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ORIGINAL PAPER

Peptide YY inhibits transcription and replication of hepatitis B virus by suppressing promoter/enhancer activity

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Abstract

Hepatitis B virus (HBV) infection is a noteworthy cause of liver diseases, especially cirrhosis and hepatocellular carcinomas. However, the interaction between the host and HBV has not been fully elucidated. Peptide YY (PYY) is a 36-amino-acid gastrointestinal hormone that is mainly involved in the regulation of the human digestive system. This study found that PYY expression was reduced in HBV-expressing hepatocytes and HBV patients. Overexpression of PYY could significantly inhibit HBV RNA, DNA levels, and the secretion of HBsAg. In addition, PYY inhibits HBV RNA dependent on transcription through reducing the activities of CP/Enh I/II, SP1 and SP2. Meanwhile, PYY blocks HBV replication independent on core, polymerase protein and ε structure of pregenomic RNA. These results suggest that PYY can impair HBV replication by suppressing viral promoters/enhancers in hepatocytes. Our data shed light on a novel role for PYY as anti-HBV restriction factor.

Keywords Hepatitis B virus · Peptide YY · HBV promoters, enhancers

Introduction

Hepatitis B virus (HBV) belongs to the Hepadnaviridae family and is a main cause of acute or chronic viral hepatitis, cirrhosis and hepatocellular carcinoma (HCC). More than 292 million people are estimated to be chronically infected with HBV and over 880,000 annual deaths are the result of hepatitis B-related complications such as HCC and liver cirrhosis [1, 2]. HBV firstly attaches to cellular heparan sulfate proteoglycan (HSPG) and then binds to sodium taurocholate co-transporting polypeptide (NTCP) through large envelope protein (LHB) [3]. After HBV infection, the viral relaxed circular DNA (rcDNA) genome in nucleocapsid is transported into the nucleus and converted into covalently closed circular DNA (cccDNA) [4]. The HBV genome is a 3.2 kb, circular, partially double-stranded DNA with four open reading frames (ORFs) including pre-C/C, pre-S/S, P, and X [5]. The pre-C/C ORF encodes the hepatitis B e antigen (HBeAg) and hepatitis B core antigen (HBcAg); the pre-S/S ORF encodes the hepatitis B surface antigen (HBsAg); the P gene encodes the viral polymerase; the X gene encodes hepatitis B virus X protein (HBx) [6]. HBV transcripts are under the control of four promoters, such as core promoter (CP), preS1 promoter (SP1), preS2 promoter (SP2), HBV X promoter (XP), and two enhancers (enhancer I and II, Enh I/II) [5]. The pregenomic (pg) RNA acts as the messenger ribonucleic acid of capsid protein and reverse transcriptase (P protein) and is encapsulated and reverse transcribed into the progeny rcDNA through specific P-RNA interactions [7]. Finally, mature nucleocapsids containing rcDNA can be enveloped and secreted from cells as infectious virions, or rcDNA can be transported into the nucleus to amplify the cccDNA library, which is the only transcriptional template that supports HBV replication [6]. The HBV polymerase (pol) recognizes a stem-loop structure (epsilon, ε) at the 5′

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terminus of pgRNA to recruit core proteins to encapsidate the pol/pgRNA complex in the nucleocapsid and reversely transcribes at the nucleocapsid to produce progeny virus rcDNA [8].

Peptide YY (PYY), a 36-amino-acid peptide, is secreted primarily by L-cells residing in the intestinal mucosa of the ileum and large intestine [9]. Otherwise, as a gastrointestinal hormone, PYY plays important roles in the physiological functions of the digestive tract, including the regulation of gastrointestinal movement and intestinal mucosal secretion [10]. Therefore, PYY can regulate food intake and insulin secretion, especially in the field of obesity and diabetes [11]. PYY has antiproliferative and proapoptotic effects in Barrett's cancer cells [12]. PYY also has been shown to inhibit the growth of pancreatic, breast, esophagus, and gastric cancer in vitro [13]. In addition, PYY is a negative regulator of bone mass and strength [14]. Only a few reports suggest that PYY was increased in patients with decompensated liver cirrhosis [15]. However, the interaction between PYY and HBV has not been clarified.

In this study, we identified that the inhibitory effect of PYY on HBV replication via suppressing the activities of CP/Enh I/II, SP1 and SP2. Specifically, this process is independent of the ε structure, core and pol protein. These findings highlight a potential target to control HBV infection.

Materials and methods

Cell culture, plasmids and transfection

Huh7 and HepG2 cells were cultured in Dulbecco's modified Eagle medium (DMEM; Biological Industries), supplemented with a 10% fetal bovine serum (FBS, Gibco) and 1% antibiotic mix (Gibco) of penicillin (100 U/mL) and streptomycin (100 mg/mL). The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. The total amount of transfected plasmids in each sample was normalized by supplementation with empty vectors. Transfection of siRNAs or plasmids was also performed using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Interferon-alpha (IFN-α) and actinomycin D (ActD) were obtained from MedChemExpress and Sigma-Aldrich, respectively. siRNAs were purchased from RiboBio Company (Guangzhou, China). The expression vectors are listed in the Table S1. The pUC1.2xHBV/NL is a 1.2-fold HBV genome (isolate C_JPNAT, genotype C, accession number AB246345) that was cloned into the HindIII/EcoRI site of pUC19, termed pUC1.2xHBV. The plasmid pUC1.2xHBV/NL was constructed by deleting 562 nt (219–782) from the first codon of the HBV preCore coding frame of pUC1.2xHBV, and then inserting the NL gene (513 nt) from pNL2.1 N1061 NL [16]. pCMV1.2xHBV/NL, HBV-NL (HindIII/EcoRI fragment in pUC1.2HBV/NL) was subcloned into pCMV expression vector [17].

Patients and samples

In this study, the cDNAs of adjacent tissues of HCC resection for 14 tissues without HBV infection and 25 tissues infected with HBV were collected from the First Hospital of China Medical University, the clinical information of HBV patients was listed in Table S3. As for the liver samples, the inclusion criteria of the study were as following: (1) primary hepatocarcinoma was diagnosed before surgery, and HCC was confirmed with pathology; (2) Without preoperative radiotherapy or chemotherapy. The exclusion criteria: (1) Postoperative pathology of non-HCC, including cholangiocarcinoma, etc.; (2) Early hepatocarcinoma or small cancer foci that is only enough for pathological diagnosis. The informed consents were obtained regarding the use of their samples for further studies. And we obtained cDNA samples that reverse-transcript from total RNA of abovementioned HCC resection and determined the level of PYY coding cDNA by a real-time quantitative polymerase chain reaction (qPCR). The results were normalized by the internal control GAPDH.

Luciferase determination

HBV/NL reporter genes in this study were derived from nano luciferase (NL) in pNL1.1. (Promega) [17]. Luciferase activity in harvested cells was determined using the Nano-Glo Luciferase and Luciferase Assay Kit (Promega). The firefly luciferase reporter plasmids pSP1 (HBV SP1 promoter), pSP2 (HBV SP2 promoter) and pHBV (HBV CP/Enh I/II) were constructed as previously reported [18, 19].

qPCR

Total RNA was extracted by TRIsure and treated with DNase I (TaKaRa) to eliminate the transfected plasmids and genomic DNA. RNA was reversely transcribed with Reverse Transcription Kit (Vazyme) following the manufacturer's instruction. qPCR analysis was performed as described previously [20]. Briefly, qPCR analysis was performed with ChamQ Universal SYBR qPCR Master Mix (Vazyme) on corresponding systems (ABI 7500), and the results were normalized by the internal control GAPDH. Cytoplasmic HBV DNA was purified as previously described [20, 21]. Briefly, cells were resuspended with PBS, then added 1 × Complete EDTA free and 1% NP40 on ice for 20 min. The supernatant after centrifugation was adjusted with 10 mM $MgCl_2$ and treated with DNase I for 1 h at 37 °C. Protein was digested with proteinase K in the presence of 1% SDS and 50 mM NaCl. After incubation at 55 °C overnight, viral DNA was

isolated by phenol/chloroform extraction and ethanol precipitation. The HBV DNA copy numbers were absolutely quantified using the standard curve based on the pPB plasmid. The conditions are as following: ① denaturation at 50 °C for 2 min followed by 95 °C for 10 s (one cycle); ② 95 °C for 10 s, 60 °C for 30 s (40 cycles); ③ melting at 95 °C 15 s, 60 °C for 60 s, followed by 95 °C for 15 s. The samples were triplicated within each biological replicate. The primer sequences are listed in the Table S2.

Western blot analysis

Western blot was performed as described previously [20]. Cell lysates were separated by 12% SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membranes (Merck Millipore). Signals were visualized and captured using Odyssey instrument. The antibodies used in this study are mouse anti-GAPDH (SolelyBio, TA08) and rabbit anti-GFP (Cell Signaling Techniques, 2956S) and the second antibody Goat anti-Mouse IgG (Immunoway) and Goat anti-Rabbit IgG (Abcam). The results were normalized by internal control GAPDH.

Quantification of HBsAg

The supernatants were collected 72 h after transfection. Then the level of HBsAg was determined previously described [20, 21], utilizing Human HBsAg ELISA Kit (Lvye Biotechnology) and TECAN infinite M200PRO, following the manufacturer's instruction.

Statistical analysis

GraphPad software was used for statistical analysis. A two tailed Student's *t* test was performed to compare the means of two groups. A one-way ANOVA was performed and comparisons between specific groups were done using Dunnett's multiple comparison test to compare the means of three or more groups. All data are presented as the mean ± SD. *P*-values < 0.05 were considered statistically significant.

Results

PYY negatively regulates HBV RNA

To screen differentially expressed genes induced by HBV replication, Huh7 cells were transfected with pPB, followed by RNA-seq analysis. The analysis identified 148 host genes significantly expressed ($P < 0.05$) deposited in the BioProject database of the National Center for Biotechnology Information (Accession number PRJNA856828), 13 of up-regulated and 29 of down-regulated genes of which were filtered out based on more than 2-fold change [20]. EPN3, SEMA5B, UBD, KLRF1, TAS2R38 SLC25A22 and LPA have been studied before [20]. To further verify the important function of remaining genes on viral replication, Huh7 cells were transfected with siRNAs targeting top 1 up-regulated (SH2B2) and top 4 down-regulated genes (PYY, ZNF334, SCART1, ZDHHC1), along with an HBV replicon plasmid (pPB) or HBV pgRNA reporter system (pUC1.2xHBV/NL) [16] (Fig. 1a, b). Compared to the siRNA negative control (siNC) transfectant, the siPYY transfectant showed the highest expression level of HBV RNA (Fig. 1a, b). Meanwhile, the RNA level of PYY was down-regulated in HBV-expressing cells and HBV-infected liver tissues (Fig. 1c, d), which is consistent with the data of RNA-sequencing (PRJNA856828).

To verify the effect of PYY on HBV RNA levels, we knocked down PYY expression in Huh7 cells (Fig. 2a), and HBV RNA, HBV DNA and HBsAg expression levels were up-regulated, consistently (Fig. 2b–e; Sfig. 1a). In addition, pUC1.2xHBV/NL was used to reflect the pgRNA expression level. We found that siPYY consistently increased the NL activity compared to the siNC (Fig. 2f; Sfig. 1b).

To elucidate the functional roles of PYY in HBV replication, Huh7 cells were transfected with EGFP-PYY and pPB plasmids. We found that PYY significantly decreased the expression levels of HBV RNA, HBV DNA and HBsAg, compared to the EGFP (Fig. 3a–f; Sfig. 2). We obtained consistent results in the HepG2 cell line (Sfig. 3). The consistent results were obtained when we utilized another reporter system, 1.3 × WT HBV-Luc [22] (Fig. 3g). These results demonstrate that PYY can repress HBV replication.

PYY reduces HBV RNA in a transcription-coupled manner and transcriptionally inhibits HBV promoter/enhancer activities

To investigate the inhibitory mechanisms of PYY on HBV RNA, we examined whether PYY-mediated HBV RNA down-regulation is coupled with transcription using a transcription inhibitor actinomycin D (ActD) [23]. Our data showed that ActD decreased viral RNA in the EGFP transfected group, but not the PYY transfectant (Fig. 4a). In our previous study, activation-induced cytidine deaminase (AID) could reduce HBV transcripts to suppress HBV replication [24]. And in this research, our results suggest that PYY-mediated HBV RNA down-regulation depends on transcription, similar to AID [24]. Subsequently, we first assessed the potential effect of PYY on viral promoter activity. As shown in Fig. 4b, the luciferase reporter assays demonstrated that HBV CP/Enh I/II, SP1 and SP2 activities were inhibited, but not pGL3-basic, when PYY was expressed. The experiment was also repeated in non-hepatic cells 293T, and it was similar to the results of

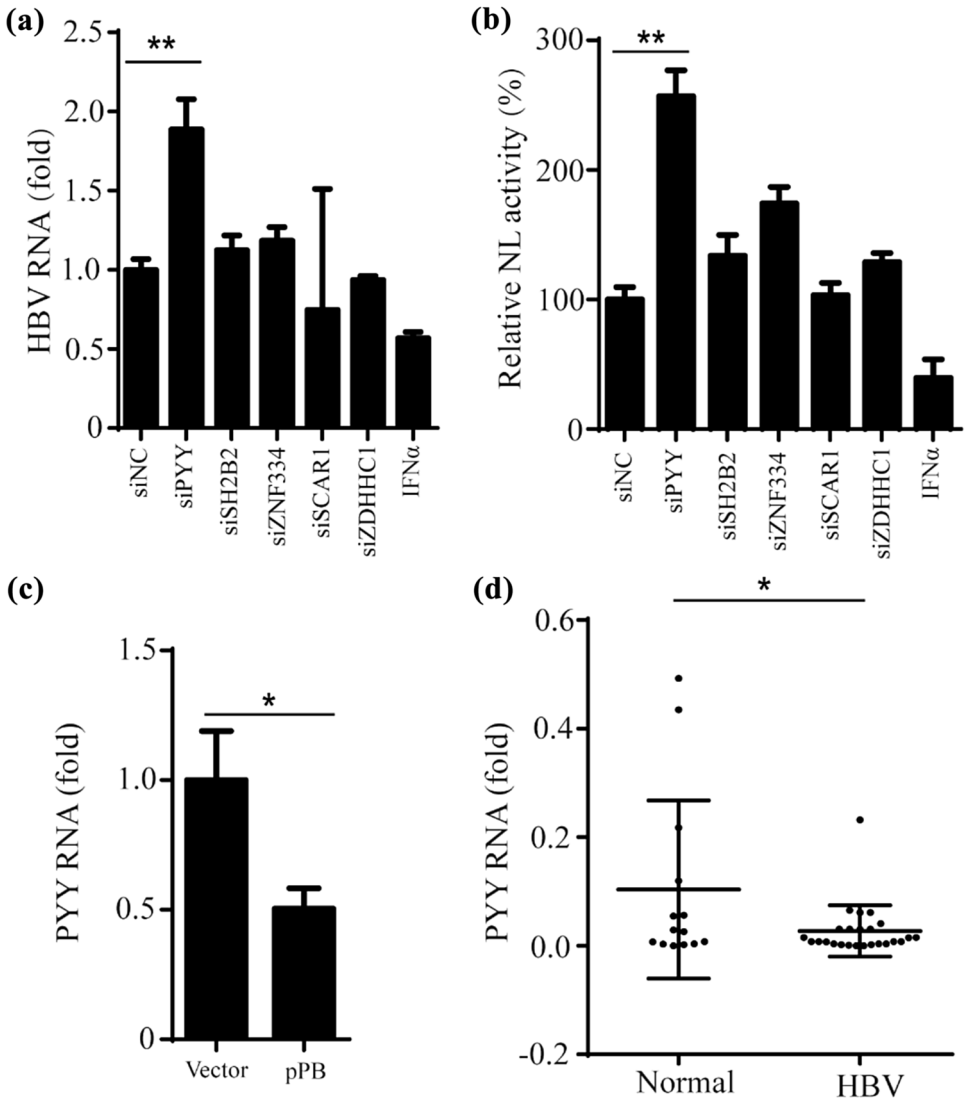


Fig. 1 PYY screening and expression levels in HBV-expressing Huh7 cells and liver tissues. **a** Huh7 cells were transfected with pPB and the indicated siRNAs or the negative control (siNC); 3 days after transfection, total RNA was extracted and HBV RNA expression levels were quantified by qPCR. The RNA level of the siNC sample was defined as value 1. **b** Huh7 cells were transfected with pUC1.2xHBV/NL, pcDNA-CP and the indicated siRNAs or siNC, and luciferase detection was performed 3 days after transfection. The NL activity of the siNC sample was defined as value 100. **c** The vector group of Huh7 cells were transfected with empty plasmid, and the pPB group of Huh7 cells were transfected with pPB plasmid. Cells were harvested 3 days later. The expression level of PYY RNA was detected by qPCR. The expression level of PYY RNA in the vector group was set to 1. **d** The cDNA of adjacent tissues of hepatocellular carcinoma resection for 14 tissues without HBV infection and 25 tissues infected with HBV were collected from the First Hospital of China Medical University. The expression levels of PYY RNA were detected by qPCR. The expression levels of PYY RNA in the normal group were set to 1. $^{**}P < 0.01$; $^{*}P < 0.05$

Huh7 cells (Sfig. 4). Therefore, it demonstrates that PYY reduces HBV RNA through inhibiting viral promoter/enhancer activity at the transcriptional level.

PYY-mediated HBV RNA reduction does not rely on viral core, pol protein or ε structure

HBV pgRNA encapsidation requires the dynamic interactions among three viral components, specifically the pgRNA, pol, and core protein [25]. Therefore, we speculated that PYY might antagonize one or more of these three viral factors to block encapsidation. As the template for reverse transcription, pgRNA recruits the core protein and pol via its ε stem-loop structure to assemble an RNA-containing nucleocapsid [26]. We verified the necessity of core, pol protein and ε stem-loop structure for PYY-mediated reduction of HBV RNA, which was important and essential for HBV replication and AID-mediated

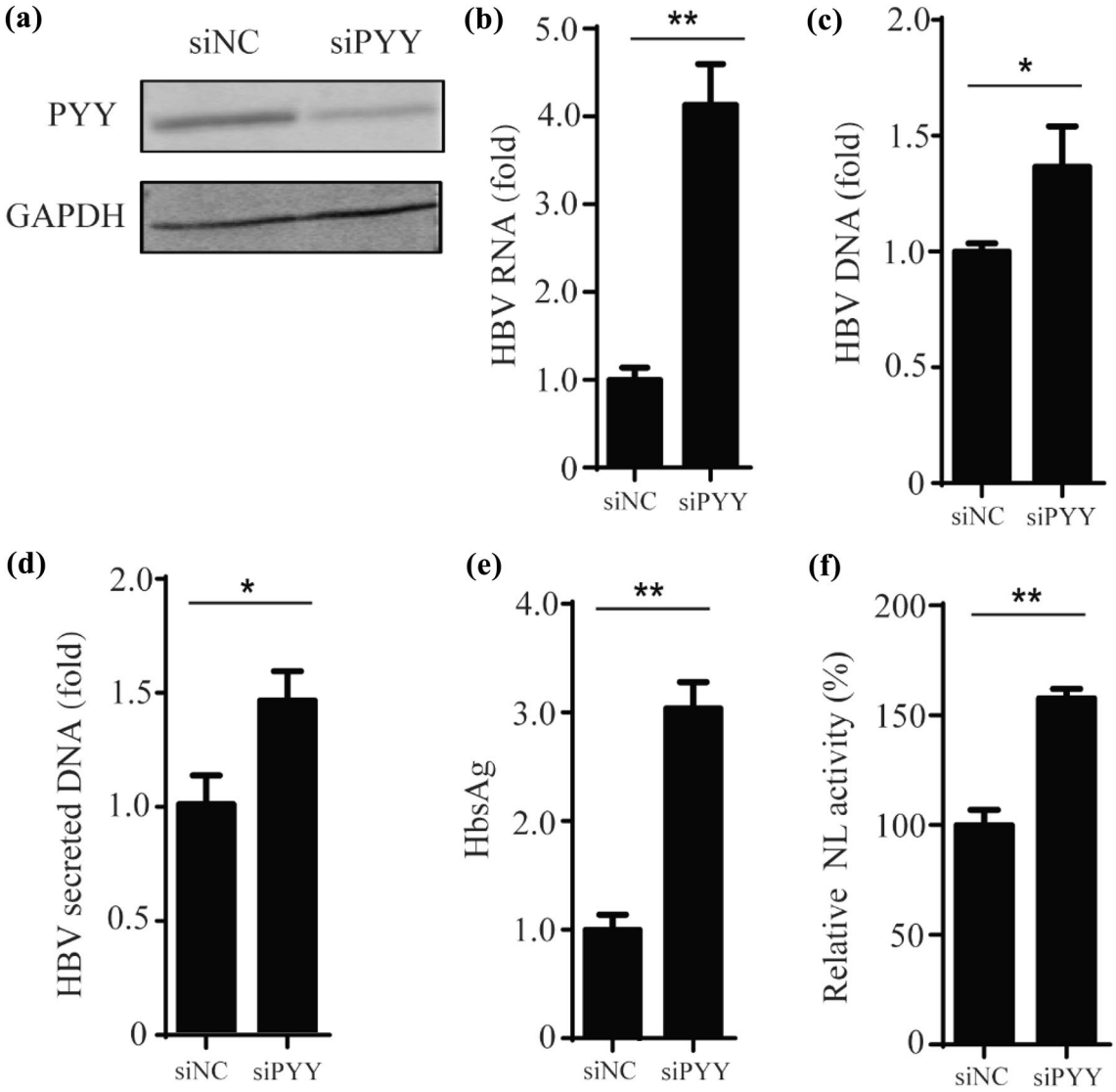
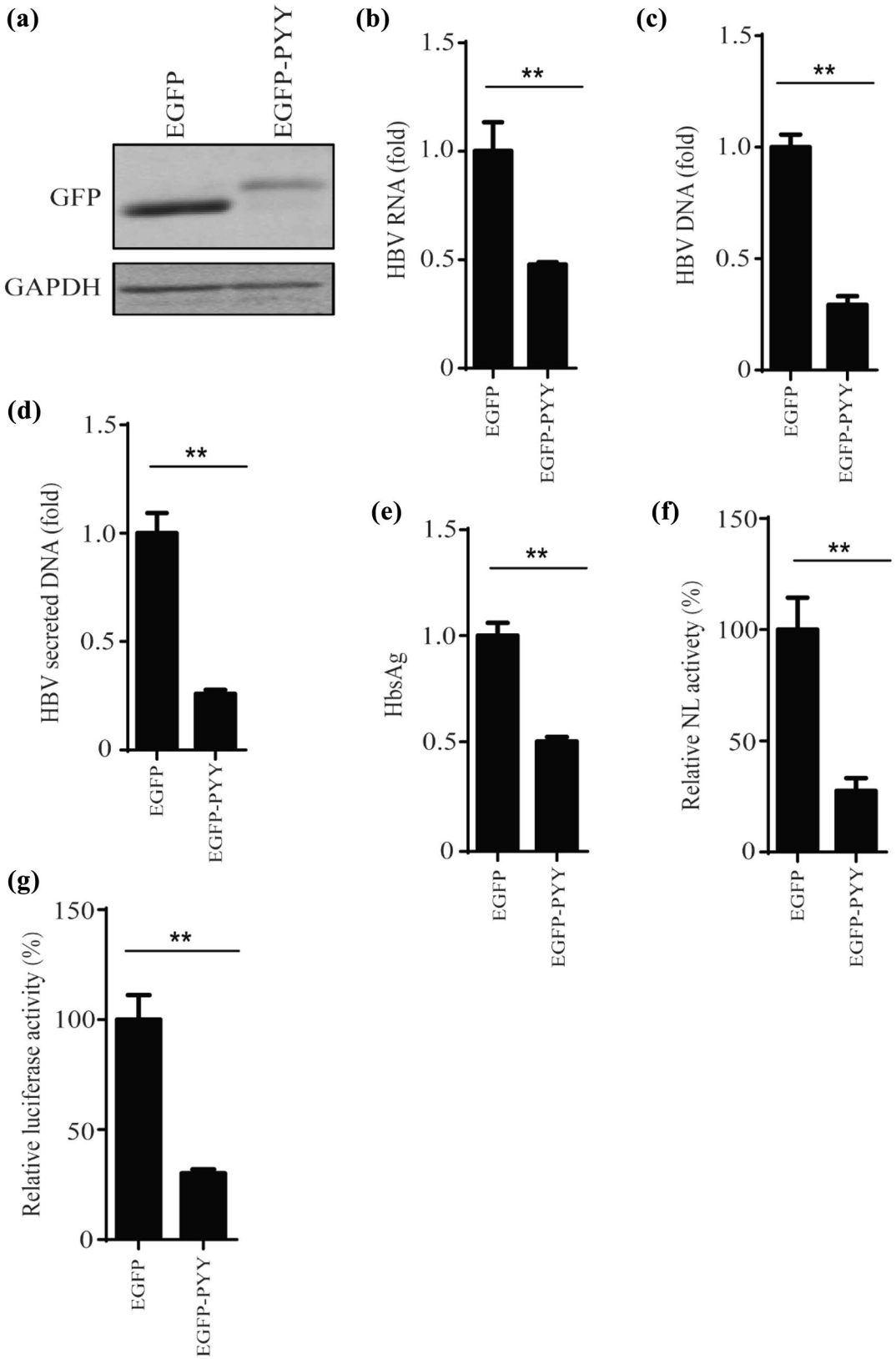


Fig. 2 Silencing of PYY promotes HBV replication. The pPB and GFP-PYY plasmids were co-transfected with siNC or siPYY into Huh7 cells, respectively; the cells and supernatant were collected after 3 days. **a** PYY protein expressions were measured by western blot analysis. **b** HBV RNA levels were quantified by qPCR. The RNA level of the siNC sample was defined as value 1. qPCR was used to detect copies of intracellular (**c**) or extracellular (**d**) HBV DNA levels. **e** ELISA was applied to measure levels of HBsAg in cell culture supernatants. The HBsAg expression level of the siNC sample was defined as value 1. **f** The pUC1.2xHBV/NL, pcDNA-CP and EGFP-PYY plasmids were co-transfected with siNC or siPYY into Huh7 cells, respectively and luciferase was detected 3 days later. The NL activity of the siNC sample was defined as value 100. $^{**}P<0.01$; $^{*}P<0.05$. Data are representative of three independent experiments

reduction of HBV RNA [17]. In this study, we observed that PYY-mediated HBV RNA reduction independent on viral core and pol proteins (Fig. 5a). Further, we utilized mutant pCMV1.2xHBV/NL vectors, whose 5′- and/or 3′-ε structures were lacking. The NL activity was reduced by PYY in the transfectants lacking either 5′- or 3′-ε, even both. This suggests that the antiviral mechanism of PYY is different from AID's (Fig. 5b). To verify the inhibitory effect of PYY on only HBV εs but not extensive RNA transcripts, the pCMV-Fluc [27] and EGFP-PYY or EGFP were co-transfected in Huh7 cells and it showed that PYY only inhibited HBV RNA levels but not suppressed CMV promoters (Fig. 5c).

Discussion

PYY is widely distributed in the gastrointestinal tract and exerts hormonal regulatory action in the upper digestive tract. PYY plays a regulatory role in cell growth. It exerts


(a)
EGFP
EGFP-PYY
GFP
GAPDH
(b)
HBV RNA (fold)
1.5
1.0
0.5
0
**
EGFP
EGFP-PYY
(c)
HBV DNA (fold)
1.5
1.0
0.5
0
**
EGFP
EGFP-PYY
(d)
HBV secreted DNA (fold)
1.5
1.0
0.5
0
**
EGFP
EGFP-PYY
(e)
HbsAg
1.5
1.0
0.5
0
**
EGFP
EGFP-PYY
(f)
Relative NL activety (%)
150
100
50
0
**
EGFP
EGFP-PYY
(g)
Relative luciferase activity (%)
150
100
50
0
**
EGFP
EGFP-PYY

◂**Fig. 3** Overexpression of PYY inhibits HBV replication in Huh7 cells. **a–e** Huh7 cells were transfected with pPB and EGFP-PYY plasmids or EGFP, and the cells and supernatants were harvested 3 days later. **a** Western blot was used to detect the protein expression level of PYY in transfected cells. **b** HBV RNA levels were detected by qPCR, and the RNA level of the EGFP sample was defined as value 1. qPCR was applied to detect copies of intracellular (**c**) or extracellular (**d**) HBV DNA levels. **e** ELISA was employed to measure levels of HBsAg in cell culture supernatants. The HBsAg expression level of the EGFP sample was defined as value 1. **f** and **g** Huh7 cells were transfected with EGFP-PYY or EGFP, together with luciferase reporter plasmids (pUC1.2xHBV/NL or 1.3xWT HBV-Luc) for 72 h, respectively. The NL or luciferase activity of the EGFP was defined as value 100. $^{**}P < 0.01$. Data are representative of three independent experiments

inhibitory action on the growth of pancreatic, breast, and esophagus cancers in vitro [13]. As for liver disease, an abnormal neuroendocrine regulation of PYY(3-36) occurs in patients with decompensated liver cirrhosis [15], but not in hepatoblastoma [28]. Moreover, it's little known about roles of PYY in infectious diseases. In this study, we investigated the roles of a gastrointestinal hormone PYY in HBV infection. The mRNA level of PYY was down-regulated in HBV-expressing cells and HBV patients (Fig. 1). Overexpression of PYY could inhibit HBV transcription and viral replication (Fig. 3; Sfigs. 2, 3), whereas silencing PYY enhanced HBV replication (Fig. 2; Sfig. 1).

HBV transcripts are under the control of four promoters and two enhancers, which interact with cellular factors to regulate HBV gene expression [29]. Our results show that pgRNA and HBsAg levels decreased significantly in PYY-expressing cells, so we further studied the interaction between PYY and CP, SP1 or SP2 promoter. To determine whether PYY down-regulated HBV expression at the transcriptional level, we performed reporter assays in which the firefly luciferase gene was under the control of individual HBV promoters (the CP/Enh I/II, SP1, SP2) in Huh7 cells. PYY significantly reduces the activities of HBV CP/Enh I/II, SP1 or SP2, thereby it inhibits the transcription and replication of HBV (Fig. 4b). We previously reported that AID recruits the RNA exosome to transcribe HBV RNA through an association with the P protein, and thereby downregulates HBV transcripts [24]. Zinc finger antiviral protein (ZAP) and ISG20 selectively degrade HBV RNA through interacting with HBV RNA terminal redundant region containing ε stem-loop structure [17, 30]. In sharp contrast, PYY did not require the ε structure, P protein or core protein for its antiviral activity (Fig. 5).

There are a few limitations that need to be addressed. First of all, our study lacks animal experiments on the

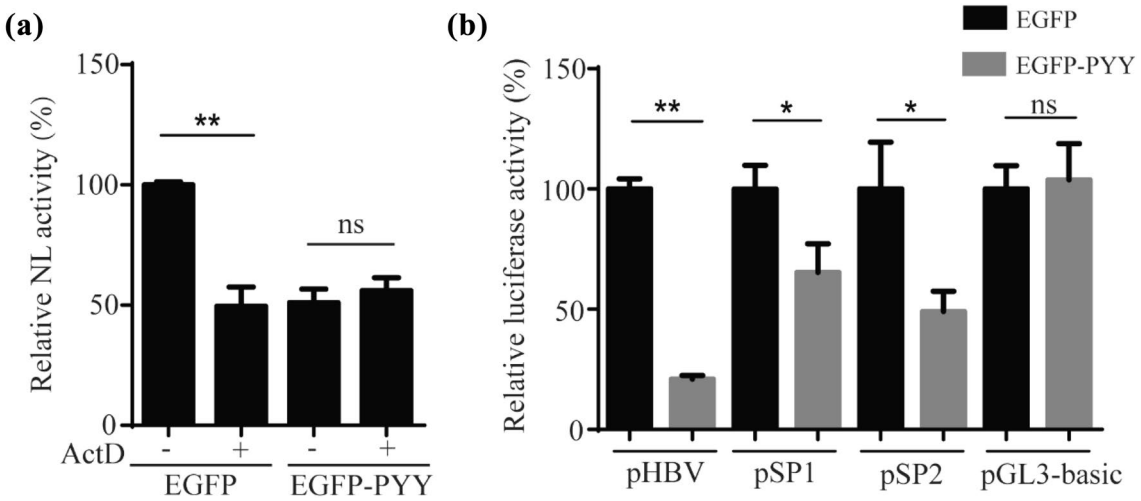


Fig. 4 PYY mediates HBV RNA reduction via attenuating activities of HBV promoters/enhancers transcriptionally. **a** Huh7 cells were transfected with pCMV1.2xHBV/NL, pcDNA-CP, EGFP-PYY (or EGFP) expression plasmids and cultivated for 3 days. ActD was added to block transcription 18 h before harvested. The NL activity of the EGFP without ActD treatment was defined as value 100. **b** Huh7 cells were co-transfected with pHBV-Luc (or pSP1-Fluc, pSP2-Fluc, pGL3-basic plasmid) and EGFP-PYY (or EGFP) expression plasmids. Luciferase detection was performed 3 days after transfection. The Luciferase activity of the EGFP sample was defined as value 100. $^{*}P < 0.05$; $^{**}P < 0.01$; *NS* not significant. Data are representative of three independent experiments

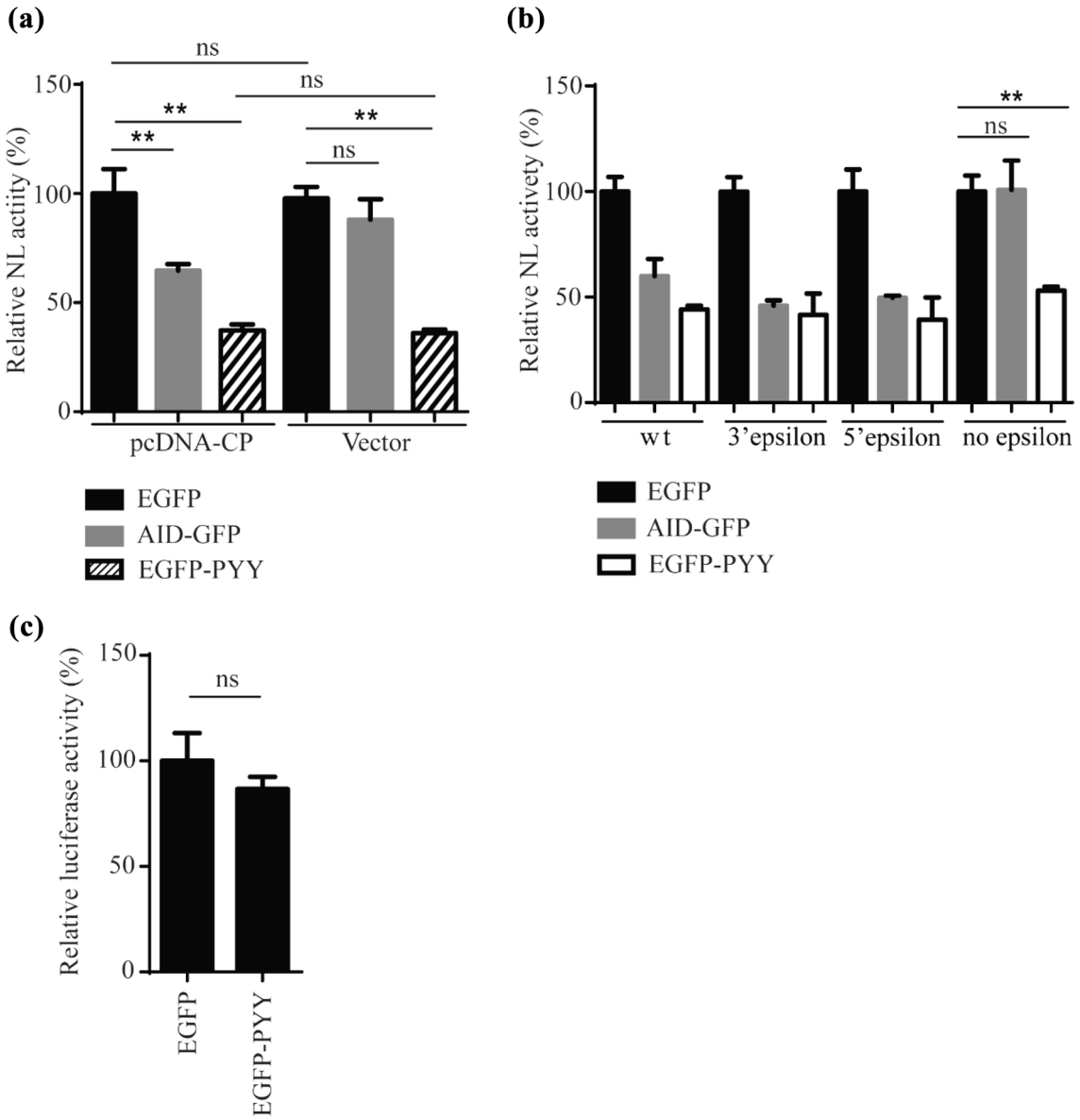


Fig. 5 PYY-mediated HBV RNA reduction is independent of viral protein expression. **a** Huh7 cells were transfected with pCMV1.2xHBV/NL with or without the helper plasmid (pcDNA-CP) and EGFP-PYY (or AID-GFP or EGFP). Cells were harvested at 3 days after transfection, and the luciferase activity was determined. The NL activity of EGFP with pcDNA-CP was defined as value 100. **b** Huh7 cells were transfected with the wild type or mutated pCMV1.2xHBV/NL (*wt* wild type, 5′-ε, 3′-ε, or no ε), along with pcDNA-CP and either EGFP, AID-GFP, or EGFP-PYY expression vector. 3 days after transfection, cells were harvested and luciferase activity was determined. The NL activities of the EGFP transfectants were defined as value 100. **c** Huh7 cells were transfected with pCMV-Fluc and EGFP-PYY or EGFP. Cells were harvested 3 days later, and the luciferase activity was determined. The luciferase of the EGFP transfectant was defined as value 100. $^{**}P < 0.01$; *NS* not significant. Data are representative of three independent experiments

inhibition of HBV replication by PYY, and the model of human chimeric liver in mice can be used. Next, an RNA-immunoprecipitation (RIP) assay can be performed to examine whether PYY interacts with HBV RNA by pull-down the PYY protein in Huh7 cells. Then, the interaction between PYY and HBx should be verified continually, of which HBx usually acts as a transcript factor to regulate HBV mRNA levels. Finally, the mRNA sequence analysis of PYY-expressing Huh7 cells may help us to further clarify the antiviral mechanism of PYY.

In this study, we demonstrated the antiviral roles of PYY negatively regulating HBV RNA level in viral

replicating hepatocytes. Furthermore, PYY inhibits HBV replication by repressing the activities of HBV promoters/enhancers, independent of the ε structure, core and pol protein. However, in clinical liver samples, the inhibitory effect of HBV on PYY expression can be further exploration to help understand the escaping mechanisms of HBV for immune response. In conclusion, the study provides important discovery to understand the interaction between host factors and HBV, which might shed new light on viral pathogenesis or antiviral strategies.

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Author contributions XX, WZ, XL, CZ and YL: data acquisition. XX, WZ, XL, BY and YS: data analysis and interpretation. XX, XT, ZJ, XF, JC and GL: manuscript preparation. GL: study conception and design, study super vision. WZ, GL: funding acquisition. All authors contributed to the article and approved the submitted version.

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Data availability The datasets supporting the conclusions of this article are available in the SRA (www.ncbi.nlm.nih.gov/sra) database.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This study was approved by the Ethics Committees of the First Hospital of China Medical University (No. AF-SOP-07-1.1-01).

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