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HBV multidrug resistant sW172* truncated variant possibly involve in the ER stress pathway during hepatocarcinogenesis

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Keywords  HBV rtA181T/sW172*, ER stress, Hepatocellular carcinoma

Running Title  HBV sW172* could activate ER stress pathway

\textbf{SUMMARY} We investigated the biological effect of HBV rtA181T/sW172* point mutation on HBsAg secretion and the potential mechanisms involved in hepatocarcinogenesis. Plasmids expressed full-long HBV wildtype and HBV rtA181T/sW172* were transfected into HepG2 HCC cell lines or were injected to C57BL/6 mice. The HBsAg and HBeAg expressions of extracellular and intracellular proteins, mice serum and liver tissues were detected by ELISA. The localization of truncate protein was characterized in vitro. The expression of ER stress gene GRP78 mRNA was determined. Significant high levels of HBsAg was observed in supernatants of cells transfected with HBV wildtype or serum of mice injected with HBV wt compared with HBV rtA181T/sW172* mutant. Reverse trend was observed in intracellular cells and intrahepatic liver cells. S wild protein only could rescue this dysfunction. HBV rtA181T/sW172* truncate surface proteins showed a more aggregated cytoplasmic pattern which also localized to the ER in comparison with HBV wt. Meanwhile, grp78 mRNA was increased in cells 72 hour post-transfected with HBV rtA181T/sW172* cells relative to HBV wt ($P=0.0154$). The HBV sW172* truncate mutant has a defect on HBsAg secretion which can lead to surface protein retention in ER, it may contribute to the hepatocarcinogenesis through activating the ER stress signaling pathways.
INTRODUCTION

Chronic infection with Hepatitis B virus (HBV) is common, globally affecting more than 350 million people, and frequently leads to serious consequences such as cirrhosis and hepatocellular carcinoma (HCC) (1,2). Treatment of chronic hepatitis B carriers with long-term nucleos(t)ide analogues (NAs), including lamivudine (LMV), adefovir (ADV), telbivudine (ETV), and clevudine (LdT) has significantly increased the rate of anti-HBe seroconversion and therefore reduced the impact of chronic hepatitis B (CHB) on liver disease. However, therapies using NAs have been confronted with viral resistances which are often associated with worsening of liver disease (2).

HBV is a partially double-stranded DNA virus containing a genome of 3.2 kb in size, which contains four open reading frames encoding viral polymerase, the core and e antigen, the HBx protein and the pre-S/S gene encoding the three surface antigens (i.e. the large [pre-S1 + pre-S2 + S], middle [pre-S2 + S] and small [S only] surface proteins). The HBV S gene is completely overlapped by the polymerase gene. As a consequence, mutations in the polymerase gene may produce changes in the overlapping S gene. Altogether the widespread use of NAs as a treatment for chronic hepatitis B infection and the resulting selection of these combination polymerase and S gene HBV mutants would have important public health implications (3,4).

Among them, amino acid (a.a.) substitution at position 181 (rtA181T) in the HBV polymerase gene also generates a stop codon in the surface reading frame (sW172stop), leading to the truncation of the surface proteins. This point mutation was recently reported in chronic HBV patients with LAM or ADV viral breakthrough (5-9). Recent studies have indicated that rtA181T/sW172* mutant has a dominant negative secretion effect as well as an increased oncogenic potential through its transactivation activity (4,10-13). The other potential mechanism that can be speculated is these truncated protein cause ER stress and cell damage by their intracellular retention (12,14). Accumulation of unfolded or misfolded proteins in the ER (i.e. ER stress) induces the unfolded protein response (UPR) (15,16), which in hepatocytes may lead to HCC (17,18). But so far, this speculation is still inconclusive.

In this study, We generated a mouse model of HBV replication with hydrodynamic injection method and attempted to investigate the effect of HBV rtA181T/sW172* point mutation on HBV
surface protein expression, secretion and hepatocarcinogenesis in vitro and vivo. As a result, we confirmed that in vitro and vivo, the HBV rtA181T/sW172* truncate proteins present a weak capacity of surface protein expression and secretion, which can lead to the surface protein’s accumulation in the ER lumen of hepatocellular carcinoma cell lines, and therefore lead to the constitutively overexpression of glucose-regulated protein (grp) 78 gene, a ER stress signaling pathways related gene that can be induced by physiological and environmental stress conditions (16,19). Our results provide a novel potential mechanism which implied ER stress may participated in the oncogenic potential of HBV rtA181T/sW172* truncated variant related hepatocarcinogenesis.

MATERIALS AND METHODS

HBV expression vectors constructions:

The pwt-HBV plasmid was constructed based on the C type of the HBV genome (GenBank: AB644286). It was cleaved with Pst I, then blunt-ended and digested with EcoRI, the resulting product was a 1.2-genome-length HBV DNA. The plasmid vector pZac2.1 vector (a kind gift from Dr Gao, UPenn, USA) was cleaved with BglII, then blunt-ended and digested with EcoRI, then the 1.2-genome-length HBV DNA was cloned into pZac2.1 vector, pZac-HBV-wt was generated.

To express truncate mutant HBV surface proteins, pZac-HBV-172*w were generated. The mutagenesis was carried out through PCR-mediated site-directed mutagenesis. PCR was performed using pZac-HBV-wt as the template, The primers which contained the target mutation site were F1(HBV12F) and R1(HBV688R) (Table 1).The PCR product was inserted into EZ-T vector (GenStar Biosolutions Co.Ltd, Beijing, China) is named pHBV-St-T. The XhoI-SpeI fragment from pHBV-St-T was cloned into the XhoI and SpeI which are also sites in pZac-HBV-wt. The final plasmids were named pZac-HBV-172* which carried the rtA181T/sW172* truncate mutation.

To express wildtype S proteins only, pCDNA3.1-S-wt was also constructed. PCR was performed using pZac-HBV-wt as template. The fragment was used to derive PCR primers (Table1.F2, R2and F2, R3), incorporating BamHI and AgeI restriction sites. Then the fragment was cloned into the BamHI and AgeI digested plasmid pCDNA3.1V5HisTOPO (Invitrogen, USA). The final plasmids pCDNA3.1-S-wt was generated. All constructs were confirmed by sequencing.
Cell cultures and transfections:
HepG2 cells and HuH-7 cells were grown in Dulbecco’s modified Eagle medium (Hyclone, Thermo Fisher Scientific Inc., USA) supplemented with 10% fetal bovine serum (GIBCO, Invitrogen, USA) at 37°C in 5% CO2. Cells were seeded at 70% confluence in six-well cell culture plates. Transient transfection of the plasmids into HepG2 cells and HuH-7 cells were performed using Neofectin™ DNA In Vitro Transfection Reagent (Mid-Atlantic BioLabs, West Bethesda, MD) according to the manufacturer’s protocol. Co-transfections with green fluorescent protein reporter plasmid were performed to normalize transfection efficiency. All experiments were performed in triplicate.

A mouse model of hydrodynamic injection with HBV wt and HBV rtA181T/sW172*:
In order to establish the mouse model for HBV replication, a total of 16 μg of pZac-HBV-wt or pZac-HBV-172* expression plasmids was injected individually into the tail vein of 6 week-old C57BL/6 male mice in a volume of saline equivalent to 9% of the body mass of the mouse (e.g., 1.8 ml for mouse of 20 g). The total volume was delivered within 5–8 seconds. Each injection group contained 5 mice. Mice peripheral blood were collected before injection, 3 days and 7 days after injection, besides, mice liver tissues were also separated for the subsequent detection. All mice using for this experiment were purchased from the Department of Laboratory Animal Science at Peking University Health Science Center. All mice received humane care under the Institutional Review Board in accordance with Animal Protection Art of Peking University.

ELISA assays for HBsAg and HBeAg detection:
For intracellular proteins, cells were lysed in 50 mmol/L Tris-HCl [pH 8], 1 mmol/L ethylenediaminetetraacetic acid [EDTA], 1% NP-40, and PMSF protease inhibitor (AMRESCO, USA). Mice liver tissues were pestled and lysed using reagents mentioned above to get hepatic proteins. For extracellular proteins, cell culture supernatants and mice serum were used directly. The HBsAg and HBeAg were quantified using commercially available microparticle enzyme immunoassay kits (AxSYM, Abbott Laboratories, IL, USA).

Immunofluorescence assays:
Immunostaining was performed to detect the subcellular localization of HBV surface proteins in pZac-HBV-wt and pZac-HBV-172* transfected HuH-7 cells. 48 hours after transfection, cells were incubated with prewarmed 1μM ER tracker Red DPX (Invitrogen, USA) for approximately
15 minutes at 37°C, then fixed with 4% formaldehyde for 2 minutes at 37°C. After that, cells were washed twice and blocked in 3% BSA PBS for 2h at 37°C. Cells were first incubated for 1 hour at 37°C with the goat polyclonal anti-HBs antibody (Keyuezhongkai Biotech Co., Ltd, Beijing, China, 1:250 dilution), and second for 1 hour with FITC-conjugated rabbit anti-goat antibody (Invitrogen, USA, 1:100 dilution). To visualize the nuclei, cells were stained with DAPI (200 ng/mL). Confocal microscopy was performed using Leica TCS SP2 Laser Scanning Spectral Confocal System. If the cells showed apparent HBV surface proteins overlapped with ER staining, we called it “aggregated cell”, 100 cells were counted and then the percentage of the aggregated cells were calculated.

**RNA extraction and Real time RT-PCR:**

Total RNA was extracted from HepG2 cells transfected with pZac-HBV-wt, pZac-HBV-172*, or pZac-Basic (mock controls) separately for 48 hours by using Trizol reagent (Invitrogen, USA) according to the manufacturer’s protocol. And cDNA was reverse transcribed from 3.0 μg of total RNA with random hexamer primers using an Maxima® First Strand cDNA Synthesis Kit (Fermentas, MBI) as recommended by the supplier. qRT-PCR was performed on the LightCycler® 480II real-time PCR system (Roche, USA) using the QuantiTect SYBR Green PCR kit (Roche, USA). The 20 μl reaction mix contained 200 nM of each primer, 100 μl of LightCycler 480 SYBR green I master mix (Roche), and 1 μl of template cDNA. The primers for the grp78 gene were F ( 5'- gctgtatattcagcc-3' ) and R ( 5'-ttcatcttgccagcc-3' ), and those used for glyceraldehyde-3-phosphate dehydrogenase (gapdh) gene were F ( 5'- agaaggctggggctcatttg-3' ) and R ( 5'-agggccatccagctc-3' ). For the gene grp78 and gapdh, the amplifications consisted of 5 min at 95°C followed by 40 cycles, each consisting of denaturing for 30 s at 95°C, annealing for 30 s at 60°C, and elongation for 30 s at 72°C. All PCR were performed in triplicate using cDNA synthesized from the same batch and starting amount of total RNA. Negative controls containing no cDNA template were included for each gene within each PCR run. Amplification specificity for each gene was confirmed by a single distinct melting curve. PCR products were separated using 1.5% agarose gel electrophoresis to confirm the presence of a single band at the expected amplification size.

**Data analysis:**

All statistical analysis was performed using GraphPad Prism V5.0 software (GraphPad Software
Inc., San Diego, CA). Data were expressed as mean±SD. Comparisons between groups were performed using Mann–Whitney U-test. *P*-values<0.05 were considered statistically significant.

RESULTS

The effect of HBV rtA181T/sW172* mutant on HBsAg expression and secretion:

Culture supernatants and cell lysates from 24 hour, 36 hour, 48 hour and 72 hour post-transfected HepG2 cells were collected to detect the expression levels of HBsAg for extracellular and intracellular proteins. The target region for HBsAg detection locates in the N terminal of the surface gene ensures that the assay is not impacted by this sW172* drug-resistant mutant. In each time point, cells transfected with HBV wt experienced dramatically high levels expression of HBsAg compared to HBV rtA181T/sW172* mutant in supernants, the HBsAg expression of two plasmids co-transfected in equal amounts was between HBV wt or HBV rtA181T/sW172* transfected individually (Fig.1A, 24h: *P*<0.0001; 36h: *P*=0.0004; 48h: *P*<0.0001; 72h: *P*=0.0001). By contrast, the intracellular HBsAg levels of HBV rtA181T/sW172* was higher than HBV wt transfected cells (Fig.1B, 24h: *P*=0.0132; 36h: *P*=0.004; 48h: *P*=0.0003; 72h: *P*<0.0001). In addition, we combined the intracellular and extracellular HBsAg together to evaluate the total HBsAg expression levels in different transfection groups, we found that HBV wt expressed dramatically high levels of total HBsAg compared with HBV rtA181T/sW172* (Fig.1C, 24h: *P*=0.0002; 36h: *P*<0.0001; 48h: *P*<0.0001; 72h: *P*=0.0002). Finally, we analyzed the distribution profiles of extracellular and intracellular HBsAg between HBV wt and HBV rtA181T/sW172*. In HBV wt group, extracellular HBsAg took mainly part (nearly 75%~95%)in the total HBsAg secretion. On the contrary, for HBV rtA181T/sW172* mutant, intracellular HBsAg contributed most (nearly 80%~94%) of HBsAg expression (Fig.1D). Taken together, HBV rtA181T/sW172* mutant has a defect in HBsAg expression and secretion which could lead to HBV surface proteins intracellular accumulation. Additionally, this defect was much more obvious as time went by (Fig.1A, B, C, D).

Since clinically HBV wt and HBV rtA181T/sW172* exist as a mixture in drug-resistant HBV chronic infected patients, we also detected the amount of HBsAg in mix population of HBV wt and HBV rtA181T/sW172*. HepG2 cells were cotransfected with pZac-HBV-wt and pZac-HBV-172* in different ratios but with total equal amounts (Fig.2). In supernatants, cells transfected with pZac-HBV-wt alone harvested the maximal HBsAg secretion in supernatant, whereas there was little HBsAg secreted from pZac-HBV-172* transfected alone. Along with the
increasing amounts of HBV rtA181T/sW172* and decreased amounts of HBV wt, the level of HBsAg remarkably decreased. When two plasmids coexisted in equal amounts, HBsAg expression level was lower than the simple sum of HBV wt and HBV rtA181T/sW172* transfected alone in the same transfection amounts (Fig.2A). Results indicated that HBV rtA181T/sW172* mutant has a dominant negative secretion effect. This showed a coincidence with previous reports (4,10). Reverse trend was observed in intracellular cell lysates (Fig.2A), which once again proved HBV rtA181T/sW172* mutant’s defect in HBsAg secretion and subsequently intracellular accumulation.

In the next, we also detected the HBeAg expression levels in the supernatants and cell lysates in HepG2 cells were cotransfected with pZac-HBV-wt and pZac-HBV-172* in different ratios. Different from HBsAg, both intracellular and extracellular HBeAg in different transfection ratios groups showed similarly expression levels, but intracellular HBeAg was far less than the supernatants HBeAg (Fig.2B). This result also verified that the significant HBsAg expression and secretion differences were not due to the transfected efficiency differences between pZac-HBV-wt and pZac-HBV-172*, in other words, our results truly reflected the different HBsAg secretion capabilities between HBV wt and HBV rtA181T/sW172*.

HBsAg and HBeAg expression in serum and intra-hepatocyte of mice injected with HBV wt and HBV rtA181T/sW172*:

To examine whether the HBV sW172* HBsAg secretion defect existed in vivo. We generated a mouse model by injecting pZac-HBV-wt and pZac-HBV-172* plasmids into C57BL/6 mice with hydrodynamic methods which can directly lead to the specific expression of target proteins in liver. We tested the HBsAg and HBeAg secretion and expression in mice serum and hepatocyte 0 days, 3 days and 7 days after injection. Before injection, the background of HBsAg in the tested mice serum is quite low. 3 days after injection, the HBsAg and HBeAg reached into the peak (Fig.3A, B). HBV wt infected mice showed very obviously high HBsAg in serum compared with HBV rtA181T/sW172* injected group (HBV wt: 1746 IU/ml Vs HBV sW172*: 0.69 IU/ml; \( P=0.0379 \)) (Fig.3A). 7 days after injection, although the HBsAg and HBeAg levels were decreased, but serum HBsAg expression of HBV wt was still significantly higher than HBV rtA181T/sW172* mice.
The HBV wt infected mice HBsAg secretion was 264 IU/ml, and HBV rtA181T/sW172* injected mice HBsAg expression was in the low limit of detection (Fig.3A). The HBeAg secretion levels were highest 3 days-post injection and down-regulated 7 days-post injection (Fig.3C). Both HBV wt and HBV rtA181T/sW172* injected mice HBeAg secretions were not obviously different, HBV rtA181T/sW172* injected mice showed slightly higher HBeAg than HBV wt injected mice (Fig.3C). 7 days after injection, mice liver tissues were separated, grinded and lysed to obtain intrahepatic proteins. HBV rtA181T/sW172* injected mice showed significant higher levels of HBsAg than HBV wt injected mice (HBV wt: 56 IU/ml Vs HBV sW172*: 332 IU/ml) (Fig.3B). Meanwhile, HBV rtA181T/sW172* injected mice showed a slightly higher levels of HBeAg than HBV wt injected mice (Fig.3D). The HBeAg expression profiles indicated indicated there were no difference between pZac-HBV-wt and pZac-HBV-172* plasmids expressed efficiency in vivo.

Combined with the above results, in vivo experiment provided further proof that HBV rtA181T/sW172* mutant relative to the HBV wt existed defects in HBsAg secretion but not HBeAg secretion, thus resulted in a large number of truncated surface proteins retained in hepatocytes.

**S wild type protein alone can rescue HBV rtA181T/sW172* mutant’ defect in HBsAg secretion:**

Since this drug-resistant point mutation which overlaps polymerase and envelope genes can lead to the disorder in surface proteins secretion, we investigated whether S wild protein could rescue this dysfunction. All transfection groups have the equal amounts of full-length HBV rtA181T/sW172*, as the S wild protein increased, the HBsAg secretion level significantly increased (Fig.4A). When HBV rtA181T/sW172* and S wild protein were in equal amounts, the HBsAg expression was almost 290-fold relative to HBV rtA181T/sW172* alone (Fig.4A). The rescue effect was not simply due to the increased amounts of S wild protein transfected into the cells, for the reason that the amounts of HBsAg in the HBV rtA181T/sW172* and pcDNA3.1-S-wt cotransfection with the ratio 1:1 group (17.68 IU) was much more higher than HBV rtA181T/sW172* (0.06 IU) or pcDNA3.1-S-wt (4.94 IU) transfected individually (Fig.4A). Meanwhile, same HBsAg expression trend was also observed in the intracellular cell lysate. So we can get the conclusion that S wild protein alone also could help to increase the HBsAg expression level both intracellular and extracellular cells (Fig.4B). As is expected, HBeAg levels both in
supernatants and cell lysates did not show any significant difference among these groups. In other words, S wild protein alone could not effect HBeAg secretion and expression of HBV rtA181T/sW172* (Fig.4C, D).

**Accumulation of intracellular surface protein may lead to ER stress signal activation:**

Because of the intracellular retention of surface protein, we studied the subcellular localization of wt and truncate surface proteins to see whether the truncate surface proteins accumulated in the endoplasmic reticulum. The HuH-7 cells were transfected transiently with the pZac-HBV wt and pZac-HBV-172*, labeled by ER tracker and stained for HBsAg by immunofluorescence at 48 hour post-transfection and visualized by confocal microscopy. Cells transfected with the HBV wt showed the diffused cytoplasmic staining pattern for the presence of HBV surface proteins which localized to the endoplasmic reticulum (Fig.5A). However, cells transfected with the HBV rtA181T/sW172* mutant plasmid showed an aggregated cytoplasmic pattern of HBV surface proteins which also localized to the endoplasmic reticulum (Fig.5A). We also quantified the aggregated cells in HBV wt or HBV rtA181T/sW172* transfection groups, HBV rtA181T/sW172* transfected cells showed much more aggregated cells than HBV wt transfected cells ($P<0.0001$) (Fig.5B). Results suggested an endoplasmic reticulum accumulation of envelope proteins for HBV rtA181T/sW172* truncated mutation as compared with HBV wt.

Presumably, the accumulation of truncated envelope proteins in endoplasmic reticulum may lead to the ER stress signal activation. In order to evaluate the transcription and translation of ER stress genes in cells transfected with HBV wt or HBV rtA181T/sW172* plasmids, we analyzed the expression ofgrp78 gene which is typical markers of ER stress. The analysis by real time RT-PCR indicated that grp78 mRNA was found to be induced 1.5-fold ($P=0.0154$) in cells 72 hour post-transfected with HBV rtA181T/sW172* cells relative to HBV wt transfected cells (Fig. 5C). Above studies may provide suggestion of the oncogenic potential of HBV rtA181T/sW172* surface protein truncations via ER stress and induction of the UPR.

**DISCUSSION**

The clinical prolonged usage of nucleos(t)ide analogs (NAs) for the long-term treatment of chronic hepatitis B (CHB) patients arise with the emergence of drug-resistant HBV mutants which can lead to the treatment failure and progression to liver disease. The rtA181T/sW172* multi-drug resistant mutant selected by the several NAs has raised much more concerns for its decreased
susceptibility to LMV, LdT, ADV and TDF (8,10,20). Its dominant negative effect on wild-type HBV virion secretion and oncogenic potential through transactivation activities were also reported (10-12). Here, in our study, we used ELISA quantitatively detective method to confirm the secretion defect of this truncated mutant and its dominant negative effect on wild type surface proteins secretion in mix population of wild and mutant HBV. In addition, S wild protein alone can rescue this secretion and expression defect. However, we can speculate S wt play a major role in rescuing the secretion of HBV rtA181T/sW172* because of the remarkable increase of extracellular HBsAg showed in Fig. 4A. As we know, all of the information required to produce an HBsAg particle resides within the S protein. Like typical membrane proteins, the HBV envelope proteins are synthesized at the endoplasmic reticulum (ER). The C-terminal hydrophobic 57 amino acid of S are believed to be embedded in the ER membrane. Foreign domains fused to the C terminus of S are oriented towards the ER lumen (21). Deletion of the C-terminal hydrophobic domain results in a stable, glycosylated, but nonsecreted chain. However, when coexpressed with wild-type S protein this mutant polypeptide can be incorporated into particles and secreted, indicating that the chain is still competent for some of the distal steps in particle assembly (22). The truncated mutant due to the stop codon in the 172 site of surface gene can lead to loss the functional C-terminal hydrophobic peptide, thus result in not only the secretion of subviral particles but also the envelopment of mature virion. In natural condition, subviral particles can be found highly over expressed relative to virions and reach a 10000-fold higher concentration in serum. Subviral particles and virions carry identical surface antigens (HBsAg), although the protein composition is not identical. It is assumed that the massive HBsAg overproduction serve as a mechanism of evading the host immune system that is advantageous for the virus (23). In a certain sense, this mutant ‘s disability of producing virion particles help HBV itself surviving and escaping control by the immune system.

We also confirmed the abrogated HBsAg serum secretion and increased HBsAg rentation in liver but no change of HBeAg profiles in the animal model injected with HBV rtA181T/sW172* mutant strain, these data were coincident with other group’s study (24). Combined with the results in vivo and in vitro, we futherly proved that HBV rtA181T/sW172* mutant relative to the HBV wt existed defects in HBsAg secretion but not HBeAg secretion, thus resulted in a large number of viral particles retained in hepatocytes.
Whereas, the sW172* mutant also shows dysfunction of subviral particles release leading to consequence of abundant surface proteins retention intracellularly. Since the HBV envelope protein synthesis and secretion process in the ER, most truncated mutant proteins accumulate in the ER lumen, this can trigger the unfolded protein response (UPR) owing to the overloading of the ER with virus-encoded proteins, which could represent one of the ancient evolutionary pressures for linking ER stress to cell suicide in avoiding the replication and spread of virus (25,26). In our study, HBV sW172* truncated mutant proteins was found to display more aggregate ER lumen distribution in comparison with HBV wild proteins, and we also obtain the results of high expression levels of molecular chaperones GRP78 (also known as Bip) in cells 72 hour post-transfected with sW172* cells compared to wt HBV transfected cells. GRP 78 plays a role in many functions of the ER, including assisting protein folding, targeting misfolded proteins for proteasomal degradation, serving as a sensor for ER stress, and contributing to ER calcium stores (25,26). Taken together, our results reflect the intracellular accumulation of truncated protein may lead to the activation of ER stress signaling pathway. Previous reports have indicated that pre-S mutant HBsAg accumulate in ER, resulting in strong ER stress, oxidative DNA damage and genomic instability, combined effects could potentially lead to hepatocarcinogenesis over the decades of chronic HBV infection. The presence of pre-S mutants in sera was reported to carry a high risk of developing hepatocellular carcinoma (HCC) (27,28). Meanwhile, the oncogenic potential of HBV sW172* was also confirmed through its ability to transactivate the c-myc and SV40 promoters (12,29). So far, there is no report about this truncated mutant ‘s oncogenic potential for initiating ER stress and oxidative damage. It can be concluded that HBV sW172* truncated mutant not only can influence the envelopment of HBV nucleocapsid and virion secretion but also may cause cell stress and damage by their intracellular retention. ER stress induces the unfolded protein response (UPR), which in hepatocytes can lead to hepatocellular carcinoma.

In conclusion, the prolonged usage of NA s for CHB therapies could significantly suppress the viral genome replication, but it also could select for multi-drug resistant mutants that may contribute to the worsen of liver disease even hepatocellular carcinoma through inducing activation of ER stress signaling pathways. For this reason, the CHB patients should take careful consideration before the NAs usage. Moreover, the tendency for developing new anti-viral drugs
should target other steps of HBV lifecycle which could not only promote virologic clearance but also prevent the emergence of oncogenic potential drug resistant mutants.

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Conflict of interest None to declare.

REFERENCES
FIGURE LEGENDS

Figure 1. HBV rtA181T/sW172* mutant has a defect in HBsAg expression and secretion. The HBsAg expression levels in HepG2 cells culture supernatants and cell lysates of HBV wt and HBV 172* 24 hour, 36 hour, 48 hour and 72 hour post-transfection were detected by ELISA. (A) The HBsAg expression levels in culture supernatants (B) The HBsAg expression levels in cell lysates. (C) The total HBsAg expression levels (Supernatant+Intracellular). (D) The ratio of HBsAg distribution in supernatants and cell lysates (Supernatant+Intracellular=100%). Results are shown as the means±SD of three independent experiments (**P <0.01, *P <0.05).

Figure 2. HBV rtA181T/sW172* mutant has a dominant negative effect on the HBsAg expression of HBV wt. HepG2 cells were cotransfected with pZac-HBV-wt and pZac-HBV-172* in different ratio. The HBsAg and HBeAg expression levels in HepG2 cells culture supernatants and cell lysates from 48 hour post-transfection were detected by ELISA. (A) The HBsAg expression levels in culture supernatants and cell lysates (B) The HBeAg expression levels in culture supernatants and cell lysates. Results are shown as the means±SD of three independent experiments.

Figure 3. HBsAg and HBeAg secretion in mice serum and hepatocyte. The HBsAg and HBeAg expression levels of peripheral blood and liver tissue proteins from C57BL/6 mice 0 day, 3 days, 7 days after hydrodynamic injection of HBV wt and HBV sW172* were detected by ELISA. (A) The HBsAg expression levels in mice serum. (B) The HBsAg expression levels in hepatocyte. (C) The HBeAg expression levels in mice serum. (D) The HBeAg expression levels in hepatocyte. Results are shown as the means±SD of three independent experiments (*P<0.05).

Figure 4. S wild protein could assist HBV rtA181T/sW172* mutant to increase the HBsAg secretion. All transfection groups have the equal amounts of full-length HBVsW172*, the amounts of plasmid pcDNA3.1-S-wt which could only express the S wild protein were gradually increased. The HBsAg expression levels which only transfected pcDNA3.1-S-wt or pZac-HBV172* individually were also analyzed. 48 hour-post transfection, culture supernatants and cell lysates were prepared for the detection of HBsAg and HBeAg by ELISA. (A) HBsAg expression in the supernatants. (B) HBsAg expression in the intracellular cell lysates. (C) HBeAg
expression in the supernatants. (D) HBeAg expression in the intracellular cell lysates. Results are shown as the means±SD of three independent experiments.

**Figure 5. HBV rtA181T/sW172* induces the ER stress compared with HBV surface proteins.**

(A) Immunofluorescence analysis was performed in the HuH-7 cells transfected with HBV wt, HBV sW172*. Different cytoplasmic staining pattern were shown in pictures. ER tracker Red DPX (red) were used as markers for endoplasmic reticulum. Cells transfected with HBV wt and HBV sW172* mutant were stained with goat anti-HBS. Anti-goat antibody conjugated with FITC (green) and ER tracker red DPX (red) were then simultaneously incubated with the cells. Overlay, the merged images of HBS and ER tracker staining. (B) Statistical aggregated cells percentages in HBV wt and HBV172* groups visualized under the microscope vision. (C) Total RNA from cells transfected with pZac-HBV wt and pZac-HBV172* after 36 hour, 48 hour and 72 hour were used to assess grp78 mRNA level using real time RT-PCR. Levels were normalized to cellular gapdh mRNA abundance and concentrations from Huh7 cells transfected with pZac-Basic were set as the basis for the comparative results. Results are shown as the means±SD of three independent experiments (***P<0.001, *P<0.05).
### Table 1. Oligonucleotide primers used in the study

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<th>Primer</th>
<th>Sequence (5'-3')</th>
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<td>ATTCCACCAAGCTCTGCTAGACCCCA</td>
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<td>TGGCACTAGTAAACTGAGTCAGGAG</td>
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(A) DAPI  HBS  ER tracker  Overlay

HBV wt

HBV 172*  

(B)

(C)

Aggregated cells percentage

Relative gp78 mRNA

Vector control  HBV wt  HBV 172*

HBV wt  HBV 172*

36h  48h  72h

** ** ***