ORIGINAL PAPER
Polish Journal of Microbiology
2024, Vol. 73, No 2, 217–235
https://doi.org/10.33073/pjm-2024-021

Guanylate-Binding Protein 1 (GBP1) Enhances IFN-α Mediated Antiviral Activity against Hepatitis B Virus Infection

YADI LI[1], HAIYING LUO[1], XIAOXIA HU[1], JIAOJIAO GONG[1, 2], GUILI TAN[1, 2], HUATING LUO[3], RUI WANG[1], HAO PANG[1, 2], RENJIE YU[1] and BO QIN[1]*

[1] Department of Infectious Diseases, Chongqing Key Laboratory of Infectious Diseases and Parasitic Diseases, The First Affiliated Hospital of Chongqing Medical University, Chongqing, China
[2] Central Laboratory, The First Affiliated Hospital of Chongqing Medical University, Chongqing, China
[3] Department of Geriatrics, The First Affiliated Hospital of Chongqing Medical University, Chongqing, China

Submitted 7 February 2024, accepted 8 May 2024, published online 14 June 2024

Abstract

Interferon-alpha (IFN-α) is a first-line drug for treating chronic hepatitis B (CHB). Guanylate-binding protein 1 (GBP1) is one of the interferon-stimulating factors, which participates in the innate immunity of the host and plays an antiviral and antibacterial role. In this study, we explored how GBP1 is involved in IFN-α antiviral activity against HBV. Before being gathered, HepG2-NTCP and HepG2 2.15 cells were transfected with the wild-type hGBP1 plasmid or si-GBP1, respectively, and followed by stimulation with Peg-IFNα-2b. We systematically explored the role of GBP1 in regulating HBV infection in cell models. Additionally, we also examined GBP1 levels in CHB patients. GBP1 activity increased, and its half-life was prolonged after HBV infection. Overexpression of GBP1 inhibited the production of HBsAg and HBeAg, as well as HBs protein and HBV total RNA levels, whereas silencing of GBP1 inhibited its ability to block viral infections. Interestingly, overexpressing GBP1 co-treatment with Peg-IFNα-2b further increased the antiviral effect of IFN-α, while GBP1 silencing co-treatment with Peg-IFNα-2b partly restored its inhibitory effect on HBV. Mechanistically, GBP1 mediates the anti-HBV response of Peg-IFNα-2b by targeting HBs. Analysis of clinical samples revealed that GBP1 was elevated in CHB patients and increased with Peg-IFNα-2b treatment, while GBP1 showed good stability in the interferon response group. Our study demonstrates that GBP1 inhibits HBV replication and promotes HBsAg clearance. It is possible to achieve antiviral effects through the regulation of IFN-α induced immune responses in response to HBV.

K e y w o r d s: chronic hepatitis B, antiviral, hepatitis B virus, hepatitis B surface antigen, guanylate-binding protein 1 (GBP1), pegylated interferon α-2b

Introduction

Chronic infection with the hepatitis B virus (HBV) remains a significant global public health concern, as it has the potential to contribute to the generation and progression of liver disease (Yuen et al. 2018). Approximately 257 million people worldwide suffer chronic HBV infections, which account for 3.5% of the global population and cause nearly 1 million deaths annually (Xu et al. 2022). Although anti-HBV drug research and development has reduced mortality from complications of chronic hepatitis B, the viral carriage remains high (Nguyen et al. 2020). The stable presence of covalently closed circular DNA (cccDNA) in liver cells is the biggest obstacle to the complete cure of chronic hepatitis B (CHB) patients (Du et al. 2021). The inability of current antiviral drugs to effectively target cccDNA has emerged as a primary obstacle to achieving radical treatment, preventing virus rebound, and avoiding disease recurrence in patients with CHB after drug withdrawal (Salerno et al. 2020). Presently, the closest outcome to cure is the clearance of HBV DNA accompanied by HBsAg loss, known as "functional cure" (Shih et al. 2018). HBsAg loss and HBsAg seroconversion to anti-HBs may improve liver histopathology, increase survival rates, and reduce liver disease complications such

* Corresponding author: B. Qin, Department of Infectious Diseases, Chongqing Key Laboratory of Infectious Diseases and Parasitic Diseases, The First Affiliated Hospital of Chongqing Medical University, Chongqing, China; **e-mail: qinbo@cqmu.edu.cn**
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as hepatocellular carcinoma (Ge et al. 2015; Tseng et al. 2020; Tseng et al. 2022). Nucleotide analogues (NAs) and interferon alpha (IFN-α) are first-line agents for the treatment of CHB (Islam et al. 2023). NAs, which act as reverse transcriptase inhibitors, can effectively suppress the viral load of HBV. However, it had little effect on cccDNA, resulting in lower rates of HBeAg seroconversion and HBsAg loss. Due to the high relapsing rate after cessation of NAs, most patients require long-term or even lifelong treatment. A type I interferon with direct antiviral and immunomodulatory properties, IFN-α can effectively inhibit HBV transcription and enhance immune function (Yuen et al. 2021; Yuen et al. 2022). HBeAg seroconversion and HBsAg clearance were higher following IFN-α therapy, and a long-term response can be maintained. More and more evidence confirmed the efficacy of IFN-α in treating CHB. However, only a small number of patients with CHB benefit from IFN-α (Liu et al. 2020; Huang et al. 2022; Wong et al. 2022). Thus, an in-depth study of the mechanism of interferon action on HBsAg is of great significance for optimizing the therapeutic strategy of interferon in the future and improving the clinical cure rate of CHB.

Guanylate-binding proteins (GBPs), members of the GTPase family, are the most abundant of the interferon (IFN-γ)-induced immune proteins (Tretina et al. 2019; Zhang et al. 2021). The involvement of GBP in the host is necessary for mediating innate immunity, as it exerts an antiviral effect against a wide range of exogenous pathogens, including bacteria and viruses (Mohammadi et al. 2020). GBPs can target intracellular pathogens and mediate host defense responses through inflammasome, oxidation, and autophagy (Feng et al. 2022). The guanylate-binding protein 1 (GBP1), which has a molecular weight of around 67 KD, plays many roles in biology, including antiviral and anti-tumor mechanisms (Honkala et al. 2019). Human GBP1 (hGBP1) was discovered to suppress the negative-stranded RNA elastic vesicular stomatitis virus and the microRNA virus encephalomyocarditis virus (EMCV) in the early 1990s (Prakash et al. 2020). In addition, mouse GBP1 (mGBP1) was also found to have the same antiviral action (Tessema et al. 2023). It had been demonstrated that GBP1 inhibited hepatitis C virus replication by hydrolyzing GTPase in knockout and overexpression cell models (Itsui et al. 2009). This antiviral mechanism of GBP1 was also confirmed in subsequent studies on dengue virus (Mariappan et al. 2023), influenza virus (Nordmann et al. 2012), hepatitis E virus (Glitscher et al. 2021), and swine fever virus (Li et al. 2016). Furthermore, researchers have observed that GBP1 can inhibit the spread of pathogenic microbial infections by activating inflammasomes, inducing pyroptosis, or participating in autophagy (Fisch et al. 2019). In another study, it was reported that the GBP1 and ISG15 genes were more inhibited in HBeAg (−) patients than in HBeAg (+) (Lebossé et al. 2017). Despite this, little is known about GBP1's role in anti-HBV activity.

This study unveils the unique role of GBP1 in enhancing IFN-α-mediated antiviral activity against HBV, emphasizing its distinctive contributions to inhibiting HBV replication and promoting HBsAg clearance. In contrast to previous research, our focus on GBP1's specific impact within the context of IFN-α therapy for chronic hepatitis B highlights its novel and clinically significant implications. Our results revealed that GBP1 inhibited HBV replication and promoted HBsAg clearance. Our findings provide new insights into the function and role of GBP1, an ISG induced by HBV infection, and may serve as a predictor of the efficiency of CHB IFN-αtherapy.

Experimental

Materials and Methods

Drugs and plasmids. Peg-IFNα-2b (Xiamen Tebao Company, China) was packed into a 1.5 ml EP tube and stored at 4°C. Cycloheximide (CHX) was purchased from MedChemExpress (USA) (HY-12320), dissolved by DMSO at a final concentration of 125 mg/ml, and stored at −80°C. The plasmids of 3 × Flag-GBP1, 3 × GST-GBP1, and 3 × GST-GBP1 mutations were constructed in our laboratory. GBP1 short-chain interferon RNA (si-GBP1) or non-targeted siRNA (si-control) were purchased from Beijing Entomobiology (Beijing Tsingke Biotech Co., Ltd., China). After verification, the best titer was selected for follow-up experiments.

siRNA was introduced to infected cell lines. The cells were harvested at the necessary time points to detect viral markers. A list of primer sequences used in the experiment is provided in the Appendix. HBsAg (NB100-62652; Novus Biologicals, USA), HBcAg (ab8637; Abcam Ltd., UK), Anti-GBP1 (15303-1-AP; Prointech Group, Inc., China), Anti-GST (66001-2; Prointech Group, Inc., China), Anti-HBs (NB100-62652; Novus Biologicals, USA), Anti-β-actin (81115-1-RR; Prointech Group, Inc., China), and Anti-Histone H3 (AF0009; Beyotime Biotechnology, China) were purchased.

Cell culture and virus infection. HepG2-NTCP and HepAD38 cell lines were given by the Infection Laboratory of Chongqing Medical University, and HepG2 2.15 cells were purchased from (China Center for Type Culture Collection (CCTCC)). HepG2-NTCP cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich®, Merck KGaA, Germany) supplemented with 10% fetal bovine serum (FBS; Gibco™; Thermo Fisher Scientific, Inc., USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco™; Thermo

Fisher Scientific, Inc., USA) in an incubator with 5% CO_2 at 37°C. HepG2 2.15 and HepAD38 cell lines with 400 μg/ml G418 (BioFroxx, Germany) were used to screen the cell lines producing hepatitis B virus to maintain replication of the virus. Following the manufacturer's instructions, DNA transfection agents (Invitrogen™, Thermo Fisher Scientific, Inc., USA) were used to transfect genes.

In siRNA experiments, GBP1 short-interfering RNA (siRNA) specifically silenced GBP1 expression in HepG2 2.15 cells. Two siRNA constructs targeting GBP1 (si-GBP1-1 and si-GBP1-2) were utilized, along with a non-targeted siRNA (si-control) as a control. Transfections were performed using a transfection reagent according to the manufacturer's instructions (e.g., lipofectamine RNAiMAX). The optimal siRNA concentration and transfection conditions were determined through pilot experiments. Cells were harvested at various time points post-transfection for downstream analyses (Ren et al. 2018).

HepG2 2.15 cells were transfected with plasmids containing wild-type human GBP1 (hGBP1) or mutant GBP1 constructs using a transfection reagent (e.g., Lipofectamine 3000) following the manufacturer's protocol. The plasmids included 3×Flag-hGBP1, 3×GST-hGBP1, and mutants (e.g., R48A, S73A, Q137A, D184N, R240A, R244A), which were constructed by cloning techniques such as restriction enzyme digestion and ligation. The plasmid constructs were verified through sequencing.

For HBV infection experiments, HepG2-NTCP cells were infected with HBV particles at a specific concentration (e.g., 2×10^3 genome equivalents per cell) for a defined duration (e.g., 24 hours) under conditions conducive to viral entry. Following infection, cells were washed with phosphate-buffered saline (PBS) to remove unbound virus particles, and culture media were replaced with fresh media supplemented with appropriate additives.

Cells were collected at various time points post-transfection or post-infection to assess the effects of siRNA knockdown, plasmid overexpression, or viral infection on GBP1 expression, HBV replication, and viral protein production. Time points were selected based on previous studies and preliminary experiments to capture key stages of the experimental processes and dynamic changes in molecular markers.

Enzyme-linked immunosorbent assay (ELISA). Cells were transfected with the DNA plasmid, siRNA or stimulated with Peg-IFNα-2b. Cell culture supernatant was collected, and HBsAg and HBeAg were detected using ELISA kits (Shanghai Kehua Bio-engineering Co., Ltd., China).

Western blot analysis. After plasmid transfection or drug treatment, cells were collected and lysed with radioimmunoprecipitation assay (RIPA) buffer (Roche, Germany). Protein concentration was determined using a protein analysis reagent (Beyotime Biotechnology, China). In each sample, 30 μg of protein was separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (GE Healthcare UK Ltd., UK). A chemiluminescence reagent was used to develop the images. The density of the target protein band was normalized to β-actin. Each experiment was repeated three times.

qPCR for RNA Expression Detection. TRNzol Universal Reagent (DP424; TIANGEN Biotech(Beijing) Co., Ltd., China) and FastKing RT kit (KR116-02; TIANGEN Biotech(Beijing)Co., Ltd., China) were used to isolate cellular total RNA and synthesize cDNA. Total RNA was reverse transcribed using iTaq™ Universal SYBR® Green Supermix (1725122; Bio-Rad, USA) in QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems™, Thermo Fisher Scientific, Inc., USA). The expression level was standardized to β-actin, and three independent amplifications were performed for each sample. Relative expressions for each target gene were calculated using the $2^{-\Delta\Delta Ct}$ approaches.

Immunofluorescence assay. After transfection, the cells were grown on glass sheets for 48 h and fixed in 4% polymethanol. HBs (Anti-HBs, Novas) and FLAG-Tag (monoclonal mouse Flagh 66008-3 murine IgX Proteintech) antibodies were diluted at a ratio and incubated overnight at 4°C. The cells were then rinsed with PBS solution and treated with a fluorescein-coupled second antibody (Alexa Fluor 488 to 555 donkey-a-m/Rbg antibody; Beyotime Biotechnology, China) for 2 hours. Finally, the cells were stained with 1 μg/ml DAPI and dried in the dark. Images were obtained using NIS-Elements Viewer software (NikonA1; Nikon Instruments Inc., Japan).

Molecular docking. High Ambiguity Driven Biomolecular DOCKing (HDOCK; http://hdock.phys.hust.edu.cn) was used to dock proteins. The protein structure with the highest overall score ranking and confidence and the lowest binding energy was screened. The lower the energy indicated, the more stable the binding. The interaction between GBP1 and HBs sub-proteins was analyzed using PLIP software (Adasme et al. 2021).

Participants. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee (Reference number: 3-2022) of the First Affiliated Hospital of Chongqing Medical University, Chongqing, China. Sixty-eight adult CHB patients treated with Peg-IFNα-2b were recruited at the infection Department of the first affiliated Hospital of Chongqing Medical University from October 2020 to October 2022. They were included in the experiment after being authorized by our hospital's ethical committee and receiving informed permission

from all subjects and parents. Patients were categorized into treatment group (n = 34) and functional cure group (n = 34), and 17 healthy controls were involved at the same time. The inclusion criteria included: (i) CHB confirmed for more than 6 months; (ii) HBeAg negative and HBsAg positive; (iii) HBV DNA levels below the lower limit of detection; (iv) patients in the treatment group without previous treatment for IFNα; (v) patients in the functional cure group who stopped for more than 3 months; (vi) HBsAb negative or positive; (vii) no other disease history.

Analysis of serum HBV markers and liver function. Blood samples collected for peripheral blood mononuclear cells (PBMC) extraction and serum separation were stored at 4°C. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), white blood cell (WBC), total bilirubin (TB), and direct bilirubin (DB) were detected using an automatic analyzer (Roche Diagnostics, Switzerland). Biochemical immunoassays for the ARCHITECT *i2000* system were used to detect HBsAg levels in serum (Abbott Laboratories, USA). Serum GBP1 levels were measured using an ELISA kit (Jianglai Biotechnology, China). The GBP1 level in PBMC was determined by qRT-PCR (Sansure Biotech Inc., China).

Statistical analysis. SPSS® 26.0 software (IBM® SPSS® Statistics for Windows, version 26.0, IBM Corp., USA), GraphPad Prism version 10 (GraphPad Software, USA, www.graphpad.com), and MedCalc Statistical Software version 22.023 (MedCalc Software Ltd., Belgium; https://www.medcalc.org) were utilized for statistical analysis. Data are presented as mean ± standard. A one-way analysis of variance was used in the multigroup comparison, and a Student's *t*-test was used to compare two samples. $p < 0.05$ was considered a significant difference.

Results

HBV infection upregulates GBP1 expression. An innate immune factor, GBP1, plays a role in the host's response to an infection. The role of GBP1 in host antiviral immunity has been reported recently (Li et al. 2016; Glitscher et al. 2021; et al. Fisch et al. 2019), but it is not clear whether it is involved in anti-HBV infection. To explore GBP1 level changes in response to HBV infection, the mRNA and protein levels of GBP1 were performed in HBV-infected HepG2-NTCP cells. As a result of our study, GBP1 protein levels increased significantly ($p < 0.05$), while mRNA levels did not change significantly (Fig. 1A and 1B). The expression of HBV total RNA and HBs protein characterized the successful establishment of HBV infection. Fig. 1A and 1B showed that the infected group had significantly ($p < 0.05$) higher levels of HBV total RNA and HBs

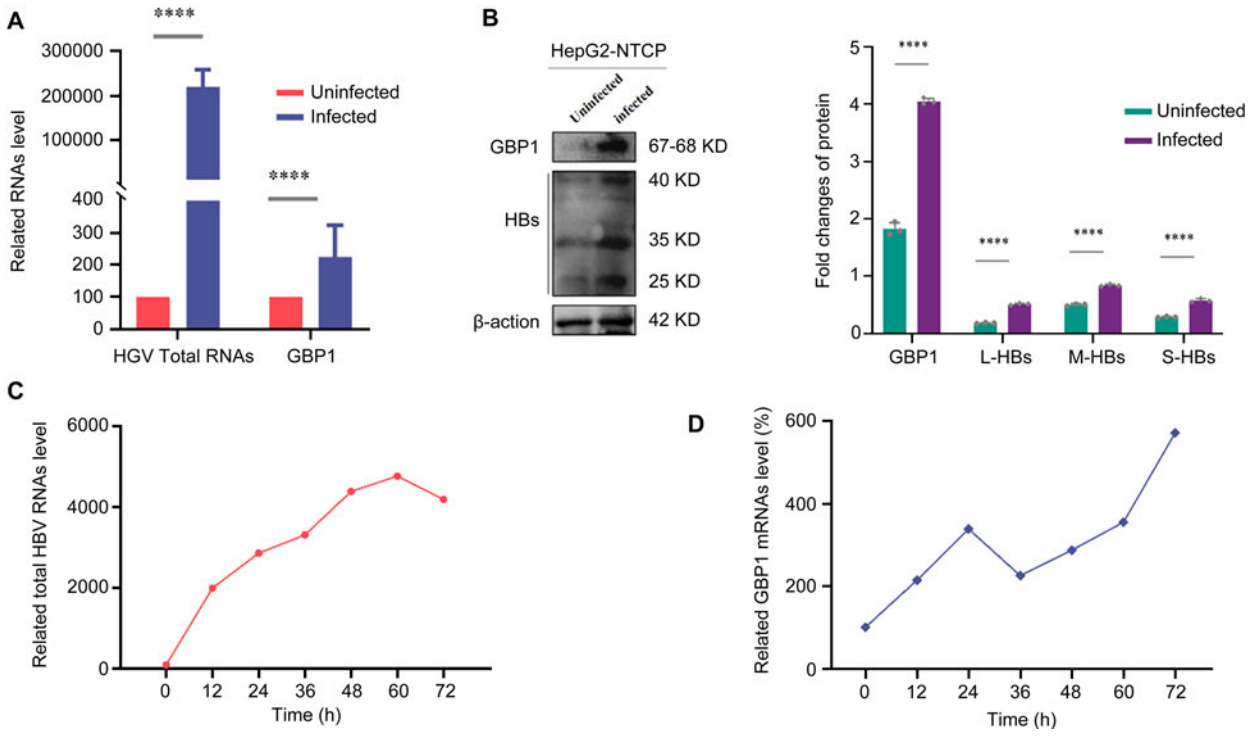


Fig. 1. HBV infection upregulated GBP1 expression.

A) The levels of HBV total RNA and GBP1 mRNA were determined by qRT-PCR in uninfected and infected HepG2-NTCP cells; B) HepG2-NTCP cells uninfected and infected were analyzed by Western blot for GBP1 and HBs expression; C–D) changes in the expression of HBV total RNA (C) and GBP1 mRNA (D) at different times of HBV infection.

Data are presented as means ± SD; ***$p < 0.001$.

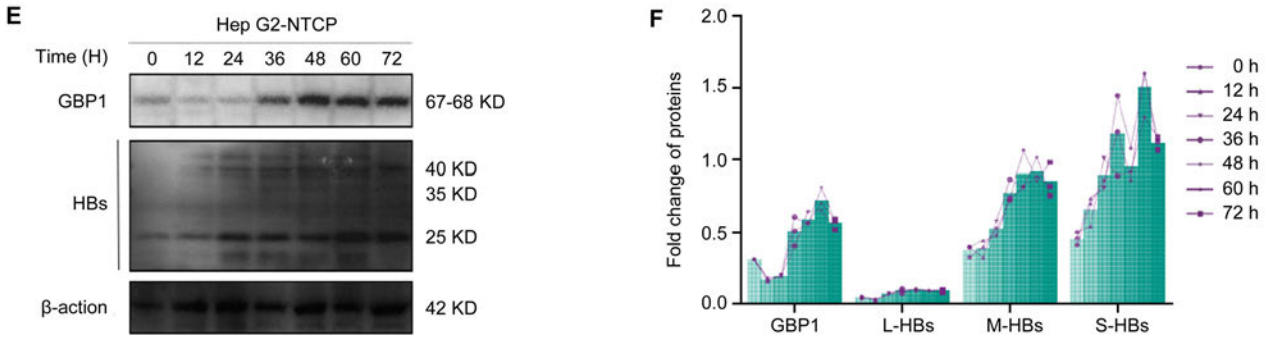


Fig. 1. HBV infection upregulated GBP1 expression.
E–F) changes in the expression of GBP1 and HBs proteins at different times of HBV infection.
Data are presented as means ± SD; ***$p < 0.001$.

protein than the uninfected group. Then, we infected HepG2-NTCP cells with HBV particles for 72 hours and detected changes in GBP1 mRNA and protein levels every 12 hours. Surprisingly, HBV total RNA declined 60 hours after infection, while GBP1 mRNA and protein and HBs protein levels remained fluctuant (Fig. 1C-1F). The above results suggested that HepG2-NTCP cells could be infected with HBV, and HBV infection could enhance GBP1 expression.

GBP1 can modulate the anti-HBV response of Peg-IFNα-2b. Currently, IFNα can improve HBsAg clearance and even HBsAb levels, thus achieving the ideal target of clinical functional cure (Wu et al. 2019). IFNα inhibits HBV transcription and replication by increasing the production of numerous interferon-stimulating factors (ISGs). GBP1 is one of the early ISGs induced by interferon (Mirpuri et al. 2010). Our results confirmed that GBP1 expression was upregulated. To further survey the mechanism by which GBP1-mediated Peg-IFNα-2b suppresses HBV infection, we used the wild-type hGBP1 plasmid and 1,000 U/ml Peg-IFNα-2b to intervene in HBV-infected cells and observed changes in viral markers and GBP1 expression. After treatment with overexpressing GBP1 or peg IFNα-2b alone or in combination, HBsAg and HBeAg were reduced (Fig. 2A), this suggested that GBP1 promoted the antiviral ability of Peg-IFNα-2b. HBV total RNA was significantly lower in the Peg-IFNα-2b, GBP1, and GBP1 + Peg-IFNα-2b groups than those in the control group, while GBP1 mRNA was higher in the Peg-IFNα-2b (Fig. 2B and 2C). Additionally, it was proven that compared to the control group, GBP1 protein levels increased, and HBs protein levels decreased in the Peg-IFNα-2b, GBP1, and GBP1+Peg-IFNα-2b groups (Fig. 2D and 2E). As shown in our results, overexpressing GBP1 co-treatment with Peg-IFNα-2b further increased the expression of GBP1 and reduced HBV total RNA and HBs protein levels. These results indicated that GBP1 had anti-HBV infection effects, and IFNα treatment could enhance its antiviral activity.

HBV infection prolongs the half-life of GBP1. To investigate the effect of prolonged Peg-IFNα-2b stimulation on GBP1, we simulated persistent HBV

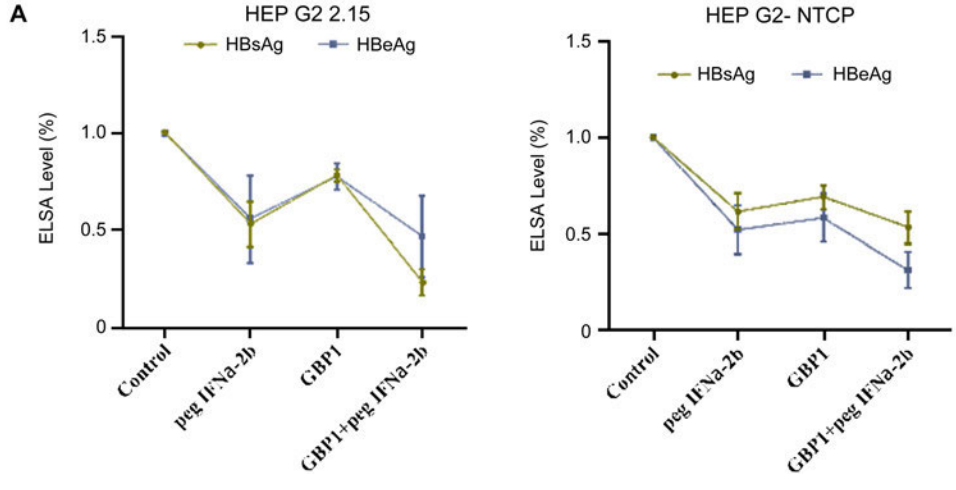


Fig. 2. GBP1 potentiated the anti-viral function of IFN-α.
A) Contents of HBsAg and HBeAg were determined by ELISA in HBV-infected HepG2 2.15 and HepG2-NTCP cells.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

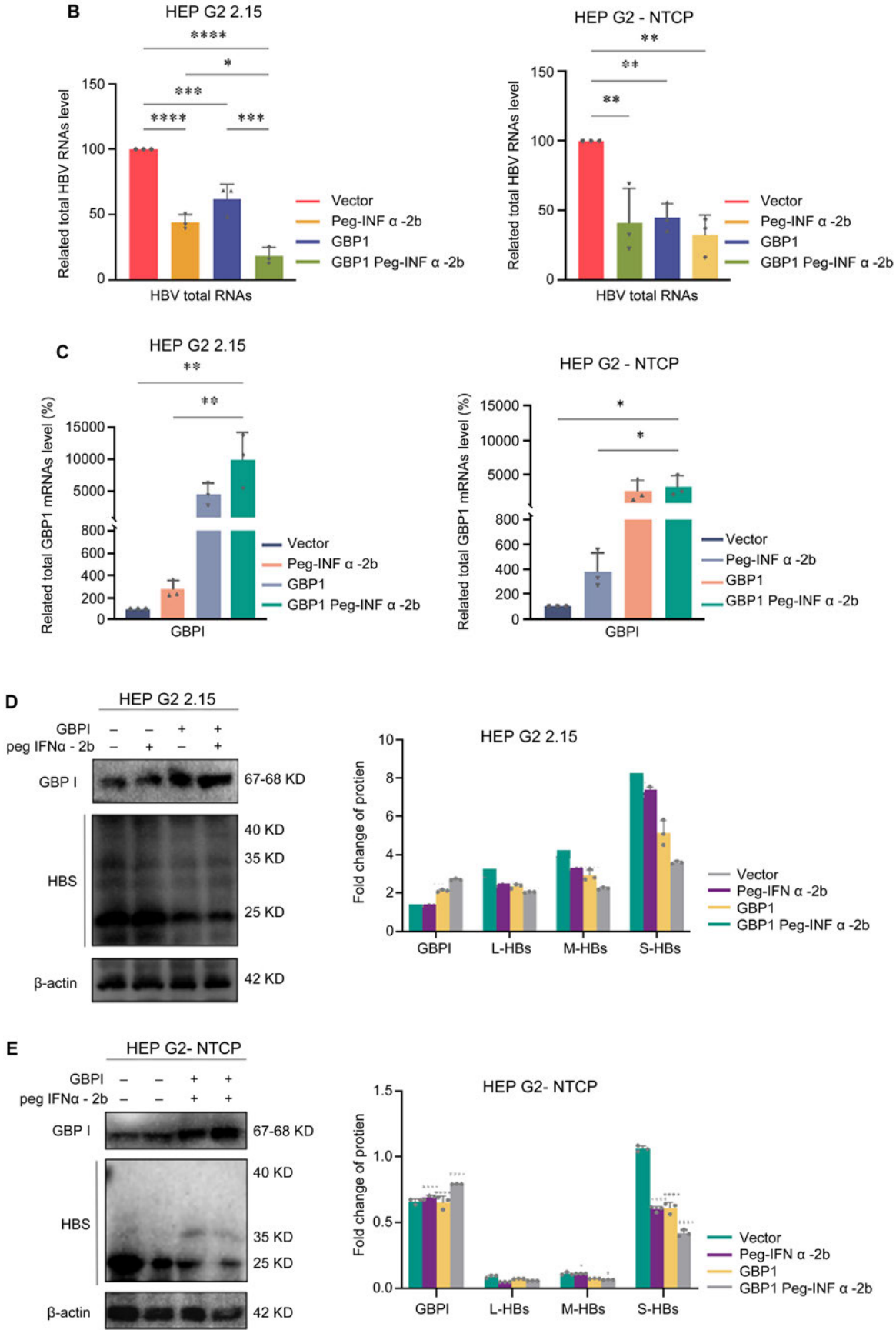


Fig. 2. GBP1 potentiated the anti-viral function of IFN-α.

B–C) relative changes in the levels of HBV total RNA (B) and GBP1 mRNA (C) in HBV-infected cells; D–E) levels of GBP1 (D) and HBs (E) proteins were examined in HBV-infected cells. Data are presented as means ± SD;

$^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$.

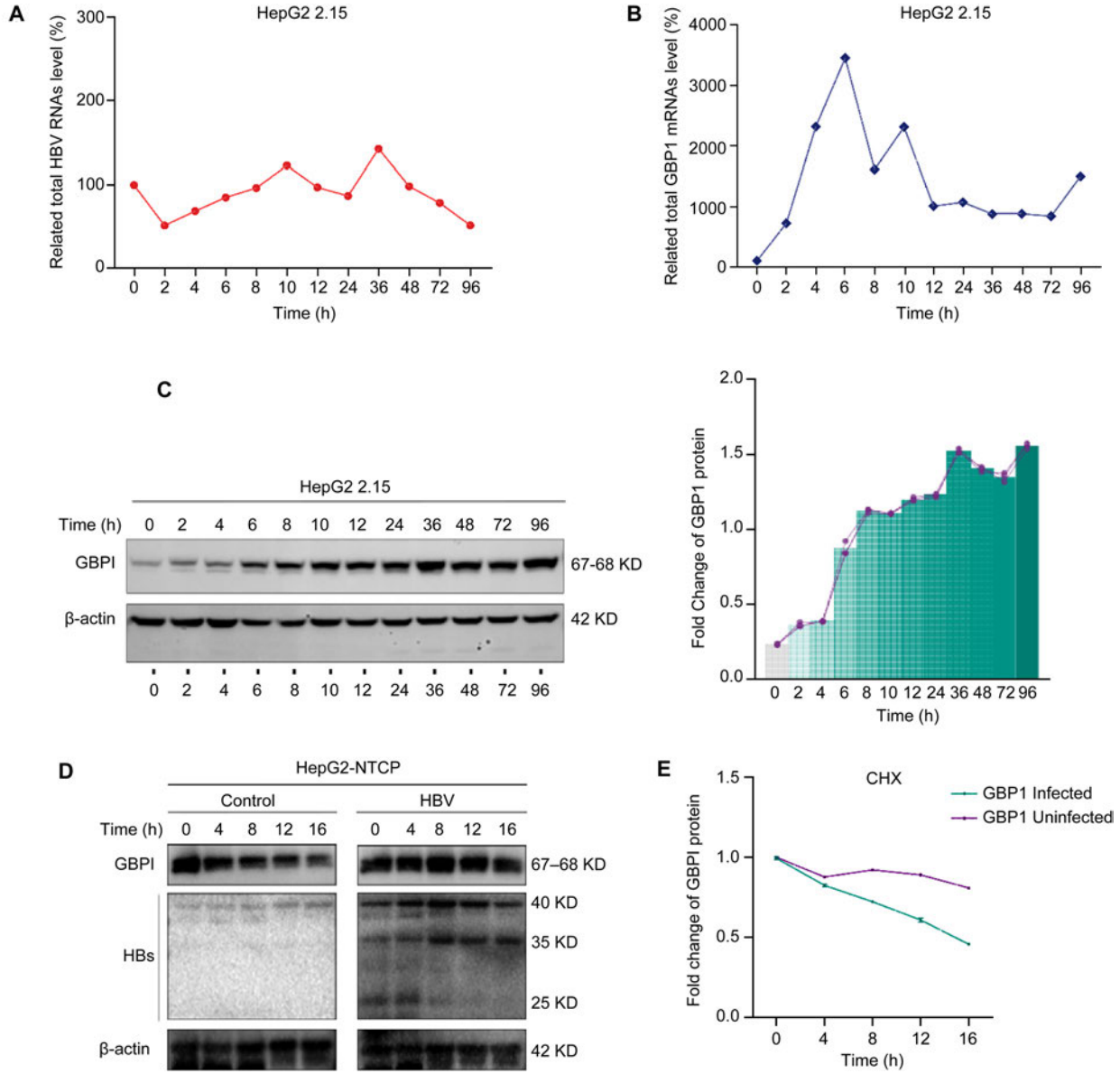


Fig. 3. HBV infection prolonged the half-life of GBP1 protein.

A–B) Relative changes in the levels of HBV total RNA (A) and GBP1 mRNA (B) in HBV-infected and stimulated HepG2 2.15 cells with Peg-IFNα-2b for 96 hours; C) levels of GBP1 protein were examined in HBV-infected HepG2 2.15 cells and stimulated with Peg-IFNα-2b for 96 hours; D–E) levels of GBP1 protein were determined in uninfected and infected HepG2-NTCP cells treated with cycloheximide (CHX). Data are presented as means ± SD.

particle infection in HepG2 2.15 cells with 1,000 U/ml Peg-IFNα-2b for 96 hours. Our results showed that there were no significant changes in HBV total RNA levels during this time period, and they were also not co-related with the GBP1 mRNA level (Fig. 3A and 3B). GBP1 protein levels increased with the duration of Peg-IFNα-2b stimulation until 36 hours, with a short decrease and again increase (Fig. 3C).

At the same time, we found that changes in GBP1 mRNA and protein levels were not completely consistent. With increased cell fusion and prolonged drug action, GBP1 protein levels were elevated in the early stage up to 6 hours but decreased in the latter phase. There may be an inconsistency in variation caused by the half-life of GBP1. To further verify whether HBV infection affected on the stability of GBP1 protein, we added the protein synthesis inhibitor cycloheximide to treat the cells. We detected changes in the half-life of GBP1. The half-life of GBP1 was not observed in infected HepG2-NTCP cells, whereas the half-life of GBP1 was 16 h in uninfected HepG2-NTCP cells (Fig. 3D and 3E). HBs protein contents were used to characterize HBV infection quantitatively. These findings indicated that HBV infection prolonged the half-life of GBP1.

GBP1 silencing promotes HBV replication. To further verify our hypothesis, GBP1 short-chain interferon RNA (si-GBP1-1, si-GBP1-2) or non-targeted siRNA

(si-control) were used to silence GBP1 expression in HepG2 2.15 cells specifically. *In vitro*, silencing of GBP1 increased HBsAg and HBeAg secretion (Fig. 4A). Transfection with siRNA constructs targeting GBP1 (si-GBP1) reduced GBP1 mRNA and protein expression relative to the si-control group while upregulating HBV total RNA and HBs protein expression (Fig. 4B and 4C). To gain a better understanding of the role of GBP1 in Peg-IFN-α-2b anti-HBV infection, HepG2 2.15 and HepG2-NTCP cells were transfected with the wild-type hGBP1 plasmid or si-GBP1, and subsequently co-treated with Peg-IFNα-2b. Our results showed that the overexpression of GBP1 inhibited HBsAg and HBeAg production, declined HBV total RNA and HBs protein levels, and increased GBP1 expression in the HepG2 2.15 and HepG2-NTCP cells – the silencing of GBP1

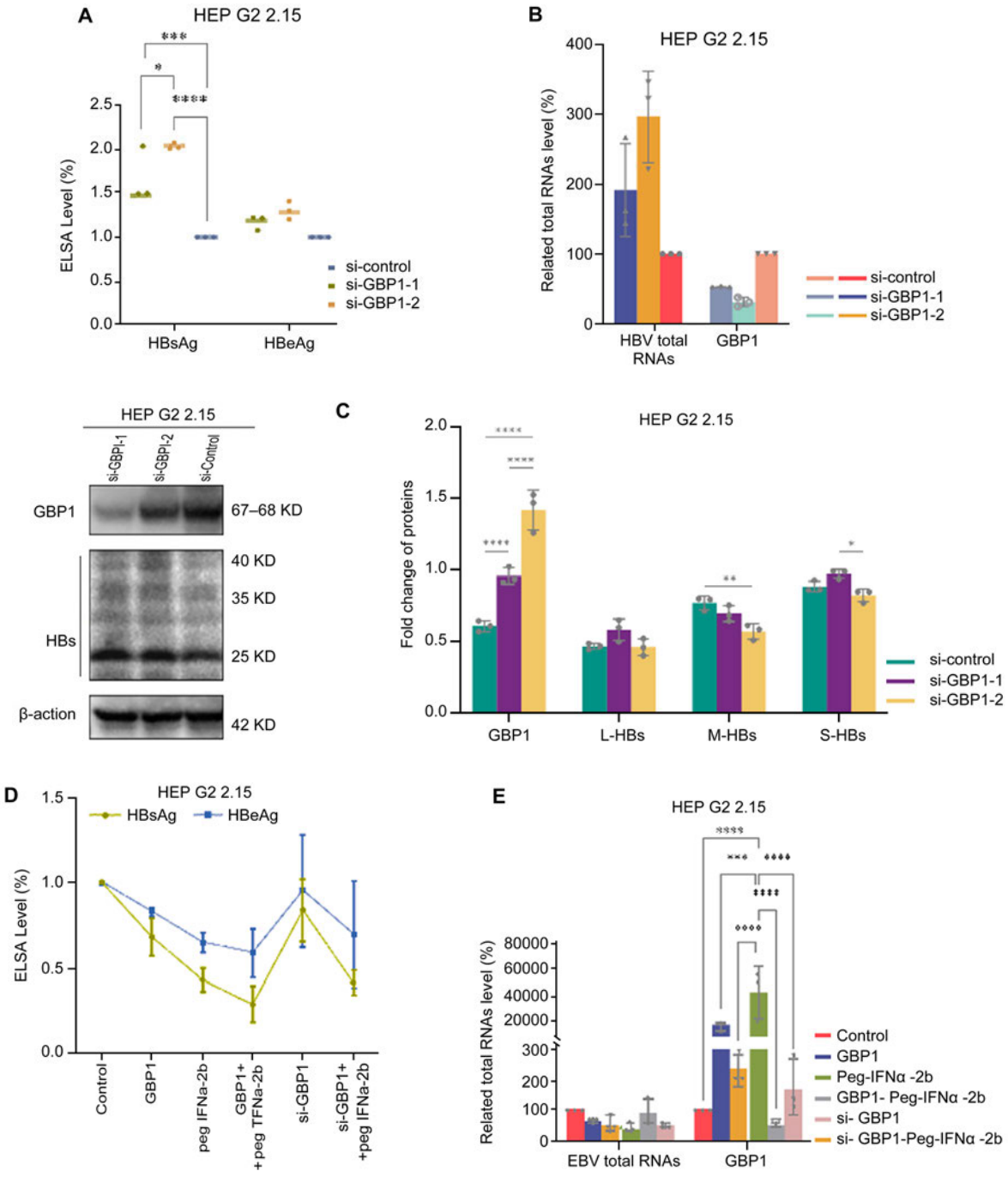


Fig 4. GBP1 silencing promoted HBV replication.

A–C) HepG2 2.15 cells transduced with specific siRNAs (si-control, si-GBP1-1, and si-GBP1-2) silence endogenous GBP1: ELISA kits (A), qRT-PCR (B), and Western blot (C) were employed to examine the indicated genes expression; D–F) before being gathered, HepG2 2.15 cells were transfected with the wild-type hGBP1 plasmid or si-GBP1, and followed by stimulation with IFN-α (1,000 U/ml): ELISA kits (D), qRT-PCR (E), and Western blot (F) were employed to examine the indicated genes expression.

Data are presented as means ± SD; *$p < 0.05$, **$p<0.01$, ***$p < 0.001$, ****$p < 0.0001$.

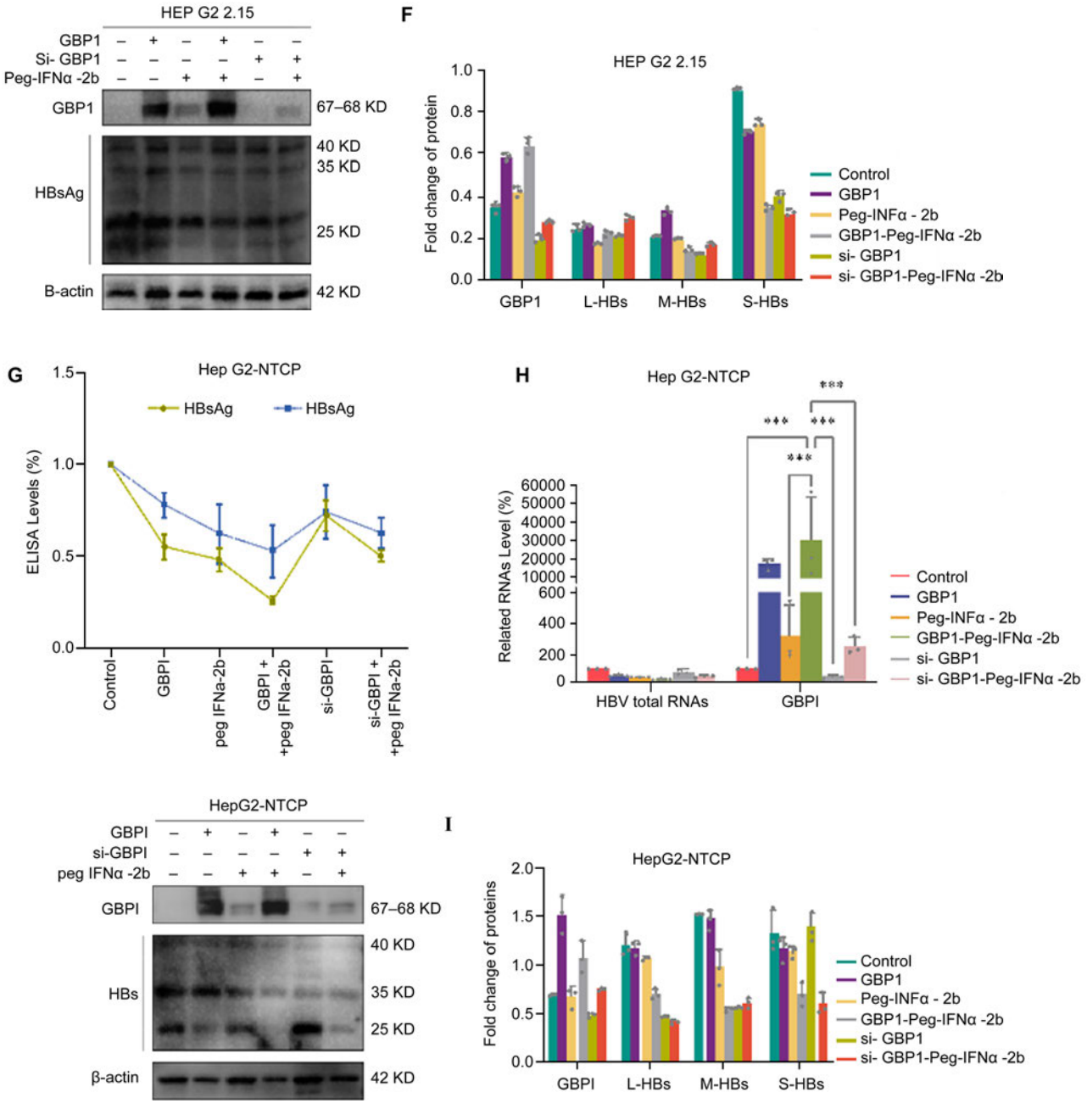


Fig 4. GBP1 silencing promoted HBV replication.

D–F) before being gathered, HepG2 2.15 cells were transfected with the wild-type hGBP1 plasmid or si-GBP1, and followed by stimulation with IFN-α (1,000 U/ml): ELISA kits (D), qRT-PCR (E), and Western blot (F) were employed to examine the indicated genes expression; G–I) transfected HepG2-NTCP cells were treated with 1,000 U/ml IFN-α for 48 h after transfection with wild-type or si-GBP1 plasmids: ELISA kits (G), qRT-PCR (H), and Western blot (I) were employed to examine the indicated genes expression.

Data are presented as means ± SD; $^{*}p < 0.05$, $^{**}p<0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

inhibited GBP1 against viral infection and reduced GBP1 expression (Fig. 4D–4I). Strikingly, GBP1 overexpression in combination with Peg-IFNα-2b further verified the previous conclusion. Meanwhile, our results showed that GBP1 silencing in combination with Peg-IFNα-2b partly restored mRNA and protein expression of GBP1 and restored its inhibitory effect on HBV (Fig. 4D–4I). These results further suggested that GBP1 also appeared to play a positive role in regulating the IFNα-induced immune response.

GBP1 can affect HBsAg expression and intracellular distribution. By immunofluorescence assay, we more visually observed the effect of GBP1 on HBsAg expression and its intracellular distribution. Notably, we found that GBP1 overexpression inhibited HBsAg activity, whereas GBP1 silencing promoted HBsAg expression. Interestingly, Peg-IFNα-2b co-treatment further enhanced GBP1 function. These results suggested that GBP1 might act on HBsAg and exert antiviral effects.

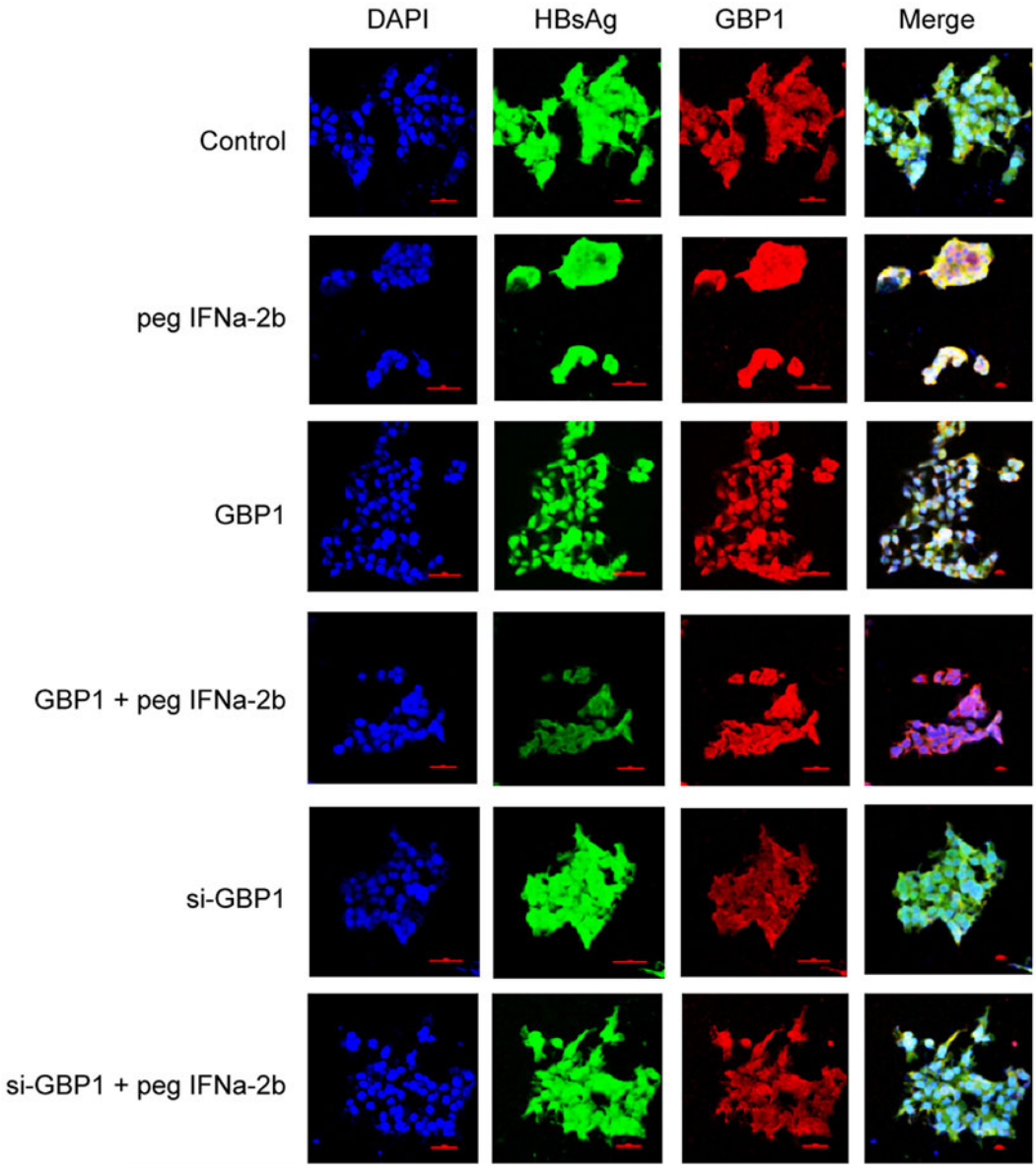


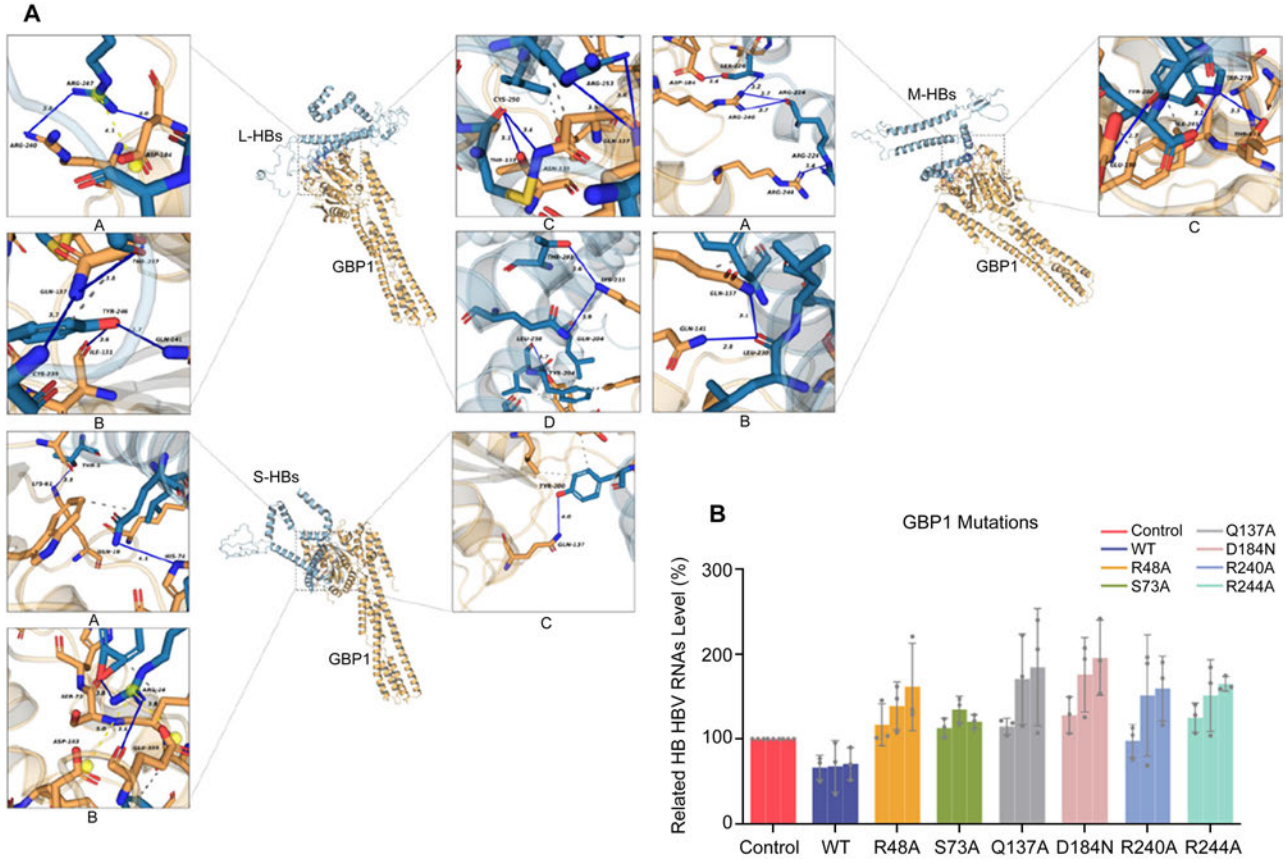
Fig. 5. GBP1 influenced the expression and distribution of HBsAg.

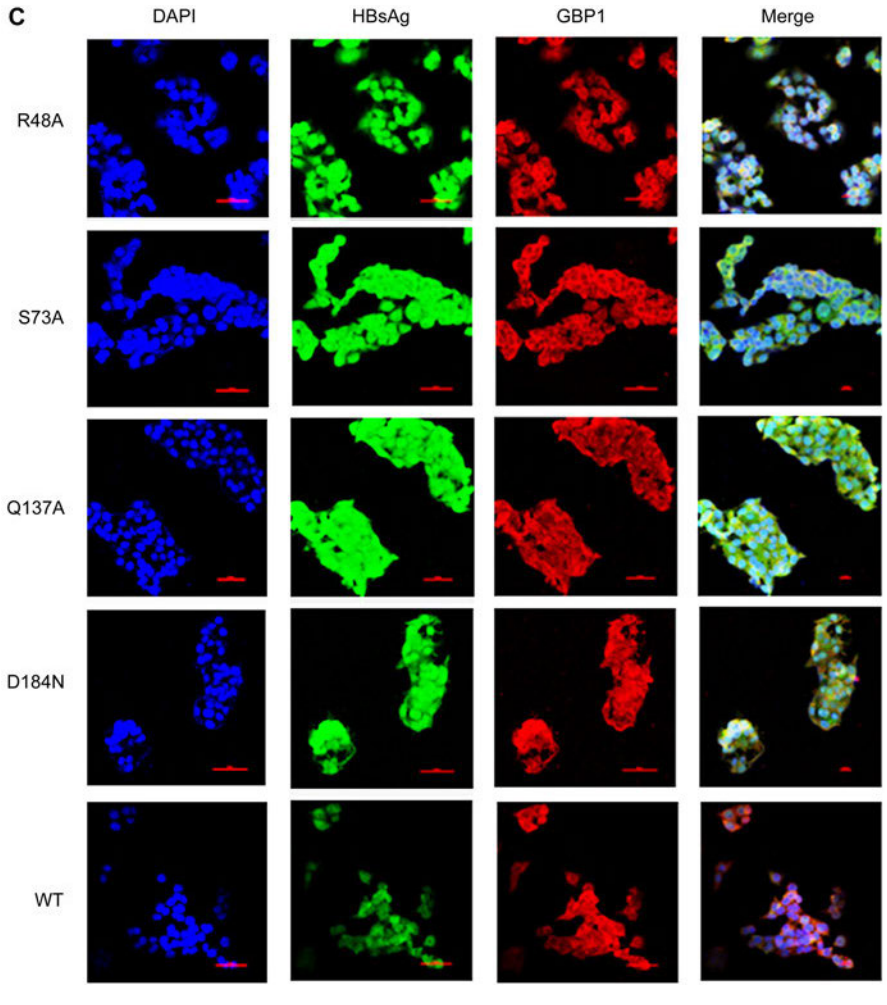
Endogenous localization of HepG2 2.15 cells was detected by immunofluorescence labeling with monoclonal anti-HBsAg (green) and anti-GBP1 (red) antibodies. Nuclei were counterstained with DAPI (blue). Scale bar: 50 μm.

Prediction and verification of binding sites between HBsAg and GBP1. HBsAg expression level is one of the most effective indicators to assess the efficacy of IFNα (Piratvisuth et al. 2013). Our results confirmed the inhibitory effect of GBP1 on HBsAg. To analyze the interaction between GBP1 and HBsAg in more detail, we attempted to find the interaction site between GBP1 and HBV by docking the wild-type hGBP1 with the structural molecules of the L, M, and S proteins of HBsAg. We found that the binding sites of GBP1 to HBs were concentrated in the N-terminal enzyme active region (1-278AA), while the sites of HBs binding to GBP1 were mostly concentrated in the main protein (S) region (Fig. 6A). In the protein structure of GBP1, it was confirmed that 4,873 sites and 181,184 TLRD sequences impacted the activity of GTPase (Raninga

Fig. 6. Prediction and verification of binding sites between HBsAg and GBP1.

A) The molecular docking technique was used to predict important amino acid sites for the interaction of three sub-proteins of HBs with hGBP1; B) mutants with only the top six amino acid sites with the same docking site in the three groups were introduced into HepG2 2.15 cells. Cellular HBV total RNA, 3.5 kb RNA, and preCRNA expression levels were determined by qRT-PCR; C) the four hGBP1 amino acid mutants (R48A, S73A, Q137A, and D184N) screened were transfected HepG2 2.15 cells. Endogenous localization of HepG2 2.15 cells was detected by immunofluorescence labeling with monoclonal anti-HBsAg (green) and anti-GBP1 (red) antibodies. Nuclei were counterstained with DAPI (blue), scale bar: 50 μm; Data are presented as means ± SD.


A
L-HBs
GBP1
M-HBs
GBP1
S-HBs
GBP1
B
GBP1 Mutations
Control
WT
R48A
S73A
Q137A
D184N
R240A
R244A
Related HB HBV RNAs Level (%)
300
200
100
0
Control
WT
R48A
S73A
Q137A
D184N
R240A
R244A


C
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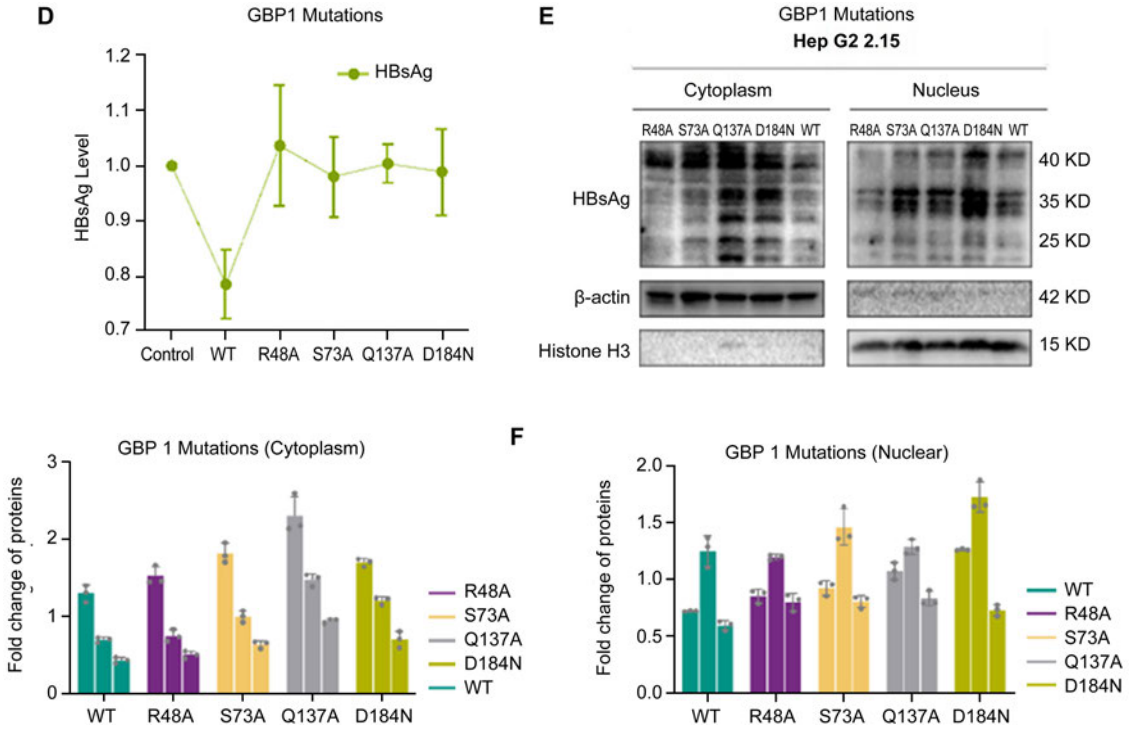

Fig. 6. Prediction and verification of binding sites between HBsAg and GBP1.

D) HepG2 2.15 cells were transfected with four hGBP1 amino acid mutants (R48A, S73A, Q137A, and D184N) for 48 h. The expression of HBsAg was analyzed by ELISA kits; E–F) representative Western blot of HBs in the nucleus and cytoplasm of HepG2 2.15 cells transfected with four hGBP1 amino acid mutants (R48A, S73A, Q137A, and D184N) for 48 h.

Data are presented as means ± SD.

et al. 2021). Therefore, we proposed that the activity of the functional amino acid site of GBP1 was associated with protein expression or translocation of HBs. To test this hypothesis, we constructed point mutant plasmids R48A, S73A, Q137A, D184N, R240A, and R244A, transfected HepG2 2.15 cells with the above plasmids, and detected the expression of HBV total RNA, 3.5 kb RNA, and preCRNA by qRT-PCR analysis. Our results showed that the above amino acid site mutations counteracted the inhibitory effect of GBP1 on HBs to varying degrees, especially the S protein, which aligned well with our molecular docking results.

Although, the R48A and S73A sites were not clearly reflected in the molecular docking results, based on the experimental results, their mutations increased the expression of HBs (Fig. 6B). Then, we selected the four mutants with the most significant effect on HBs activation for further experiments. We observed that GBP1 might primarily exert its biological function in the cytoplasm, whereas HBs seem to be expressed in whole cells. Also, immunofluorescence studies showed prominent expression of R48A and S73A mutants in the cytoplasm, whereas Q137A and D184N appeared to decentralize the location of GBP1 in the cytoplasm, which might affect the aggregation of GBP1 in the cytoplasm (Fig. 6C). To assess the potential inhibitory impact of the GBP1 mutant plasmids on HBs, we analyzed the nuclear and cytoplasmic proteins derived from HepG2 2.15 cells that had been transfected with the mutant plasmids. Our results showed that WT-GBP1 inhibited HBs better within the cytoplasm compared to the nucleus. Next, mutations at amino acid sites counteract the inhibitory effect of GBP1 on HBs. In the nucleus, the expression of M-HBs was enhanced in all groups of mutant plasmids, with the exception of R48A. R48A and S73A mutants exhibited greater contributions to L-HBs restoration in the cytoplasm, while Q137A and D184N mutants contributed to the expression of all three sub-proteins of HBs, particularly Q137A. Similarly, the findings from ELISA-based detection of HBsAg secretion yielded a congruent conclusion. ELISA analysis revealed a significant reduction in HBsAg levels in the GBP1-overexpressing cells compared to the control group (mean OD values: 0.2 ± 0.05 in GBP1-overexpressing cells vs. 0.5 ± 0.08 in control cells; $p < 0.01$). Similarly, HBeAg levels were markedly decreased in cells transfected with si-GBP1 compared to cells transfected with si-control (mean OD values: 0.15 ± 0.03 in si-GBP1-treated cells vs. 0.3 ± 0.06 in si-control-treated cells; $p < 0.05$) (Fig. 6D–6F). Together, these results suggested that GBP1 inhibited its expression by directly binding to HBs.

GBP1 expression in the peripheral blood of patients with chronic hepatitis B. *In vitro*, we confir-

Table I
Clinical characteristics of patients.

Group	Health control	CHB (Untreated)	CHB (Treated for 3 months)	CHB (Treated for 6 months)	CHB (Treated for 6 months)
Cases	17	34	34	34	34
Age (years)	26.12 ± 4.30	40.68 ± 1.61	40.68 ± 1.61	40.68 ± 1.61	42 ± 10.12
Sex (M/F)	9/8	17/17	17/17	17/17	22/12
Serum HBsAg level (IU/ml)	NA	1785.85 (384.09, 5282.87)	1018.02 (61.53, 2682.86)	197.855 (17.11, 2108.22)	NA
ALT (U/l)****	19.41 ± 5.41	27.17 ± 2.75	61.21 ± 5.89	44.44 ± 5.95	38 (19, 52)
AST (U/l)****	17.65 ± 5.67	25.05 ± 1.61	65.00 ± 8.67	39.68 ± 4.43	27 (21.75, 45.75)
WBC (10^9/ml)****	3.55 ± 0.86	5.74 ± 0.23	3.29 ± 0.20	3.89 ± 1.64	5.10 ± 1.44
TB (μmol/l)*	4.96 ± 1.22	12.56 ± 1.09	11.24 ± 0.73	9.65 ± 0.66	10.3 (8.23, 13.03)
DB (μmol/l)****	2.37 ± 0.88	4.49 ± 0.27	5.34 ± 0.30	4.36 ± 0.24	4.47 ± 1.80
Serum GBPI level (ELISA)****	1.31 ± 0.23	1.99 ± 0.30	1.94 ± 0.41	1.95 ± 0.40	1.94 ± 0.34
PBMC GBPI level (PCR)***	2.24 ± 0.53	1.82 ± 0.24	7.89 ± 1.76	5.87 ± 0.64	3.46 (2.47, 5.74)

ALT – alanine aminotransferase, AST – aspartate aminotransferase, F – female, M – male, HBsAg – HBV surface antigen, WBC – white blood cell, TB – total bilirubin, DB – direct bilirubin, HC – healthy control, NA – not applicable; data are presented as means ± SD; *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$

med the inhibitory impact of GBP1 on HBV, and the induction of GBP1 expression through IFNα stimulation. Additionally, we predicted the specific amino acid site involved in the interaction between GBP1 and HBs. To evaluate whether GBP1 was linked to the HBV infection process and interferon efficacy, peripheral blood samples from 68 adult CHB patients treated with Peg-IFNα-2b and 17 healthy controls were collected. Patients treated with Peg-IFNα-2b were categorized into the treatment group (n = 34) and the functional cure group (n = 34), and the peripheral blood of the treatment group pre-treatment, after 3 and 6 months of treatment, and the functional cure group were collected for testing.

Table I presents the clinical characteristics of the patients. As shown in Table I and Fig. 7B, in patients

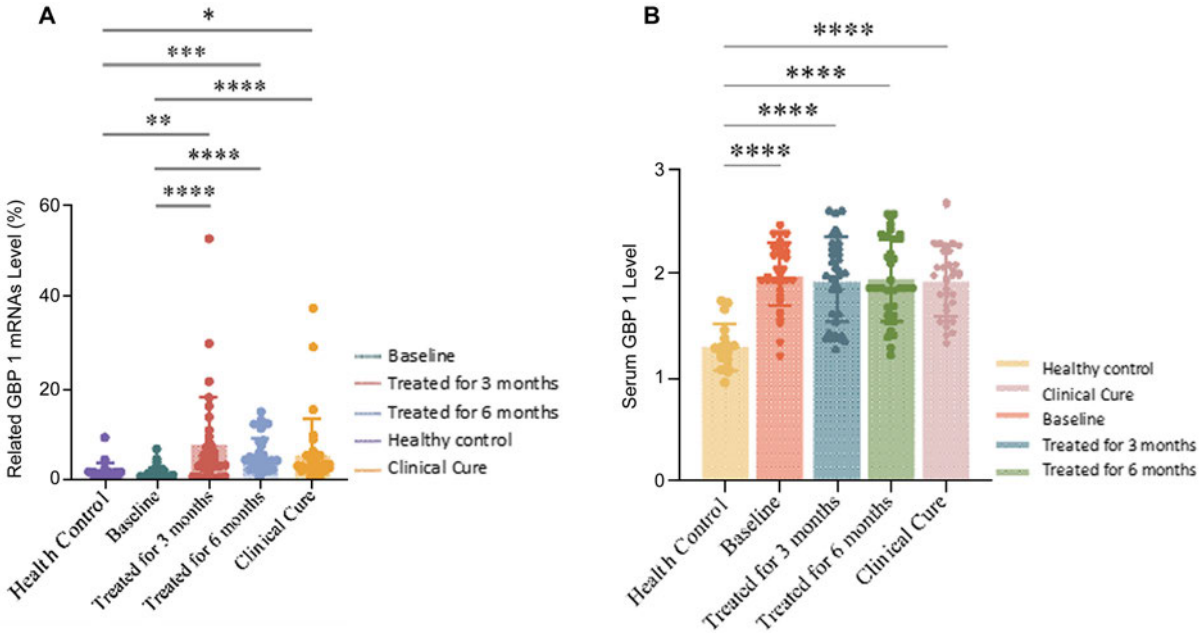

Fig. 7. GBP1 expression in the peripheral blood of patients treated with Peg-IFNα-2b and healthy controls.
A–B) Relative expression levels of GBP1 in Peg-IFNα-2b-treated patients and healthy controls. Cellular GBP1 mRNA expression levels were determined by qRT-PCR (A). Serum levels of GBP1 (B) were detected by ELISA.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

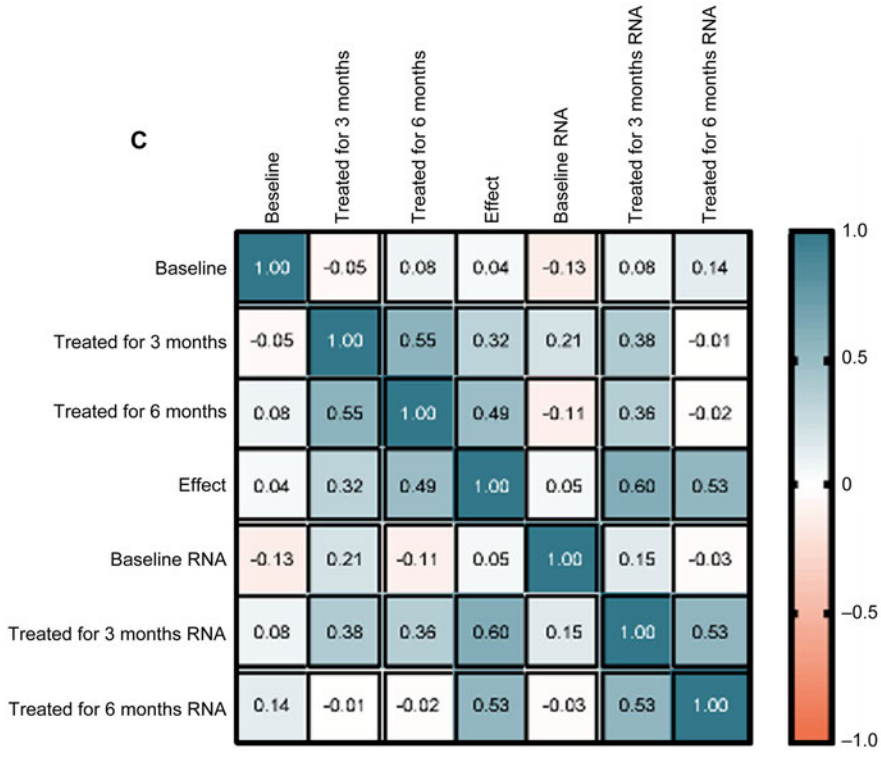

Fig. 7. GBP1 expression in the peripheral blood of patients treated with Peg-IFNα-2b and healthy controls.
C) Correlation between GBP1 levels and Peg-IFNα-2b efficacy using Spearman's correlation analysis in Peg-IFNα-2b-treated patients.
Data are presented as means ± SD; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$.

with functional cure and those with pre-treatment, 3 months post-treatment, and 6 months post-treatment GBP1 expression levels were considerably higher than healthy controls ($p<0.05$). However, the treatment and functional cure group did not differ statistically significantly ($p>0.05$). The GBP1 mRNA levels of the patients before treatment exhibited a slight decrease compared to the healthy control group. However, this was reversed after 3 months of treatment, with a significant increase ($p<0.05$) in mRNA levels. Although this trend was mitigated in the subsequent 6 months, the GBP1 mRNA levels were still higher than pre-treatment levels. There was no significant difference ($p<0.05$) in the expression levels of GBP1 mRNA in the functional cure group compared to the treatment group, though they were higher than those in the healthy control and pre-treatment groups (Table I and Fig. 7A). In addition, we further assessed the correlation between GBP1 levels and Peg-IFNα-2b efficacy using Spearman's correlation analysis and found that GBP1 mRNA levels at 3 months of treatment were consistent with the assessment of early patient response (Fig. 7C).

We then compared the patients' clinical features and GBP1 levels in the treatment group at 3 months and 6 months post-treatment. According to the treatment recommendations for chronic hepatitis B, patients were divided into two groups based on the rapid decline in HBsAg (HBsAg < 200 IU/ml or decline > 1 $\log_{10}$ IU/ml at 12 or 24 weeks) as an early response criterion. Our results showed that 20 patients responded early, and 14 were non-responders. GBP1 mRNA was significantly ($p<0.05$) different between the non-response and response groups at 3 months of treatment, and the difference decreased at 6 months, but mRNA levels in the response group were consistently above those in the non-respond group (Table II and Fig. 8A). Although not statistically significant, the response group showed greater serum levels of GBP1 than the non-response group (Table II and Fig. 8B). In clinical practice, evaluating treatment efficacy in patients with chronic hepatitis B often involves the combination of multiple indicators. Therefore, we believe that the inclusion of GBP1, along with other indicators, in assessing patient response to IFNα holds a certain level of importance in guiding subsequent drug regimens. Initially, we compared GBP1 mRNA and protein levels to determine their diagnostic value in predicting the early response to IFNα treatment. Our findings revealed that GBP1 levels at 3 months and 6 months post-treatment exhibited superior diagnostic significance compared to pre-treatment levels (Fig. 8C). We then further assessed the predictive accuracy by combining GBP1 with other indexes at 3 and 6 months after treatment. At 6 months, combined GBP1, GBP1 mRNA, HBsAg, ALT, AST, and WBC levels were more predictive of early response to IFNαthan at 3 months (Fig. 8D).

Table II
Clinical characteristics of patients with different effects.

Group	Non-response			Response		
	Untreated	Treated for 3 months	Treated for 6 months	Untreated	Treated for 3 months	Treated for 6 months
Cases	14			20		
Age (years)	42.14 ± 9.09			39.65 ± 9.69		
Sex (M/F)	8/6			9/11		
Serum HBsAg level (IU/ml)	2560.04 (1207.33, 6259.34)	1732.10 (801.27, 3413.51)	1942.69 (1257.71, 3423.27)	1133.55 (150.81, 3840.16)	380.82 (23.77, 1509.33)	102.30 (1.38, 199.06)
ALT (U/l)	30.50 (16.25, 44.50)	49.50 (33.75, 86.50)	37.50 (18.25, 6.50)	18.30 (14.00, 27.75)	63.00 (34.25, 80.00)	32.00 (21.25, 50.00)
AST (U/l)	23 (16.50, 33.75)	45.00 (29.50, 76.25)	27.00 (19, 52)	22.00 (18.00, 27.25)	50.00 (33.25, 84.00)	39 (19.75, 48.50)
WBC (10^9/ml)*	6.01 ± 1.47	3.17 ± 1.06	3.87 ± 1.57	5.55 ± 1.24	3.38 ± 1.28	4.73 ± 3.77
TB (μmol/l)	13.19 ± 7.94	11.21 ± 4.51	10.40 ± 4.45	12.12 ± 5.17	11.25 ± 4.12	9.12 ± 3.39
DB (μmol/l)	4.54 ± 1.77	5.09 ± 1.45	4.14 ± 1.21	4.45 ± 1.43	5.51 ± 1.91	4.51 ± 1.51
Serum GBPI level (ELISA)	1.97 ± 0.31	1.76 ± 0.40	1.73 ± 0.34	2.01 ± 0.31	2.06 ± 0.37	2.10 ± 0.37
PBMC GBPI level (PCR)	1.41 (1.11, 1.84)	1.77 (1.00, 3.63)	2.98 (2.58, 4.58)	1.35 (0.94, 2.72)	6.97 (4.70, 13.27)	5.63 (4.49, 10.89)

ALT – alanine aminotransferase, AST – aspartate aminotransferase, F – female, M – male, HBsAg – HBV surface antigen, WBC – white blood cell, TB – total bilirubin, DB – direct bilirubin; data are presented as means ± SD; *$p < 0.05$

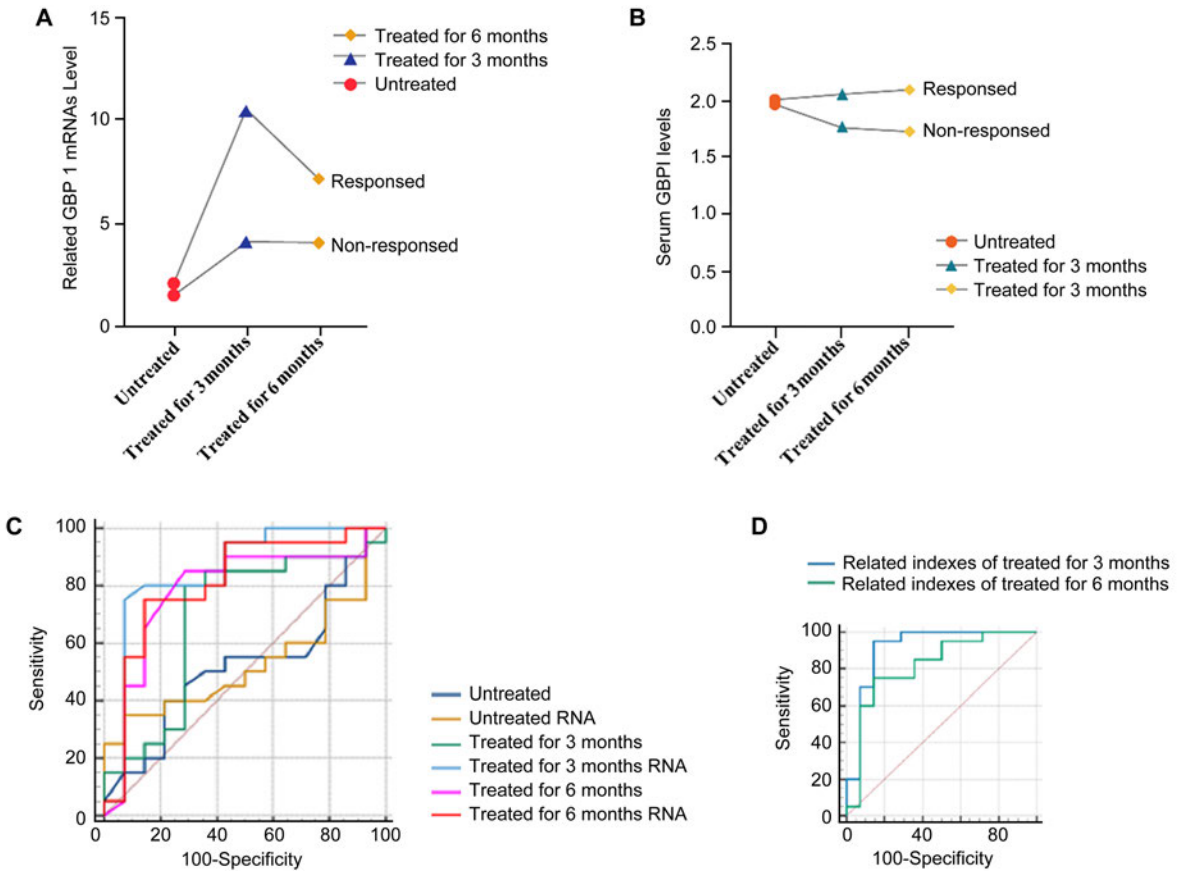

Fig. 8. GBP1 expression and its diagnostic and prognostic value in non-response and response patients.

A–B) Relative expression levels of GBP1 in non-response and response patients. qRT-PCR was performed to detect GBP1 mRNA expression (A). Serum levels of GBP1 (B) were detected by ELISA; C) ROC curve for evaluating the diagnostic and prognostic value of GBP1; D) ROC curve for evaluating the diagnostic and prognostic value of GBP1 in combination with other indicators. Data are presented as means ± SD.

Discussion

Most CHB patients respond poorly to IFNα treatment, severely limiting its clinical applicability. Individualized therapy will require a better knowledge of the virologic and host variables that influence interferon effectiveness (Lin et al. 2023). As a result, identifying the key genes responsible for interferon efficacy in CHB patients is crucial. Some studies have proved that GBP1 is related to host innate immunity and the defense of viruses and bacteria in vivo and *in vitro* models and participates in programmed cell death (Anderson et al. 1999). It has been found that the enzyme activity region of GBP1 plays a key role. In our current study, by integrating data on antiviral effects, molecular docking, and hematology, we demonstrated that GBP1 overexpression promoted HBeAg seroconversion and HBsAg clearance, inhibited HBV replication, and eventually exerted antiviral effects. Besides, our results indicate that GBP1 plays a positive role in regulating the immune response induced by IFN-a.

Previous studies have reported that GBP1 exerts its anti-HCV and HEV effects by directly targeting virus proteins or virus-inactivated lysosome compartments. Despite this, there has been little research on the role of GBP1 in the replication and spread of HBV. To explain the mechanism of GBP1 in the HBV life cycle, we first demonstrated that GBP1 expression was increased in a time-dependent manner in HBV-infected cells, indicating that HBV induces GBP1, but this induction was not completely consistent at the mRNA and protein levels. Moving forward, the CHX experiment confirmed that HBV prolonged the half-life of GBP1, and changes in GBP1 ubiquitination might explain this inconsistency. These results support the conclusion that GBP1 belongs to the viral-stress-inducible gene family (Sen and Peters 2007). After interferon stimulation, GBP1 expression is upregulated, a well-known characteristic of ISGs. IFNs induce the expression of ISGs, and these ISGs encode proteins that inhibit viral replication at different stages (Schoggins et al. 2011). However, it has been found that HBV is insidious and is not recognized by the pattern recognition receptor (PRR) (Suslov et al. 2018). HBV escapes the intrinsic immune system and the antiviral effects of IFNα-induced ISGs (Mutz et al. 2018). IFN-α has nevertheless been proven effective in treating chronic HBV infection, and it is widely used (Viganò et al. 2018). Therefore, it is crucial to elucidate the molecular mechanism of IFNα interaction with some ISGs during HBV infection. It is helpful to reveal the action of the poor efficacy of IFNα and find the targeted genes that promote the efficacy of patients. In our current research, we found that the overexpression of GBP1 inhibited HBsAg and HBeAg production and declined levels of HBV total RNA and HBs protein.

In contrast, silencing of GBP1 inhibited GBP1 against viral infection. Surprisingly, overexpressing GBP1 co-treatment with Peg-IFNα-2b further increased the antiviral effect of IFN, while GBP1 silencing co-treatment with Peg-IFNα-2b partly restored its inhibitory effect on HBV. As a result, we believe that GBP1 is a positive regulator of HBV-related IFN-a-induced immunity.

Currently, HBsAg quantification is the most commonly used predictor of Peg-IFN-a efficacy. The baseline HBsAg level or the trend of changes during treatment can guide regimen adjustment and predict durable responses after discontinuation. Serum HBsAg can be produced by cccDNA, which theoretically can indirectly reflect its activity, thereby evaluating and predicting the effectiveness of antiviral therapy (Thompson et al. 2010; Piratvisuth et al. 2013). In the study, we surveyed that GBP1 overexpression inhibited HBsAg activity, whereas GBP1 silencing promoted HBsAg expression. We then investigated whether GBP1 could influence IFNα efficacy by targeting HBs. Combined with the epidemic characteristics of the HBV genotypes in China, the protein structures of HBs and hGBP1 of the HBV gene were selected from the UniProt protein library (UniProt Consortium 2024), and the protein binding sites of L/M/S-HBs and GBP1 were predicted by molecular docking. The current study demonstrated that GBP1 targeted HBs via direct binding. According to the mutation sites from previous literature, mutant plasmids were constructed and transfected into HBV-infected HepG2 2.15 cells. The results revealed that mutations of GBP1 reversed the inhibitory effect of GBP1 on HBV total RNA. R48A and S73A mutants exhibited more significant contribution to L-HBs restoration in the cytoplasm, while Q137A and D184N mutants contributed to the expression of all three subproteins of HBs. It is worth mentioning that R48A, S73A, Q137A, and D184N mutants are located in the N-terminal GTP domain of GBP1, indicating that the N-terminal GTP domain of GBP1 plays a significant role in inhibiting HBs. Therefore, GBP1 can inhibit the expression of HBs, thereby resisting HBV infection.

L-HBs are involved in viral invasion, particle formation, and virus retention in the endoplasmic reticulum. Excess L-HBs may cause hepatocyte endoplasmic reticulum overload, resulting in chronic high stress, cell inflammation, and cell death. We thus speculate that the increase in L-HBs caused by GBP1 mutants may lead to the overloading of hepatocyte endoplasmic reticulum and ultimately lead to hepatocyte death (Bruss and Ganem 1991; Blanchet and Sureau 2006; Block et al. 2007). This kind of death differs from previous researchers' argument that GBP1 activates inflammasomes and induces cell death (Santos et al. 2020; Dickinson et al. 2023). It may be harmful and can induce liver cirrhosis and even hepatocellular carcinoma. It

was worth noting that Q137A and D184N appeared to influence GBP1 subcellular location. Studies of D184N found that D184N caused GBP1 to exist as a monomer and reduced nucleotide binding by nearly half (Li et al. 2017). Other studies have not reported changes in the distribution of the two mutants in the cytoplasm. Therefore, the following study on the subcellular localization of the mutant may enable us to analyze GBP1 specificity for HBs further.

Finally, we verified that the levels of GBP1 were increased in HBV-infected patients. We found that GBP1 was stable during Peg-IFNα-2b treatment, but this stability was more pronounced in the response group. In addition, we found that the expression of GBP1 was positively correlated with the efficacy of interferon therapy. ROC curves verified the potential value of GBP1 and HBsAg in predicting interferon efficacy and found that their combination can improve the prediction of the efficacy of early interferon therapy. Chronic infection with HBV affected GBP1 expression. Future studies will need to determine whether GBP1 plays a role in the antiviral effect of the host immune response. Therefore, it is essential to identify patients who may respond to Peg-IFN-a based on patient characteristics or serological indicators before treatment to maximize patient benefits.

To sum up, our present study investigated the mechanism of GBP1 upregulation by HBV. Overexpressing GBP1 inhibited replication of HBV, which helped to clarify CHB pathogenesis. In addition, GBP1 targets HBsAg and promotes the anti-HBV effect of IFN-α, providing a novel mechanism for the poor therapeutic response to IFN-α.

Abbreviations

HBeAg – hepatitis B e Antigen
HBsAb – hepatitis B surface Antibody
HBsAg – hepatitis B surface antigen
HCC – hepatocellular carcinoma
ISG – interferon-stimulated gene
qRT-PCR – Quantitative PCR
ROC – Ras of complex protein

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethical statement

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University, Chongqing, China (Reference number: 3-2022).

Author contributions

Y.L.: methodology, software, writing – original draft, writing – review and editing; H.L.: formal analysis, data curation; X.H.: data curation; J.G.: formal analysis, visualization; G.T.: data curation; H.L.: formal analysis, visualization; R.W.: data curation; H.P.: data curation; R.Y.: formal analysis, visualization; B.Q.: conceptualization, funding acquisition.

Funding

This study was supported by the General Project of the Chongqing Natural Science Foundation of China (CSTC2020JCYJ-MSXMX0221).

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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