioinformatics GO analysis showed that these target genes enriched in regulation of transcription, DNA-templated, regulation of nucleic acid-templated transcription, as well as regulation of transcription from RNA polymerase II promoter biological processes. Candidate target genes of the three miRNAs are also involved in the negative regulation of gene expression and regulation of protein phosphorylation. Indeed, La has been shown to regulate internal ribosome entry site (IRES)-mediated translation of many cellular genes, including CCND1³⁸ and LAMB1.^{35,39} La

antigen was shown to bind the X-linked inhibitor of apoptosis (XIAP) IRES element, facilitating ribosome recruitment and XIAP translation.⁴⁰ In addition, La protein is also involved in the activation of mdm2 mRNA translation by BCR/ABL,⁴¹ and enhanced translation of BiP mRNA⁴² and 5'-terminal oligopyrimidine (5'-TOP) mRNAs.⁴³ Therefore, the La inhibitor HBSC11 may regulate the translation of these genes via the three upregulated miRNAs. Further KEGG analysis revealed that the target genes of the three miRNAs were primarily enriched in the KEGG pathways, including ubiquitin-mediated proteolysis, TGF- β signaling, Wnt signaling, and p53 signaling. TGF- β has been shown to induce expression at the mRNA level of multiple TGF- β /BMP pathway genes, possibly governed by TGF- β -induced miRNA expression (eg, hsa-miR-125a-5p), thus decreasing both HBV mRNA and DNA expression.⁴⁴ Most recently, TGF- β was found to trigger HBV cccDNA degradation through AID-dependent deamination.⁴⁵

In summary, our study showed that the novel La inhibitor HBSC11 upregulated the expression of three miRNAs (miR-3912-5p, miR-6793-5p, and miR-7159-5p) in HepG2.2.15 cells. HBSC11 may affect a number of genes that are involved in the regulation of the TGF- β , Wnt, and p53 signaling pathways. These results demonstrate the involvement of these miRNAs and the modulation of specific pathways involving target genes of these miRNAs in HBSC11-mediated inhibition of HBV. The development of antiviral drugs by targeting La protein warrants further research.

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CONFLICTS OF INTEREST

No conflicts of interest to declare.

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SUPPORTING INFORMATION

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