Small Hepatitis Delta Antigen Selectively Binds to Target mRNA in Hepatic Cells: A Potential Mechanism by Which Hepatitis D Virus Down-Regulates Glutathione S-Transferase P1 and Induces Liver Injury and Hepatocarcinogenesis
Small Hepatitis Delta Antigen Selectively Binds to Target mRNA in Hepatic Cells: A Potential Mechanism by Which Hepatitis D Virus Down-Regulates Glutathione S-Transferase P1 and Induces Liver Injury and Hepatocarcinogenesis

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Abstract

Liver co-infection by hepatitis B virus (HBV) and hepatitis D virus (HDV) can result in a severe form of hepatocellular carcinoma with poor prognosis. Co-infection with HDV and HBV causes more deleterious effects than infection with HBV alone. Clinical research has shown that glutathione S-transferase P1 (GSTP1), a tumor suppressor gene, is typically down-regulated in liver samples from hepatitis-infected patients. In the present study, our data indicated that small HDV antigen (s-HDAg) could specifically bind to GSTP1 mRNA and significantly down-regulate GSTP1 protein expression. For the human fetal hepatocyte cell line L-02, cells transfected with s-HDAg, along with decreased GSTP1 expression, there was a significant accumulation of reactive oxygen species (ROS) and increased apoptotic ratios. Restoring GSTP1 expression through silencing s-HDAg via RNAi or over-expressing exogenous GSTP1 could largely recover the abnormal cell status. Our results revealed a novel potential mechanism of HDV-induced liver injury and hepatocarcinogenesis: s-HDAg can inhibit GSTP1 expression by directly binding to GSTP1 mRNA, which leads to accumulation of cellular ROS, resulting in high cellular apoptotic ratios and increased selective pressure for malignant transformation. To our knowledge, this is the first study to examine s-HDAg-specific pathogenic mechanisms through potential protein-RNA interactions.
Key words: s-HDAg, GSTP1, HCC, ROS, apoptosis.

Introduction

Hepatitis delta virus (HDV) is a small virus with a single stranded RNA genome in a circular conformation (Dastgerdi et al. 2012). HDV is a defective virus and cannot assemble new virions without the envelope proteins of hepatitis B virus (HBV) (Abbas et al. 2015; Pollicino et al. 2011; Tseng et al. 2010), but HDV can cause chronic or fulminant infections in the form of co- or super-infections in patients already infected with HBV (Wedemeyer 2010). Of the 240 million chronic HBV carriers, it is estimated that approximately 15 million are concurrently infected with HDV (Wedemeyer and Manns 2010). HDV is regarded as a deleterious pathogen because its infection commonly leads to progression of hepatic fibrosis, cirrhosis, and more severe hepatocellular carcinoma (HCC) (Dastgerdi et al. 2012). Recent epidemiological studies have shown an increased incidence of HCC in HDV co-infected individuals. One study showed that the adjusted estimated five-year risk for HCC was 13% for HDV-positive, and 2-4% for HDV-negative/HBV surface antigen (HbsAg)-positive cirrhotic patients. HDV infection increases the risk for HCC three-fold, and elevates the risk of mortality two-fold in patients with HBV cirrhosis (Fattovich et al. 2000; Fattovich et al. 1995). The mechanisms through which HDV affects hepatic cells remain to be elucidated.
HDV genomic RNA encodes two antigen isoforms, small hepatitis delta antigen (s-HDAg, 195 amino acids, 24 kDa) and large hepatitis delta antigen (l-HDAg, 214 amino acids, 27 kDa) (Abbas and Afzal 2013; Uhl et al. 2014). When transcribed, HDV first produces a 0.8 kb mRNA molecule, from which s-HDAg is synthesized. This protein is essential for initiation of HDV replication (Kuo et al. 1989). s-HDAg has two arginine-rich motifs that may be responsible for RNA binding (Hourioux et al. 1998; Huang et al. 2008; Taylor, 2009; Vietheer et al. 2005). In addition, s-HDAg is involved in post-translational modifications such as lysine acetylation, serine and threonine phosphorylation, arginine methylation, and lysine sumoylation (Abbas and Afzal, 2013; Gudima et al. 2002; Tseng et al. 2008).

Inhibition of GSTP1 expression is a well-studied mechanism in HCC tumorigenesis (McIlwain et al. 2006). This gene, located on chromosome 11, encodes glutathione S-transferase p1 (GSTP1), which can catalyze the formation of a thioether bond between glutathione (GSH) and electrophilic chemicals, and plays a key role in the detoxification and reduction of reactive oxygen species (ROS) (McIlwain et al. 2006; Niu et al. 2009). Lack of GSTP1 leads to the accumulation of ROS in mitochondria and causes them to release apoptosis-inducing factors (such as cytochrome C), which promote cellular apoptosis (Eisenberg-Lerner et al. 2009; Niu et al. 2009). GSTP1 is widely
expressed in human tissues, including the liver, but was found to be significantly down-regulated in most HCC cases. As a result, GSTP1 has been considered as a potential biomarker to distinguish HCC from other liver diseases at an early stage (Daniel 1993; Jain et al. 2012). One studied mechanism of inhibition of GSTP1 expression in HCC involves the hypermethylation of the GSTP1 gene promoter region (Yusof et al. 2003). However, reports regarding how specific the hypermethylation of GSTP1 is have ranged from 0% to 100% (Harder et al. 2008; Lee et al. 2003; Moribe et al. 2009). Obviously, hypermethylation of the GSTP1 promoter region does not account for all low GSTP1 expression cases in HCC, and there should be other causes for the observed low expression.

In our research, we found that s-HDAg protein could bind directly and specifically to GSTP1 mRNA, and that over-expression of s-HDAg in a hepatocyte cell line led to decreased GSTP1 protein levels, along with increased levels of ROS and cellular apoptosis. These results might reveal an important mechanism involved in liver injury and in hepatocarcinogenesis induced by HDV.
Materials and Methods

Materials

PrimeSTAR HS DNA polymerase was purchased from Takara Biomedical Technology (Beijing) Co., Ltd (Beijing, China). Restriction enzymes were obtained from Thermo Fisher (USA). *Escherichia coli* (*E. coli*) BL21(DE3) was from TransGen Biotech Inc. (Beijing, China). pcDNA3.1-3×Flag plasmid was constructed by our lab based on pcDNA3.1(+) vector (Invitrogen) and the 3×Flag coding sequence ATGGACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGATTACA AGGATGACGATGACAAG was inserted using Nhe I and Hind III restriction endonuclease sites. pcDNA3.1-HDV plasmid containing a dimeric HDV genome (GenBank: X04451.1) was a gift from Dr. Martin Pelchat (University of Ottowa). pET28a (+) vector (with a His-tag coding sequence upstream of the multiple cloning site) was from Novagen. His-tagged p53 protein (coded by a pET28a-p53 recombinant plasmid) and pEGFP-N1 vector (with an EGFP coding sequence downstream of the multiple cloning site) were kindly gifted by Dr. Hao Yang (Sichuan University, Chengdu, China). Ni-NTA beads were from QIAGEN (Hilden, Germany). Fetal bovine serum (FBS) was from Gibco (Australian origin). Dulbecco’s modified Eagle’s medium (DMEM), Lipofectamine®3000 reagent, TRIzol® reagent and MLV Reverse Transcriptase were from Thermo Fisher
(Beijing, China). T7 RiboMAX™ Express large-scale RNA production system was from Promega (Madison, WI, USA). pEASY®-Blunt cloning kit was obtained from TransGen Biotech Inc. (Beijing, China). FLAG® Immunoprecipitation kit was from Sigma-Aldrich (St. Louis, MO, USA). RNasin® Plus RNA inhibitor was from Promega (WI, USA). PVDF membranes (0.22 μm) were from Bio-Rad (USA). Mouse anti-6-His tag and mouse anti-β-actin antibodies were from Abcam (Shanghai, China). Mouse anti-human GSTP1 antibody was from Cell Signaling Technology (Danvers, MA, USA). Horseradish peroxidase-conjugated goat anti-mouse IgG was from Life Technologies (Carlsbad, CA, USA). Immobilon™ Western chemiluminescent HRP substrate was from Millipore (Germany). Annexin V-FITC apoptosis detection kit and ROS level detection kit were from Beyotime Institute of Biotechnology (Shanghai, China). s-HDAg-targeting siRNA and control siRNA were designed and synthesized by GenePharma (Shanghai, China).

**Molecular cloning**

s-HDAg cDNA was amplified by PCR using pcDNA3.1-HDV as template. Primers used are listed in Table 1. The PCR products were digested with BamHI and NotI and inserted into the multiple cloning sites of the pcDNA3.1(+), pcDNA3.1-3×Flag and pET28a(+) vectors separately to create pcDNA3.1-sHDAg, pcDNA3.1-3×Flag-sHDAg and pET28a-sHDAg plasmids.
GSTP1 complete coding sequence (NCBI Reference Sequence: NM_000852.3) was amplified by PCR using total cDNA of human hepatocyte strain L-02 cells as template. Primers used are listed in Table 1. The PCR products were then digested with BamHI and EcoRI and inserted into the multiple cloning site of the pcDNA3.1(+) vector to create pcDNA3.1-GSTP1 plasmid.

Using pcDNA3.1-GSTP1 plasmid as template, GSTP1 coding sequence was amplified by PCR, which introduced EcoRI and BamHI restriction enzyme cutting sites upstream and downstream separately, and also removed the termination codon. Primers used are listed in Table 1. PCR products were digested by EcoRI and BamHI and then inserted into pEGFP-N1 vector to create pEGFP-GSTP1 recombinant plasmid coding for a GSTP1-EGFP fusion protein.

All recombinant plasmids were verified by DNA sequencing.

**Purification of recombinant His-tagged s-HDAg protein**

pET28a-sHDAg plasmid was used to transform *E. coli* BL21(DE3) competent cells. Transformants were cultured in LB medium supplemented with 50 μg/mL kanamycin at 37°C until the OD$_{600}$ value of the culture reached 0.6, and then bacteria were induced with 0.5 mmol/L IPTG overnight at 25°C. For purification of His-tagged s-HDAg protein, Ni-NTA beads were used, as stated by the manufacturer's instructions.

**Cell culture and transfection**
L-02 cells were cultured in DMEM supplemented with 10 % FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, at 37°C in 5 % atmospheric CO₂. All cell transfection experiments were carried out with Lipofectamine® 3000 Reagent following the manufacturer's instructions. The siRNA targeting s-HDAg and its scrambled control were transfected into cells at a final concentration of 150 nM, according to the protocol supplied by the manufacturer.

**Construction of cDNA library and systematic evolution of ligands by exponential enrichment (SELEX)**

Total RNA extracted from L-02 cells by TRizol® was reverse transcribed to cDNA using M-MLV reverse transcriptase according to the manufacturer's protocols. Then, the cDNA was converted to double-stranded cDNA using a primer containing T7 promoter sequence and using T7 RiboMAX™ Express large-scale RNA production system. RNA was transcribed from the double-stranded cDNA and then detected by 1.8 % agarose gel electrophoresis. SELEX was performed as described previously using His-tagged s-HDAg as that target molecule (Manley, 2013). Selected double-stranded DNA was ligated into a T-cloning vector, and these constructs were sequenced.

**Testing the binding specificity of GSTP1 mRNA to s-HDAg inside cells**

L-02 cells were cultured in 10-cm plates to 80% confluency, washed with PBS,
and cultured in DMEM without FBS. Control vector (pcDNA 3.1-3×Flag) and pcDNA 3.1-3×Flag-sHDAg vector were separately transfected into L-02 cells. Four h after transfection, the medium was replaced with DMEM containing 10% FBS. Cells were harvested 48 h after transfection. For each cell sample, total RNA was isolated from one-sixth of the sample by TRlzol® reagent, and Flag-tagged protein was pulled down from the remainder with a FLAG® immunoprecipitation kit in accordance with the manufacturer’s instructions. RNA potentially bound to flag-tagged protein was then extracted from the pull-down complex using TRlzol® reagent. Total RNA and pull-down RNA were diluted to the same volume ratio and tested by reverse-transcription real-time quantitative PCR (RT-qPCR) and regular reverse-transcription PCR (RT-PCR). The specific PCR primer pairs designed for GSTP1, β-actin and GADPH mRNAs are listed in Table 2.

**Binding assays for s-HDAg protein and RNAs using His-tag pull-down**

HDV genomic DNA and GSTP1, β-actin and GADPH cDNAs with a T7 promoter were amplified by PCR using PrimeSTAR HS DNA polymerase. Primers used are listed in Table 1. In PCRs, pcDNA3.1-HDV plasmid was used as template for the amplification of HDV genomic DNA, and total cDNA of L-02 cell (reverse-transcribed with oligo(dT) primer) was used as template for amplification of GSTP1, β-actin and GADPH cDNAs. PCR products were
detected by electrophoresis on a 1.5 % agarose gel, purified by a cycle-pure kit (OMEGA Bio-Tek, Norcross, GA, USA) and then identified by DNA sequencing. Using cDNAs with T7 promoter as template, HDV, GSTP1, β-actin and GADPH RNAs were obtained using T7 RiboMAX™ Express large-scale RNA production system. Ni-NTA beads were incubated with His-tagged s-HDAg or His-tagged p53 protein for 2 h at 4°C in binding buffer (20 mM Tris-HCl, 50 mM NaCl, 5 mM MgCl₂, 10 % glycerin, 0.5 mg/mL BSA, 1 mM DTT, pH 7.2). After washing five times with the same binding buffer, the beads were divided into four equal portions and were then incubated with HDV, GSTP1, β-actin and GADPH RNAs separately in the same binding buffer with addition of RNase inhibitor for 2 h at 4°C. Pull-down RNAs were extracted from beads using TRIzol® reagent after washing five times with binding buffer containing RNase inhibitor and were diluted to the same volume as the starting RNA solutions. RT-qPCR and regular RT-PCR were carried out to detect RNA binding ratios.

**Affinity Assay**

The affinity was tested by biolayer interferometry assay performed on a biomolecular interaction analyzer (Blitz system, Pall ForteBio, Menlo Park, CA, USA) according to the manufacturer’s instructions (Data Acquisition User Guide 6.4, ForteBio). His-tagged s-HDAg was loaded on anti-penta-His biosensors
(ForteBio 18-0034) at 0.2mg/mL in 1 × binding buffer (20 mM Tris-HCl, 150 mM NaCl, 0.5 mg/mL BSA, 1 mM DTT, RNase inhibitor, pH 7.2) and the \( \beta -\text{actin} \), \( \text{GSTP1} \) or \( \text{HDV} \) RNA dissolved in the same buffer at 20 \( \mu \text{g/mL} \) was used for association assay. Test procedure was optimized as 30 s initial baseline, 120 s His-tagged s-HDAg protein loading, 30 s baseline, 120 s RNA-protein association, and 120 s dissociation. Analysis was standardized by deducting the association curve of s-HDAg protein in binding buffer.

RT-qPCR

TRIzol extracted RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase. Primers are listed in Table 2. Each experiment was performed in duplicate, and \( \beta -\text{actin} \) cDNA was used as an internal control for normalization.

Co-expression of sHDAg and GSTP1-EGFP (Enhanced Green Fluorescence Protein) fusion protein

Four pairs of plasmids (pcDNA3.1 and pEGFP-N1, pcDNA3.1-sHDAg and pEGFP-N1, pcDNA3.1 and pEGFP-GSTP1, and pcDNA3.1-sHDAg and pEGFP-GSTP1) were co-transfected into L-02 cells with Lipofectamine®3000 reagent. The plasmid ratio of each pair was 1:1, i.e. 500:500 ng for transfections in each well of 24-well plates, or 100:100 ng for transfections in each well of 96-well plates. At 48 h post transfection, cells were lysed with RIPA buffer for western-blot analysis and fluorescence imaging.
Western blot analysis

Cells were lysed in RIPA buffer, and proteins were separated by 12% polyacrylamide gel electrophoresis and electro-transferred to PVDF membranes. The membrane was blocked with 5% milk in PBS-T (137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na₂HPO₄, 2 mmol/L KH₂PO₄, 0.1 % Tween20, pH 7.2) at room temperature for 1 h. Mouse anti-human GSTP1 antibody (1/1000), mouse anti-EGFP antibody (1/1000) or mouse anti-human β-actin antibody (1/1000) was used as the primary antibody at 4°C overnight. Horseradish peroxidase-conjugated goat anti-mouse IgG was used as the secondary antibody. Detection was performed using Immobilon™ Western chemiluminescent HRP substrate and a ChemiDoc MP imaging system (BIO-RAD, USA).

ROS level analysis

ROS were measured with the non-fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) in a Reactive Oxygen Species Assay Kit (Cat# S0033, Beyotime) according to the manufacturer's instructions. DCFH-DA passively diffuses into cells and is deacetylated by esterases to form nonfluorescent 2′, 7′-dichlorofluorescein (DCFH). DCFH reacts with ROS (mainly including hydrogen peroxide and hydroxyl free radicals) to form the fluorescent product dichlorofluorescein (DCF), which is trapped inside cells (Liu et al. 2001; Myhre et
al. 2003; Tian et al. 2006). The intensity of DCF fluorescence, which reflects the ROS level, was detected using a fluorescence plate reader at wavelengths of 488 nm for excitation and 525 nm for emission.

**Cell apoptosis analysis**

Cells were stained using an Annexin V-FITC apoptosis detection kit, following the manufacturer's instructions, and detection was performed by flow cytometry at wavelengths of 530 nm and >575 nm.

**Data analysis**

All data are presented as means ± SEM representing three separate experiments. Student’s *t* test or ANOVA (analysis of variance) with LSD (least significant difference) were used for comparison. Differences were considered statistically significant when *p* values were less than 0.05.
Results

s-HDAg potentially binds a fragment of GSTP1 mRNA

We purified the His-tagged s-HDAg protein with Ni-NTA beads. SDS-PAGE showed that the purified protein corresponded to the predicted molecular weight of His-tagged s-HDAg (approximately 26 kDa, see Fig. 1A); this protein was further confirmed by western blotting using an anti-6-His tag antibody (Fig. 1B). We chose SELEX to screen RNAs that potentially bound to s-HDAg. We constructed a cDNA library using total RNA extracted from L-02 cells. An agarose gel electrophoresis assay indicated that the detected cDNAs from the pool ranged in size from 100 bp to 5 kb (Fig. 1C). The SELEX technique uncovered an approximately 300-bp fragment potentially bound to s-HDAg protein (Fig. 1D). After sequencing and alignment using BLAST, the 300 bp fragment was identified to be a fragment of GSTP1 (Fig. 1E).

s-HDAg specifically binds GSTP1 mRNA inside and outside of cells

We performed pull-down assays to identify that s-HDAg binds GSTP1 mRNA inside and outside of cells. First, we transfected flag-tagged s-HDAg cDNA into L-02 cells and pulled-down the flag-tagged proteins using immunoprecipitation with Flag-binding beads. Immunoprecipitated RNA was extracted, reverse-transcribed, and detected by regular RT-PCR (Fig. 2A) and RT-qPCR (Fig. 2B). In the s-HDAg cDNA transfection group, a sharp band was amplified
from pull-down complexes by using a GSTP1 mRNA-specific primer pair, but not by β-actin or GADPH mRNA-specific primer pairs (Lane 4 in Fig. 2A). However, from the pull-down complex in the control group, no band was amplified by using any of these primer pairs (Lane 2 in Fig. 2A). RT-qPCR also showed that among detected mRNAs, only GSTP1 mRNA was significantly enriched in pull-down complexes in the s-HDAg cDNA transfection group, whereas this enrichment was not observed in controls (Fig. 2B).

Because interactions involving cellular RNA/protein could be involved, we designed an in vitro experiment to further prove the direct interaction of s-HDAg with GSTP1 mRNA. We prepared four RNAs using an in vitro transcription kit and tested for binding between each single RNA and His-tagged s-HDAg protein under simple non-cellular conditions. Here, we used the HDV genome RNA as a positive control. The pull-down product was used as template for RT-qPCR and regular RT-PCR. As shown in Figs. 2C and D, significant binding of the target RNA was observed in the HDV and GSTP1 groups, but not in the β-actin or GADPH groups. The average percent binding (calculated as the ratio of pull-down amount to the total quantity) of each group was approximately 55% for HDV, 30% for GSTP1, 3% for β-actin and 4% for GADPH (Fig. 2D). To exclude the possibility of non-specific binding, we substituted an irrelevant protein, His-tagged p53, for His-tagged s-HDAg to repeat the experiment and no
significant binding of the target RNA was observed in each of the four groups (Figs. 2C and D). To further confirm the binding between s-HDAg protein and GSTP1 mRNA, a bio-layer interferometry assay was performed using a Blitz system. The Blitz system utilizes an optical analytical technique to analyze the interference pattern of white light reflected from two surfaces: a layer of immobilized protein on the biosensor tip, and an internal reference layer. Any change in the number of molecules bound to the biosensor tip causes a shift in the interference pattern that can be measured in real-time as a curve (Sultana and Lee 2015). Under the same RNA concentration, as shown in Fig. 2E, HDV RNA exhibited the strongest association with s-HDAg protein and the slowest dissociation. GSTP1 mRNA also exhibited a strong association with s-HDAg protein, but the dissociation seemed much more rapid than with HDV RNA. For β-actin mRNA, only a very weak association was observed. Taken together, it could be concluded that s-HDAg does specifically bind GSTP1 mRNA, regardless of whether in a complicated environment inside cells, or in simple, in vitro conditions outside of the cell.

**s-HDAg overexpression in L-02 cells causes a decrease in GSTP1 protein levels**

To investigate the biological effect of the binding between s-HDAg and GSTP1 mRNA, we transfected pcDNA3.1-sHDAg or pcDNA3.1(+) (control plasmid) into
L-02 cells and created a stable cell line (L-02-sHDAg or L-02-control) via G418 selection. We detected GSTP1 expression at both the transcriptional and protein levels. As shown in Fig. 3A, mRNA levels of GSTP1 did not change with expression of s-HDAg, compared to controls. However, the GSTP1 protein level was dramatically decreased with the expression of s-HDAg (Fig. 3B).

Next, we transfected either s-HDAg-targeted siRNA or control siRNA into L-02-sHDAg cells. RT-qPCR and western blotting were employed to detect GSTP1 mRNA and protein levels, respectively. Compared to the siRNA control, knockdown of s-HDAg through siRNA did not change GSTP1 RNA levels (Fig. 3C), but boosted GSTP1 abundance at the protein level (Fig. 3D).

Expression of s-HDAg significantly down-regulates GSTP1-EGFP fusion protein but not EGFP protein

To verify the relationship between expression of s-HDAg and GSTP1, we created a recombinant plasmid encoding a GSTP1-EGFP fusion protein. Results of fluorescence imaging and western blotting in co-expression experiments indicated that in L-02 cells, over-expressed s-HDAg significantly affected the expression of GSTP1-EGFP fusion protein. As shown in Figs. 3 E and F, compared with pcDNA3.1 control plasmid, transfection of pcDNA3.1-sHDAg plasmid led to down-regulation of GSTP1-EGFP fusion protein by approximately 40-50% (about 41% in fluorescence imaging assay and about 48% in western
blot assay). However, for simple EGFP expression, no significant change was observed between the presence of s-HDAg and the absence of this protein.

**In L-02 cells, increased ROS and apoptosis caused by s-HDAg overexpression can be reversed by silencing of s-HDAg or over-expression of GSTP1**

ROS assays showed that along with the down-regulation of GSTP1, ROS levels of the L-02-sHDAg cells was increased approximately three folds, compared to the ROS levels in the control group (Fig. 4A). Apoptosis assays showed that the apoptotic rate of L-02-sHDAg cells increased by approximately 60% compared with that of controls (Fig. 4B). However, both the ROS and apoptosis levels were significantly decreased when we transfected L-02-sHDAg cells with sHDAg siRNA, but not with control siRNA (Figs. 4A and 4B).

We then co-expressed GSTP1 by transfecting pcDNA3.1-GSTP1 into L-02-sHDAg stable cells to further confirm the molecular interaction described above. GSTP1 over-expression was detected through western blot assays (Fig. 4C). With restored GSTP1 protein level, ROS and apoptosis levels in L-02-sHDAg cells decreased to almost the levels of L-02-control cells, but this change could not be observed in L-02-sHDAg cells transfected with pcDNA3.1 control plasmid (Figs. 4 D and E).

These results demonstrated that in L-02-sHDAg cells, s-HDAg influenced cellular ROS and apoptosis levels. More importantly, ROS accumulation and apoptosis
induced by s-HDAg had a significant negative correlation with the expression level of GSTP1, and over-expression of GSTP1 by exogenous plasmid could reverse the increased ROS levels and apoptosis rate. Our results suggested GSTP1 might play a key intermediate role in the cytopathy induced by s-HDAg.
Discussion

Co- or super-infection of HDV (compared to HBV infection alone) is often associated with more aggressive liver diseases, including cirrhosis, and can finally lead to HCC; however, the mechanisms underlying this relationship are poorly understood. In this study, we investigated possible specific roles for s-HDAg in the outcome of HBV/HDV patients.

s-HDAg is the first protein synthesized in the HDV life cycle and has two arginine-rich motifs, which are regarded as the binding components for the viral RNA (Daigh et al. 2013; Lee et al. 1993). s-HDAg was considered to bind specifically to HDV RNA (Lin et al. 1990), an unbranched rod-like RNA (Wang et al. 1986), but later studies uncovered that s-HDAg could also act on complicated and simple non-HDV RNAs, for instance, an RNA derived from the K12 gene of human herpesvirus 8 (Defenbaugh et al. 2009). However, the role of the bound RNAs remains a mystery and the region of s-HDAg responsible for RNA binding is still debatable (Daigh et al. 2013; Lee et al. 1993; Zuccola et al.). In this study, we sought to screen RNA(s) that s-HDAg protein potentially combined with by using a simple cellular system without HDV genomic RNA interference. First, we used SELEX to screen for RNA(s) binding to s-HDAg and found GSTP1 mRNA as one of the potential candidates. We then performed immunoprecipitation, His-tag pull-down and affinity assays to prove that s-HDAg indeed bound to
GSTP1 mRNA specifically and directly, either inside or outside of the cell. To our knowledge, this is the first report of this binding.

What, then, is the biological effect of s-HDAg binding to GSTP1 mRNA? Here, we tested GSTP1 expression at both the RNA (Fig. 3A) and protein levels (Fig. 3B) after transfection of s-HDAg cDNA. It was clear that GSTP1 protein expression was inhibited by s-HDAg over-expression, although mRNA levels were not decreased. From this result, we concluded that s-HDAg could regulate GSTP1 expression in L-02 cells, and that this regulation would not affect GSTP1 gene transcription or the stability of GSTP1 mRNA and, further, should act at the level of mRNA translation.

As we know, microRNAs are key translation inhibitors of their target mRNAs by binding 3′ untranslated regions (3′ UTRs). Is there a possibility that the decrease in GSTP1 is caused by microRNAs that target GSTP1, which is up-regulated via sHDAg protein? To exclude this possibility, we constructed a recombinant plasmid which encoded a GSTP1-EGFP fusion protein. This plasmid lacked the natural 3′ UTR of GSTP1 and would not be regulated by inhibiting microRNAs which target GSTP1. In L-02 cells, the GSTP1-EGFP fusion protein was significantly down-regulated by s-HDAg, whereas EGFP protein alone was not. This result indicated that in L-02 cells, s-HDAg regulated GSTP1 expression, not through microRNA pathways, but more likely in a coding sequence
(CDS)-dependent manner. In addition, this experiment also excluded the possibility that the decrease in GSTP1 was a result of global expression inhibition induced by s-HDAg, because a global effect should also lead to the decrease in EGFP alone.

Previous research has proven that interactions between protein and RNA can affect the stability or translation efficiency of RNA and can influence almost every aspect of cellular function (Burd and Dreyfuss 1994). In our research, we proved s-HDAg has the ability to specifically bind $GSTP1$ mRNA and can also knock down GSTP1 expression in a GSTP1 CDS-dependent manner at the post-transcriptional level. All these clues implied that s-HDAg’s specific mRNA binding activity might play a key role in GSTP1 regulation in liver cells.

After $s$-$HDAg$ gene transfection, accompanied by the down-regulation of GSTP1, significant accumulation of ROS and increased cell apoptosis were observed in L-02 cells. Silencing $s$-$HDAg$ via siRNA or by over-expressing exogenous GSTP1 could decrease ROS levels and rescue abnormal cell apoptosis. These results implied that down-regulation of GSTP1 by s-HDAg may be an important mechanism in liver injury induced by HDV. However, this may seem somewhat incompatible with the current literature regarding the association between carcinogenesis and cellular apoptosis induced by HDV. It is generally accepted that one factor leading to carcinogenesis is a decrease in cellular apoptosis.
(instead of an increase). Many studies have shown ROS to be a key promoter of apoptosis (Chen et al. 2015; Qiu et al. 2015; Wu et al. 2015; Zhang et al. 2015). Thus, it was expected that down-regulation of GSTP1, resulting in significant ROS accumulation, would induce cellular apoptosis. However, it should be noted that not all hepatocytes undergo apoptosis. ROS accumulation causes oxidative DNA damage in proliferating cells. Continuous base mismatch and selective pressures can enrich for apoptosis-resistant proliferating hepatocytes, and also drive malignant transformation in the regenerating liver (Hikita et al. 2015). Clinical research has indicated GSTP1 activity often decreases in tumor tissue, rather than in para-tumor tissue, in hepatitis-associated HCC. HBV-induced hypermethylation of the \textit{GSTP1} promoter at CpG sites is a well-studied mechanism to explain this clinical feature (Su et al. 2007; Zhong et al. 2002; Zhou et al. 1997). However, the uncertain specificity of \textit{GSTP1} hypermethylation indicates that this mechanism cannot fully explain the decreased GSTP1 in hepatitis-related HCC (Harder et al, 2008; Lee et al, 2003; Moribe et al, 2009). In this paper, our findings reveal a novel potential mechanism of GSTP1 silencing associated with S-HDAg expression that may contribute to the development of HCC in HBV-HDV co-infection.

Structurally, s-HDAg is a typical RNA binding protein (with two RNA binding motifs) as well as being an alkaline protein, with an isoelectric point above 11.
appears that s-HDAg should bind RNAs extensively. However, previous reports and our study showed that the binding between s-HDAg protein and RNAs was more specific than expected. To date, we do not understand why s-HDAg specifically targets \textit{GSTP1} mRNA. The GC content of the s-HDAg binding fragment is not unusual, and HDV gRNA and \textit{GSTP1} mRNA share scant resemblance in terms of sequence or secondary structure. It is also difficult to understand why s-HDAg operates to down-regulate GSTP1, which would obviously impair the cells' capability to replicate HDV. Further research needs be performed to answer these questions.

In summary, to our knowledge, this is the first study to examine s-HDAg-specific pathogenic mechanisms through potential protein-RNA interactions. Our findings show that s-HDAg can bind to \textit{GSTP1} mRNA directly and down-regulate the expression of GSTP1 protein, and overexpression of s-HDAg leads to a decrease in cellular ROS consumption and an increase in cell apoptosis.
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Disclosure

The authors declare they have no competing interests as defined by *Biochemistry and Cell Biology*, or other interests that might be perceived to influence the results and discussion reported in this paper.
References


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Niu, D., Zhang, J., Ren, Y., Feng, H., et al., 2009. HBx genotype D represses GSTP1 expression and increases the oxidative level and apoptosis in HepG2 cells. Molecular oncology 3(1), 67-76.


Figure Legends

Fig. 1 *GSTP1* mRNA fragment was enriched through SELEX using recombinant His-tagged s-HDAg protein

pET28a-sHDAg plasmid was transformed into *E. coli* BL21(DE3) and 0.5 mmol/L IPTG was used to induce recombinant protein expression. sHDAg protein with a His-tag was purified using Ni-NTA beads and confirmed by western blot. Total RNA extracted from L-02 cells was used to generate the RNA pool for SELEX. s-HDAg protein worked as the bait and was incubated with the RNA pool for six rounds. In the final round, the RNA binding to s-HDAg protein was recovered and reverse-transcribed into cDNA. The cDNA was transfected into a T-vector and transformants were sequenced to identify the bound RNAs. (A) SDS-PAGE assay of purified protein using a 12 % gel. Lane 1: protein marker; Lane 2: purified recombinant protein. (B) Western blot of purified protein using anti-6-His tag antibody. Lane 1 (M): protein marker; Lane 2 (s-HDAg): purified recombinant protein. (C) cDNA library detected by electrophoresis using a 1.2 % agarose gel. Lane 1: DNA marker; Lane 2: cDNA library; (D) different cycles of SELEX. Lane 1: DNA marker; Lane 2: cycle 1 of SELEX; Lane 3: cycle 3 of SELEX; Lane 4: cycle 6 of SELEX; (E) sequence of RNA fragments enriched by SELEX.

Fig. 2 s-HDAg protein binds to *GSTP1* RNA *in vivo* and *in vitro*. 


(A) and (B) Detection of immunoprecipitated mRNAs inside cells. Cell lysates from L-02-sHDAg-flag cells or L-02-flag cells were incubated with anti-Flag antibody and RNAs binding to the antibody were pulled down, recovered, reverse-transcribed into cDNAs and assayed by regular PCR or qPCR. (A) regular RT-PCR detection: after 25 cycles of amplification separately with GSTP1, β-actin and GADPH specific primer pairs, PCR products were detected on a 1.5% agarose gels. Template of Lane 1: total RNA extracted from control group cells (transfected with pcDNA3.1-3×flag plasmid for 48 h); Template of Lane 2: RNA extracted from immunoprecipitated complexes in control cells; Template of Lane 3: total RNA extracted from sHDAg group cells (transfected with pcDNA3.1-3×flag-sHDAg plasmid for 48 h); Template of Lane 4: RNA extracted from the immunoprecipitated complexes in sHDAg group. (B) RT-qPCR detection: The enrichment percentage is the ratio of the amount of the target RNA in immunoprecipitated RNA to that in total RNA. Data are reported as average means ± SEM, from three independent experiments. *** P < 0.001.

(C) and (D) Detection of sHDAg-mRNA binding outside of cells. Target RNA fragments were transcribed from cDNA templates by specific primers with T7 promoter sequences, and then pulled-down with Ni-NTA beads associated with His-tagged s-HDAg. To exclude the possibility of non-specific binding, Ni-NTA beads associated with His-tagged p53 protein was used as a control. Pull-down
RNAs were recovered, reverse-transcribed into cDNA and assayed by regular PCR or qPCR. (C) Regular RT-PCR detection of binding outside of cells. PCR products after 25 cycles of amplification were detected on 1.5% agarose gels. Lane 1: RT-qPCR results by using RNA samples prepared by transcription system in vitro as template. Lane 2: RT-qPCR results by using pull-down products as template. (D) RT-qPCR detection of relative binding percentage among HDV gRNA, GSTP1 mRNA and β-actin mRNA. The binding percentage is the ratio of the pull-down RNA quantity to the amount of input RNA. Data are reported as average means ± SEM, from three independent experiments. *P < 0.05, **P<0.01. (E) Affinity assay between s-HDAg and RNAs. Real-time biolayer interferometry assays were performed with s-HDAg protein and RNAs using a Blitz instrument, as described in Materials and Methods.

**Fig. 3 sHDAg down-regulates GSTP1 expression in L-02 cells.**

pcDNA3.1-sHDAg or control plasmid (pcDNA3.1(+)) was transfected into L-02 cells and G418 selection was carried out to create a stable cell line (L-02-sHDAg or L-02-control). (A) Expression of GSTP1 mRNA in L-02-control and L02-sHDAg cells tested by RT-qPCR (β-actin mRNA was used as an internal control). (B) Expression of GSTP1 protein tested by western blot assay. (C) RT-qPCR assay for expression of s-HDAg and GSTP1 mRNA (β-actin mRNA was used as an
internal control) in L-02-sHDAg cells after transfection with *s-HDAg* siRNA. (D) Western blot assay for GSTP1 protein in L-02-sHDAg cells after transfection with *s-HDAg* siRNA. (E) Fluorescence intensity assay for cell lysates in s-HDAg and GSTP1-EGFP fusion protein co-expression experiment. Relative fluorescence intensity is presented as percentages by setting the fluorescence intensity of the group lacking s-HDAg as 100%. (F) Western blot assay for GSTP1-EGFP fusion protein and EGFP protein in co-expression experiments. Data are reported as average means ± SEM for three independent experiments, ***P < 0.001.

**Fig. 4** *s-HDAg increased ROS levels and apoptosis induced in L-02 cells is reversible by silencing of s-HDAg or over-expression of GSTP1*

(A) Relative ROS levels and (B) apoptotic rates tested in L-02-control cell line, L-02-sHDAg cell line or L-02-sHDAg cell line transfected with s-HDAg siRNA or siRNA control. (C) Protein levels of GSTP1 tested by western blot assays in L-02-control cell line or L-02-sHDAg cell line transfected with GSTP1 overexpression plasmid (pcDNA3.1-GSTP1) or control plasmid (pcDNA3.1). (D) Relative ROS levels and (E) Apoptosis rates tested in L-02-control cell line or L-02-sHDAg cell line transfected with GSTP1 overexpression plasmid (pcDNA3.1-GSTP1) or control plasmid (pcDNA3.1). Data are reported as average means ± SEM of three independent experiments. *P < 0.05, **P < 0.01
Fig. 1 GSTP1 mRNA fragment was enriched through SELEX using recombinant His-tagged s-HDAg protein.
Fig. 2 s-HDAg protein binds to GSTP1 RNA in vivo and in vitro.
Fig. 3 sHDAg down-regulates GSTP1 expression in L-02 cells.
Fig. 4 s-HDAg increased ROS levels and apoptosis induced in L-02 cells is reversible by silencing of s-HDAg or over-expression of GSTP1.

338x190mm (300 x 300 DPI)
### Tables

Table 1. Primers used for preparation of expressive plasmids and DNAs with T7 promoter.

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<td>s-HDAg Reverse</td>
<td>5´-TATGCGGCCCTAATGGAAATCCCCGGTTTC-3´ (NotI)</td>
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Table 2. Primers used for RNA detection
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