Abstract

Background and Aims: Continuous release and transmission of hepatitis B virus (HBV) is one of the main factors leading to chronic hepatitis B (CHB) infection. However, the mechanism of HBV-host interaction for optimal viral transport is unclear. Hence, we aimed to explore how HBV manipulates microtubule-associated protein 1S (MAP1S) and microtubule (MT) to facilitate its transport and release.

Methods: The expression of MAP1S or acetylated MT was investigated by immunofluorescence, RT-PCR, immunoblotting, and plasmid transfection. MAP1S overexpression or knockdown was performed by lentiviral infection or shRNA transfection, respectively. HBV DNA was quantified using q-PCR. Results: Significantly higher level of MAP1S in HepG2215 cells compared with HepG2 cells was detected using RT-PCR (p<0.01) and immunoblotting (p<0.001). Notably, stronger MAP1S expression was observed in the liver tissues of patients with CHB than in healthy controls. MAP1S overexpression or knockdown demonstrated that MAP1S promoted MT acetylation and reduced the ratio of HBV DNA copies inside to outside cells. Further, transfection with the hepatitis B virus X protein (HBx)-expressing plasmids induced significantly higher level of MAP1S than that in controls (p<0.0001), whereas HBVX- mutant-encoding HBV proteins (surface antigen, core protein, and viral DNA polymerase) hardly affected its expression. Conclusions: These results demonstrate that HBx induces the formation of stable MTs to promote the release of HBV particles through upregulating MAP1S. Thus, our studies delineate a unique molecular pathway through which HBV manipulates the cytoskeleton to facilitate its own transportation, and indicate the possibility of targeting MAP1S pathway for treatment of patients with CHB.


Introduction

Hepatitis B virus (HBV) infection is a global health concern, with more than 257 million chronic hepatitis B (CHB) carriers at risk of liver cirrhosis and hepatocellular carcinoma (HCC) despite the application of effective vaccines. At present, two kinds of antiviral drugs are available, namely nucleotide analogs (NAs) and interferons (IFNs). Although NAs require long-term administration, they can effectively inhibit HBV replication. IFNs have considerable side effects. These two drugs inhibit viral replication but cannot completely eliminate HBV. Continuous release and transmission of HBV is one of the main factors leading to CHB. However, the mechanism of HBV-host interaction for optimal viral transport and spread is largely unclear. In recent years, interference with nucleocapsid or virus morphogenesis and intracellular transport as a potential antiviral strategy has attracted increasing attention.

HBV is an enveloped hepadnavirus composed of S, C, P, and X genes coding for surface antigen (HBs), core protein (HBc), and viral DNA polymerase (HBp), as well as a multifunctional regulatory X protein (HBx), respectively. HBx regulates transcription not only directly at the chromatin level but also indirectly by modulating the activity of multiple transcription factors necessary for HBV replication through upregulating MAP1S. Thus, our studies delineate a unique molecular pathway through which HBV manipulates the cytoskeleton to facilitate its own transportation, and indicate the possibility of targeting MAP1S pathway for treatment of patients with CHB.

Keywords: Hepatitis B virus; Hepatitis B virus x protein; Microtubule; Microtubule-associated protein 1S.

Abbreviations: CHB, chronic hepatitis B; HBV, hepatitis B virus; HBx, hepatitis B virus x protein; MAP1S, microtubule associated protein 1S; MT, microtubule.

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and implicated in HBV-related oncogenesis.8,9 HBV core particle interacts with surface antigens on the endoplasmic reticulum to form a mature virion, which is subsequently released from the infected cell. However, the underlying mechanism by which HBV is transported and egressed is poorly understood, and the cellular factors specifically involved in this process warrant further investigation.

Depending upon the host transport system for intracellular navigation, viruses have evolved various measures to manage the cytoskeletal function. The cytoskeleton is a protein fiber network system in eukaryotic cells, whose main components include MTs, microfilaments, and intermediate filaments.10,11 MTs are mainly composed of α-tubulins and β-tubulins; one α-tubulin and one β-tubulin form a heterodimer by non-covalent binding. Multiple heterodimers meet at the beginning and end to form a protofilament, and 13 protofilibrils combine to form a hollow cylindrical MT. MTs have specific structural polarities, namely minus-end and plus-end; the minus-end is usually fixed on the MT-organizing center (MTOC) and the plus-end extends outward from the MTOC to form a dynamically growing or shrinking filament. The dynamic change of the MT ends allows MTs to continuously explore the intracellular environment, identifying intracellular cargo to be loaded, such as organelles or internalized viruses, through the process of exploration and capture. In normal cells, most dynamic MTs have a very short half-life (only a few minutes), while post-translationally modified filaments, such as acetylated (Ace-) α-tubulins, can function as dedicated highways for cargo transport.11

MT dynamics and stability are manipulated by a variety of microtubule-associated proteins (MAPs). MAP1S, a member of the MAP1 family, is widely distributed in human and mammalian cells,12 whose function is similar to that of its family proteins in that it can interact with MTs and microfilaments to stabilize the cytoskeleton structure. Viruses have evolved strategies by which they “hijack” the cytoskeleton, usually aiming to transport cellular proteins and vesicles, in favor of their active transport in infected cells.5,13,14 Previous studies showed that adenovirus infection could modulate host MT dynamics to a state that favors viral transmission.15 During human immunodeficiency virus (HIV) infection, MAP1 proteins help tether capsid protein P24 to MTs, thereby promoting HIV transport to the nucleus.16 In addition, recent confocal microscopy studies demonstrated that the HBV core interacted with tubulin and co-localized with MT, while the HBV permissiveness of HepAD38 cells was impaired upon treatment with MT inhibitor.17

However, it remains unclear whether and how HBV manipulates the microtubule system to facilitate its transport and release. To address this question, we tested the expression of MAP1S both in HBV-stable-expressing cells and during natural infection. Moreover, through MAP1S overexpression or knockdown, we analyzed the effect of MAP1S on the stabilization of MTs and release of HBV. The data obtained indicated that HBV induced the formation of stable MTs to promote the release of HBV particles through upregulating MAP1S.

**Methods**

**Ethics statement**
This study was conducted in accordance with the Declaration of Helsinki and its amendments. The experiment protocol was also approved by the ethics committee of Beijing You An Hospital, Capital Medical University (Archiving No: LL-2021-027-K, Approval No. 2021-015).

**Human tissue samples**
Six CHB tissues were obtained from the bioinformatic sample bank in Beijing You An Hospital, Capital Medical University. None of the patients received radiotherapy or chemotherapy before surgery. The MAP1S immunostaining of normal control liver tissues was performed as per the Human Protein Atlas (https://www.proteinatlas.org/).

**Cell lines**
Human hepatocarcinoma cell lines HepG2 and HepG2215 (HBV-expressing cells) were maintained by our laboratory and cultured in DMEM (Hyclone, Logan, UT, USA) with 10% fetal bovine serum, 100 U/mL penicillin and streptomycin, and 5 mmol/L glutamine (Gibco, Grand Island, NY, USA). Transfections were performed with lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions.

**Immunofluorescence**
Cells were inoculated at a density of 1×10⁵ cells/well in a 24-well plate, covered with slides overnight, and then fixed with 4% paraformaldehyde for 15 min; subsequently, they were washed three times with phosphate-buffered saline (PBS) and punched with 0.1% Triton X-100 for 20 m, blocked with 3% bovine serum albumin for 60 m, and incubated with a diluted primary antibody against MAP1S (1:500; Biorbyt, Cambridgeshire, UK) or HBs (1:500; Abcam, Cambridge, UK) overnight at 4°C. After washing three times with PBS, the cells were incubated with a secondary antibody labeled with Alexa-Fluor-594 or Alexa-Fluor-488 (1:1,000; Jackson Immunoresearch, West Grove, PA, USA) at room temperature for 60 m. The nuclei were counterstained with DAPI (Sigma-Aldrich, St. Louis, MO, USA). Fluorescence micrographs were acquired using Nikon XDS-18 inverted microscope or Nikon TE2000-S super-resolution fluorescence microscope.

**Immunoblotting**
Cells were lysed using 2× high-salt radioimmunoprecipitation assay (2× RIPA) buffer and complete protease inhibitor phenylmethylsulfonyl fluoride (PMSF). The concentration of the extracted proteins was determined using the BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). The proteins were then transferred from SDS-PAGE gel to a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). After blocking with 5% milk in PBS containing 0.05% Tween-20, the membrane was incubated with primary antibodies anti-MAP1S (1:1,000; GeneFex, San Antonio, TX, USA), anti-Ace-tubulin (1:2,000; Sigma-Aldrich), and anti-β-actin (1:1,000; Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. Later, secondary antibody (1:2,000; Jackson Immunoresearch) was added to it and the membrane was incubated for 60 m at room temperature. Finally, proteins were quantified using the ImageQuant-TL-7.0 software version 2010 (Amersham Biosciences, Piscataway, NJ, USA).

**Real-time quantitative polymerase chain reaction**
Total RNA was isolated from cells using a Trizol reagent (Thermo Fisher Scientific), according to the manufacturer’s instructions. RNA samples (2 μg) were used to perform re-
verse transcription and real-time PCR with a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) and SYBR Green Premix Ex Taq™ kit (TaKaRa Bio, Kyoto, Japan), respectively. The primer sequences were designed as follows: MAP1S forward 5′-CGCTGGAAGAATCCCTCATC-3′ and reverse 5′-GAGTGAGCCCAGTGAGAAGG-3′, GAPDH forward 5′-CGGGCTCTCCAGAACATC-3′ and reverse 5′-ATGACCTTGGCCACAGCTT-3′. The relative expression levels of MAP1S were normalized to GAPDH.

Establishment of MAP1S-overexpressing/-silencing HepG2215 cells

The lentivirus-based MAP1S overexpressing-RED-PURO (ad-MAP1S) and MAP1S-silencing-shRNA-GFP-PURO (sh-MAP1S) were constructed by Beijing Hosun Gene Corporation (Beijing, China). Ad-MAP1S infected HepG2215 cells with an MOI of 50, and the empty vector was used as a control (ad-Vector) in the presence of polybrene (5 mg/mL), according to the manufacturer's instructions. After 48 h, the cells were cultured in a medium containing puromycin (2–5 µg/mL) for about 14 days to generate a stable cell line. HepG2215 cells were transfected with sh-MAP1S in the presence of polybrene (5 mg/mL), according to the manufacturer's instructions. After 48 h, the cells were cultured in a medium containing puromycin (2–5 µg/mL) for about 14 days to generate a stable cell line. MAP1S-overexpressing/-silencing HepG2215 cells were confirmed by western blotting or RT-PCR.

Immunocytochemistry

The tissue sample sections were deparaffinized and rehydrated, and then boiled in 0.01 M sodium citrate buffer (pH 6.0) for 15 min to remove the antigen. After blocking with 1% goat serum and adding the primary antibody of MAP1S (1:500, GeneTex), the sections were incubated overnight at 4°C. Subsequently, the secondary antibody labeled with horseradish peroxidase was added and the sections were incubated for 60 min at room temperature. Signals were detected with 3,3′-diaminobenzidine tetrahydrochloride, and the sections were counterstained with hematoxylin.

HBV DNA detection

Cells were plated in a six-well plate at a density of 1×10^6 cells/well. After 24 h, the supernatant was collected and quantified (SN1). Subsequently, the cells were counted and lysed with 2× high-salt RIPA containing PMSF for 30 min. The lysed cells were centrifuged at 12,000 × g for 20 min, and the supernatant (SN2) was detected for extracellular and intracellular HBV DNA, respectively, via a fluorescence quantitative PCR analyzer ABI 7500 (Applied Biosystems, Carlsbad, CA, USA).

Plasmid transfection

Plasmids p1.3mer HBV (HBVwt) and p1.3mer HBV- (HBV⁻) and the plasmid pECE1HBX that expressed the HA-tagged HBx (HBX) were kindly gifted by Professor Jing-hsiung Jams Ou and have been described before. HepG2 cells were transfected with DNA plasmid using Lipofectamine 3000 (LC3000150; Thermo Fisher Scientific), according to the manufacturer's instructions.

Rescue experiment

The HepG2 cells were grown in six-well plates until they reached a density of 70-80% and transfected with 1 µg of HBV, HBVX⁻, and HBX plasmids using Lipofectamine 3000, according to the manufacturer's instructions, and the empty vector was used as a control. After transfection for 12 h, HBX plasmids were added to HepG2 cells transfected with HBX-DNA and the cellular proteins were extracted after 48 h for validation.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 8.3 software (GraphPad Software, Inc., La Jolla, CA, USA) using two-tailed paired t-test. A p value <0.05 was considered statistically significant. Data are presented as means±standard deviations from at least three independent experiments.

Results

HBV infection induced upregulation of MAP1S expression

To investigate whether HBV affects the expression of MAP1S, we evaluated MAP1S expression in HBV stable-expressing HepG2215 and HBV-negative HepG2 cells using fluorescence microscopy, and cells were stained for MAP1S. As shown in Figure 1A and B, the fluorescent signal was weak in the absence of HBV but bright in the presence of HBV. To confirm that HBV could indeed induce upregulation of MAP1S, we conducted immunoblotting analysis on MAP1S expression. As shown in Figure 1C and D, a significantly larger amount of MAP1S was detected in HepG2215 cells than in HepG2 cells (p<0.001). Further analysis with RT-PCR showed significantly higher level of MAP1S mRNA in HepG2215 cells than in HepG2 cells (p<0.01) (Fig. 1E). In addition, stronger MAP1S expression was observed in the liver tissues of patients with CHB than in those of healthy controls (Fig. 1F). Particularly, MAP1S expression was much stronger in HBs-positive cells than in HBs-negative cells in the same liver tissue sample of patients with CHB, and an extensive co-localization of HBs and MAP1S was detected in the hepatocytes of these patients (Fig. 1G and H). Together, these results indicated that HBV could induce the upregulation of MAP1S in human hepatocytes.

MAP1S promotes microtubule stabilization

As MAPs have been shown to change the dynamic behavior of MTs in favor of early viral infection, we reasoned that HBV infection could promote the formation of stable MTs through modulating the expression of MAP1S. To address this, we conducted MAP1S knockdown on HepG2215 cells via transfection of either MAP1S-silencing-RNA (sh-MAP1S) or sh-Vector as the control. As shown in Figure 2A–D, knockdown of MAP1S led to a reduction in the level of Ace-α-tubulin, which is one of the several post-translational modifications associated with stable non-dynamic MTs; however, unmodified protein α-tubulin was unchanged and the ratio of acetylated tubulin/α unmodified tubulin was decreased (Fig. 2E), indicating that knockdown of MAP1S did not affect the cytoskeleton and only affected microtubule acetylation modifications. By contrast, overexpress-
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Fig. 1. HBV infection induces upregulation of MAP1S expression. (A–E) Representative fluorescence images, RT-PCR, and immunoblotting were used to analyze the impact of HBV on the expression of MAP1S in HepG2 and HepG2215 cells. Scale bar in A: 10 µm. (F) Liver tissues from patients with CHB were stained with MAP1S antibody. The MAP1S immunostaining of normal control liver tissues was performed as per the Human Protein Atlas (https://www.proteinatlas.org/). Scale bar: 20 µm. (G) Representative images show immunofluorescence staining of HBs and MAP1S in HBs-negative and HBs-positive tissues from different patients with CHB, and arrows represent positive staining with indicated antibodies. Scale bar: 10 µm. (H) Representative images show the mean intensity of tissue immunofluorescence. Histograms show the mean from at least three independent experiments. Bars indicate SD. **p<0.01, ***p<0.001. CHB, chronic hepatitis B; HBV, hepatitis B virus; MAP1S, microtubule associated protein 1S.

Fig. 2. MAP1S promotes microtubule stabilization. (A) Representative immunoblot shows levels of MAP1S, α-tubulin, and acetylated α-tubulin (Ace-tubulin) in HepG2215-sh-Vector and HepG2215-sh-MAP1S cells. (B–D) Representative quantification shows levels of MAP1S, α-tubulin, and Ace-tubulin in HepG2215-sh-Vector and HepG2215-sh-MAP1S cells. (E) Representative quantification demonstrate the ratio of acetylated tubulin/α unmodified tubulin in HepG2215-sh-Vector and HepG2215-sh-MAP1S cells. (F) Representative immunoblot shows levels of MAP1S, α-tubulin, and Ace-tubulin in HepG2215-ad-Vector and HepG2215-ad-MAP1S cells. (H–I) Representative quantification shows levels of MAP1S, α-tubulin, and Ace-tubulin in HepG2215-ad-Vector and HepG2215-ad-MAP1S cells. β-actin was used as a loading control. Histograms show the mean from at least three independent experiments. Bars indicated SD. *p<0.05, **p<0.01, ***p<0.001. MAP1S, microtubule associated protein 1S.
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upregulation of MAP1S is conducive to the conversion of unmodified protein α-tubulin to acetylated MT. Compared with the control group, the amount of acetylated MTs was significantly increased while the amount of α-tubulin was reduced ($p<0.01$) (Fig. 2F–J). Taken together, these results confirm the function of MAP1S in promoting the formation of stable MTs via increase of MT acetylation.

MAP1S facilities HBV transport and release via promotion of MT stabilization

Viruses depend on the host transport systems for intracellular navigation. To determine whether MAP1S participates in the process of HBV release, we also performed sh-RNA knockdown to suppress the expression of MAP1S; the supernatant and cells were collected to quantify HBV DNA copies inside and outside the cells with RT-PCR, respectively. As shown in Figure 3A and B, a more than two-fold increase in the ratio of HBV DNA copies inside to outside cells (RatioI/O) was observed in the sh-MAP1S-treated HepG2215 cells compared with the control ($p<0.01$) (Fig. 3C and D), reflecting that overexpression of MAP1S facilitated HBV release, while knockdown of MAP1S inhibited HBV transport and release. To ensure that MAP1S indeed promotes HBV intracellular transport, we observed that MAP1S silenced HepG2215 cells with confocal microscopy and cells were stained for HBs. As shown in Figure 3E, a bright fluorescent signal of HBs around the nucleus was observed in sh-MAP1S-treated HepG2215 cells, while a relatively weak signal of HBs distributed diffusely in the cytoplasm of control cells, suggesting an accumulation of HBs inside the cells as a result of MAP1S knockdown. Thus, these results shown in Figures 2 and 3 together indicate a positive effect of MAP1S on HBV transport and release via promotion of MT stabilization.

HBV upregulates MAP1S expression via HBx activity

HBx is a regulatory protein with a dual role, namely modulating promoter activity in the nucleus and regulating sig-

Fig. 3. MAP1S facilitates HBV transport and release via promotion of MT stabilization. (A–D) Quantification of HBV DNA inside to outside of MAP1S-silencing (A, B) and MAP1S-overexpressing (C, D) HepG2215 cells. (E) Subcellular distribution of HBs in the sh-MAP1S-treated HepG2215 cells and control cells was observed with fluorescence microscopy. Scale bar: 10 µm. Histograms show the mean from at least three independent experiments. Bars indicate SD. **$p<0.01$. HBV, hepatitis B virus; MAP1S, microtubule associated protein 1S.
then harvested after 48 h for immunoblotting analysis using the indicated antibodies. (C–D) For the rescue experiment, HepG2 cells were transfected with wild-type HBV, HBX, or Vector plasmids and the HBVX mutant plasmids first, and 12 h later, HBX plasmids were transfected onto these HepG2 cells. Cells were harvested after 60 h for immunoblotting analysis. β-actin was used as a loading control. Histograms show the mean from at least three independent experiments. Bars indicate SD. *p<0.05, **p<0.01, ***p<0.001. HBV, hepatitis B virus; HBx, hepatitis B virus x protein; MAP1S, microtubule associated protein 15.

Fig. 4. HBV upregulates MAP1S expression via HBX activity. (A–B) HepG2 cells were transfected with wild-type HBV, HBX, or Vector plasmids and then harvested after 48 h for immunoblotting analysis using the indicated antibodies. (C–D) For the rescue experiment, HepG2 cells were transfected with the HBVX− plasmid first, and 12 h later, HBX plasmids were transfected onto these HepG2 cells. Cells were harvested after 60 h for immunoblotting analysis. β-actin was used as a loading control. Histograms show the mean from at least three independent experiments. Bars indicate SD. *p<0.05, **p<0.01, ***p<0.001. HBV, hepatitis B virus; HBx, hepatitis B virus x protein; MAP1S, microtubule associated protein 15.

Discussion

In this study, we found that MAP1S was a key regulator of HBV transport and production. MAP1S expression significantly increased in HBV-expressing HepG2215 cells, suggesting that HBV induced the upregulation of MAP1S. Further immunohistochemical staining of liver samples from patients with CHB demonstrated that the expression of MAP1S in HBV-positive hepatocytes was much stronger than that in HBV-negative cells, confirming that HBV induced the upregulation of MAP1S during a natural infection. The increment of acetylated MTs reflects an increase in

the population of stable MTs.12,19 The stable MT has a long half-life and can be used as a special channel for cargo transportation.5,11 Our research demonstrated that silencing of MAP1S resulted in a decrease in acetylated microtubules but increased the ratio of intracellular to extracellular HBV DNA, which indicated that lack of MAP1S reduced the stability of microtubules, damaged the intracellular transport channel and, to a large extent, led to a reduction in the release of HBV virus particles. These results are in agreement with the findings in HeLa cells, where shRNA-mediated knockdown of MAP1S resulted in MTs with a shortened lifespan.20 Likewise, Iwamoto et al.21 found that the functional combination of cell microtubules and viral capsid assembly could support effective HBV replication. Fernandez et al.10 reported that depletion of MAP1S in P4-CR55 cell lines and primary human macrophages led to a remarkable decrease in HIV-1 infectivity as a result of impaired retrograde trafficking. In further support of the role of MAP1S in the stabilization of MTs, overexpression of MAP1S led to an increase of acetylated microtubules, a decrease of intracellular HBV accumulation, and, consequently, a reduction in the ratio of intracellular to extracellular HBV DNA, indicating that MAP1S is required for MT acetylation, HBV intracellular transportation, and egress.

We reasoned that the fact that HBV secretion was not completely inhibited in sh-MAP1S-treated cells was attributable to the presence of other MAP1 proteins. As MAP1S shares the three hallmark domains of microtubule-associated protein 1 family,18 other MAP1 proteins, such as MAP1A and MAP1B, might partially compensate for the decrease in acetylated microtubules caused by MAP1S knockdown. Alternatively, it was reported that MAPs interact with molecular motors to directly interfere with the binding of MTs and cargo or to affect their overall function.17 Although the molecular motors involved in HBV trafficking have not yet been identified, increasing evidence indicates that dynein and kinesin might be involved in the process.22,23 In addition, since the movement is bidirectional, daughter HBVs could simultaneously bind to molecular motors of opposite polarity, as shown for herpes simplex virus HSV-1.24 Thus, the detailed mechanisms by which HBV interacts with molecular motors warrant further investigation.

HBV is considered a promiscuous trans-activator of viral and cellular promoters and enhancers.25–28 Our results showed that HBV could upregulate the expression of MAP1S at both mRNA and protein level. Further, transfection with the HBX-expressing plasmid induced significantly higher levels of MAP1S expression than that of the control, whereas HBV-mutant-encoding HBV viral proteins (HBs, HBc, HBp) hardly affected MAP1S expression, suggesting that HBV induced MAP1S upregulation through the activity of HBx, which itself played a crucial role in upregulating MAP1S expression. Consistently, in previous studies, HBx had been shown to target the epigenetic control of cell gene expression by interacting with chromatin-modifying enzymes.26,28 Further, a series of HBx genomic binding sites related to HBV-induced hepatocarcinogenesis have been identified.29 The biological process through which intracellular cargo is loaded onto the microtubule track, navigated along its route, and subsequently transported may be modulated by a variety of virus–host interactive mechanisms.30 Our current work and previous studies both indicated that HBV could actively manipulate its intracellular transport by using multiple mechanisms such as upregulating microtubule-associated proteins and inducing stable microtubules (our study).21,30

In conclusion, through this study, we established a unique mechanism by which HBV induces the formation of stable MTs to enhance productive infection through upregulating MAP1S. Our findings also raise the possibility of targeting the MAP1S pathway to treat patients with CHB.
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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Writing the manuscript (YG, YW), acquisition of data (YG, SZ, YH, RZ), analysis and interpretation of data (YG, YW, YZ) critical revision of the manuscript for important intellectual content (ML, BS, DC, YS). All authors read and approved the final version of the manuscript.

Data sharing statement

The data used to support the findings of this study are included within the article.

References


