ORIGINAL PAPER

An antiviral drug-resistant mutant of hepatitis B virus with high replication capacity in association with a large in-frame deletion in the preS1 region of viral surface gene

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Abstract

We amplified a full-length hepatitis B virus (HBV) genome from the serum of a chronic hepatitis B patient who experienced virological breakthrough with high HBV DNA titer following adefovir (ADV) therapy. The PCR product was cloned and sequencing of the six clones revealed an isolate of C2 subgenotype. Mutation(s) in the polymerase gene responsible for ADV resistance included rtA181T (all clones) and rtN236T (four clones). The rtA181T mutation caused the W172* nonsense mutation in the overlapping S gene. In addition, all the clones harbored another nonsense mutation in the S gene (C69*) and a 207nt in-frame deletion in the preS1 region. These clones were converted to a 1.1mer construct for transient transfection of Huh7 cells. All the clones were deficient in hepatitis B surface antigen production. Three clones had similar levels of DNA replication. Comparison with a wild-type clone of the same genotype revealed a higher intracellular level of replicative DNA for clone c4, which was reduced by putting back the deleted 207nt, but not by co-transfection with an expression construct for the three surface proteins to rescue virion production. The HBcAg expression of the c4 and c4+207nt clones was mainly in the nucleus. Co-transfection with the L/M/S proteins expression construct did not alter the distribution of core. Clone c4 showed a significantly decreased susceptibility to ADV, a mild reduction in susceptibility to lamivudine and tenofovir, but remained sensitive to entecavir. In conclusion, this is an unusual ADV-resistant HBV isolate harboring two nonsense mutations in the S gene and a large in-frame deletion in the preS1 region, but still retains a high replication phenotype, which can provide a platform for recombinant vector construction.

Abbreviations

ADV	Adefovir
CHB	Chronic hepatitis B
CMV	Cytomegalovirus
DMEM	Dulbecco's modified Eagle medium
ELISA	Enzyme-linked immunosorbent assay
ETV	Entecavir
HBeAg	Hepatitis B e antigen
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
LAM	Lamivudine
L, M, S	Large, middle, and small surface proteins
ORF	Open reading frame
P	Polymerase
PCR	Polymerase chain reaction
pgRNA	Pregenomic RNA
RT	Reverse transcriptase
TDF	Tenofovir
WT	Wild type
WTB	Wild-type clone of subgenotype B2
WTC	Wild-type clone of subgenotype C2

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Introduction

Over 257 million people worldwide are chronically infected by hepatitis B virus (HBV) and are at risk of liver cirrhosis and hepatocellular carcinoma (HCC) [1, 2]. High level of HBV DNA is a dominant risk factor associated with increased rates of cirrhosis and HCC [3]. HBV has a highly compact genome containing four overlapping open reading frames (ORFs): preC/C, polymerase (P), preS/S, and X. Currently, the principal treatment for chronic hepatitis B (CHB) involves nucleoside/nucleotide analogs. In this regard, a critical step in the HBV replication cycle involves the reverse transcription of pregenomic (pg) RNA into minus-stranded DNA, which is catalyzed by P protein through its reverse transcriptase (RT) domain. The nucleos(t)ide analogs can be incorporated into the growing minus strand, but prevent further chain elongation. However, mutations in the RT domain of the P protein can cause drug resistance [4–6], which is associated with a poor long-term prognosis [7]. Lamivudine (LAM) and adefovir (ADV) were the first approved nucleos(t)ide analogs for treatment of chronic HBV infection in China. They were only recently replaced by more effective drugs such as entecavir (ETV) and tenofovir (TDF).

The P gene is overlapped by the S region, which can be subdivided into preS1 (119 codons for genotype C), preS2 (55 codons), and S gene (226 codons), all starting with an ATG codon. Consequently, alternative translation initiation generates co-terminal large (L), middle (M), and small (S) HBs proteins, with M protein having an extra preS2 domain at the amino terminus of S protein and L protein having an extra preS1 domain. Naturally occurring large in-frame deletions in the preS gene frequently arise during chronic HBV infection, especially at the later stage [8, 9]. In the present study, we cloned full-length HBV genomes from a patient who did not respond anymore to ADV therapy. DNA sequencing revealed mutations predicted to confer ADV resistance, in addition to a large in-frame deletion in the preS1 region and two nonsense mutations in the S gene. The full-length HBV genomes were subcloned to PHY106 vector to characterize the biological properties of this drug-resistant HBV isolate by transient transfection experiments.

Materials and methods

Patient and clinical data

A 43-year-old male was diagnosed as having chronic active hepatitis B according to the Chinese guideline for prevention and treatment of CHB published in 2005. Laboratory tests highlighted the following: HBsAg+, HBeAg+, anti-HBe−, HBV DNA 8.62×10^5 IU/mL, AST 267 U/L, ALT 325 U/L, and bilirubin 13 μmol/L. The patient started LAM 100 mg per day as single dose in 2005. After a 3-month therapy, HBV replication decreased (HBV DNA $< 10^3$ IU/mL), with ALT (45 U/L) and AST (33 U/L) returned to normal. However, an HBV flare (HBV DNA 4.07×10^5 IU/mL) occurred two years after LAM monotherapy. The patient was switched to ADV (10 mg per day) monotherapy in 2007, but virological breakthrough emerged 9 months later with HBV DNA 6.37×10^5 IU/mL. Then the serum sample was collected and stored at −20 °C until use. Signed informed consent for the use of serum sample for scientific research was obtained from the patient. ETV 0.5 mg per day overlapping therapy was adopted, and the patient obtained complete and sustained virological response.

Amplification, sequencing, and cloning of full-length HBV genomes from the serum sample

Full-length HBV genome was amplified from the serum sample by PCR according to the method of Gunther et al., using phusion DNA polymerase (Invitrogen). The primers used were sense: CCGGAAAGCTTGAGCTCTTCTTTTTCACCTCTGCCTAATCA (nt 1806—1825) and anti-sense: CCGGAAAGCTTGAGCTCTTCAAAAAGTTGCATGGTGCTGG (nt 1821–1841), both with SacI and SapI sites. The PCR product was cloned to pUC19 vector via the SacI restriction site. Six clones were sequenced using previously described primers and method [10]. To identify the dominant HBV species from the serum sample, direct sequencing of the PCR product was carried out (GenBank accession number FJ518810, subgenotype C2).

Replication constructs harboring 1.1 copies of the HBV genome and site-directed mutant

The six PCR clones were digested with SapI, and the full-length HBV genome was subcloned via SapI site to the pHY106 vector downstream of the cytomegalovirus (CMV) promoter. The 1.1 copies of the HBV genome thus generated could transcribe pgRNA under the CMV promoter to ensure efficient genome replication [11]. For comparison, wild-type HBV genomes of subgenotype B2 (GenBank accession number AY220698) and subgenotype C2 (GenBank accession number AF411411) previously cloned in our laboratory were subcloned to pHY106 to create WTB and WTC [12, 13]. Overlap extension PCR was used to put back the 207nt deletion from a wild-type isolate (subgenotype C2) in the preS1 region of clone c4, thus generating c4+207nt.

For transfection, plasmid DNA of the 1.1mer construct was purified by Qiagen Midi kit.

Cell culture, transfection, and detection of HBV genome replication

Huh7 cells were seeded at 5×10^5 cells/well in six-well plates. Transfection was performed at 80–90% cell confluence using lipofectamine 2000 (Sigma). Cells were harvested 72 h after transfection. Southern blot analysis of intracellular capsid-associated DNA was performed as described [14]. Briefly, core particles were precipitated from cell lysate by polyethylene glycol, followed by nuclease digestion, proteinase K digestion, and DNA extraction. DNA was separated in agarose gels and transferred to nylon membrane. The nearly full-length probe DNA was obtained from a cloned genome of HBV by nested PCR amplification to eliminate the vector sequence and labeled with [32P] dCTP by random priming. The blots were washed at 62 °C with $2 \times$ SSC—0.1% SDS solution.

Trans-complementation to rescue virion secretion

The 0.7mer expression construct for L, M, and S surface proteins has been described [15]. 1 μg of the 1.1mer construct of c4, c4+207nt, and WTC was transfected alone or together with increasing concentrations of the 0.7mer L/M/S construct using Lipofectamine 3000 reagent (Invitrogen) into Huh7 cells. The total amount of DNA transfected was brought to 1.9 μg/well using pcDNA3.1zeo(-). Western blot analysis was performed as described previously [16]. HBcAg was detected using 1:1000 dilution of a custom made rabbit antibody against core protein (a kind gift from Dr. Haitao Guo, Indiana University, USA). Surface proteins were detected by a horse polyclonal antibody (anti-ad/ay; Novus). HBsAg secreted to culture supernatant was measured by an enzyme-linked immunosorbent assay (ELISA) kit (KHB, Shanghai, China) with proper dilution to prevent signal saturation. Virions were immunoprecipitated from 1.5 mL of culture supernatant by a rabbit anti-HBs antibody (anti-ad/ay) and an anti-preS1 (GenScript), followed by DNA extraction and Southern blot analysis.

Immunofluorescence microscopy to investigate the subcellular distribution of HBcAg and HBsAg

For immunofluorescence microscopy, 1 μg of the 1.1mer construct of c4, c4+207nt, and WTC was transfected alone or together with 0.3 μg of 0.7mer L/M/S expression construct using Lipofectamine 3000 reagent into Huh7 cells. The total amount of DNA transfected was brought to 1.9 μg/well using pcDNA3.1zeo(-). 2 days post transfection, cells were fixed onto the collagen-coated coverslips using 4%

paraformaldehyde (Merck) in PBS, for 15 min at room temperature. The coverslips were permeabilized with 0.25% Triton x-100 for 10 min. After blocking with 3% bovine serum albumin (BSA, fraction V, Sigma) in PBS for 30 min, cells were exposed to a mixture of a polyclonal rabbit anti-HBc antibody (Dako) at a 1:50 dilution, β-actin antibody (Proteintech) at a 1:100 dilution, and a horse polyclonal anti-HBs antibody at a 1:50 dilution at 4 °C overnight. Coverslips were washed in PBS three times for 5 min at room temperature and incubated with secondary antibodies (Cy3-affinipure goat anti-mouse IgG, H+L, YEASEN; 488-affinipure goat anti-rabbit IgG, H+L, YEASEN) for 1 h at 4 °C. Cells were washed as described above and embedded in 50 mg/mL DABCO/moviol (Sigma Chemical Co. and Hoechst, respectively). Nuclear DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma). Cells were visualized by a laser confocal scanning microscope (Leica TCS SP8).

Drug sensitivity of the clinical HBV isolates

The 1.1mer construct of clone c4, c4+207nt, and WTC were transfected into Huh7 cells. The next day, cells were fed with fresh medium containing different concentrations of LAM, ADV, ETV, or TDF. Seventy-two hour after drug treatment, intracellular HBV DNA was quantified by Southern blotting. The inhibition of HBV replication by LAM, ADV, ETV, and TDF were quantified using Image J software and regression analyses were used to determine the IC50 of each treatment.

Results

PCR, cloning, and sequencing of HBV genomes from a clinical isolate associated with ADV resistance

We amplified the full-length HBV genome from a serum sample of a patient experiencing virological breakthrough against ADV. The serum sample obtained after 9 months of ADV monotherapy was positive for both HBeAg and HBsAg, and had a HBV DNA titer of 6.37×10^5 IU/mL. The PCR product was cloned to pUC19 vector and then subcloned to PHY106 vector to generate the 1.1mer construct. The PCR product and the six PCR clones (c1, c2, c3, c4, c6, and c8) were closely related, with 0–15 nt per genome variability. Phylogenetic analysis revealed their classification into the C2 subgenotype. The PCR product and all the six clones had A1762T/G1764A core promoter mutations. They also harbored a 207nt deletion covering nt 2971–3177, which led to removal of 69 residues (42th–110th) in the preS1 domain of L protein as well as the spacer of P protein (Fig. 1). The G670A (PCR product and all six clones) and A836C
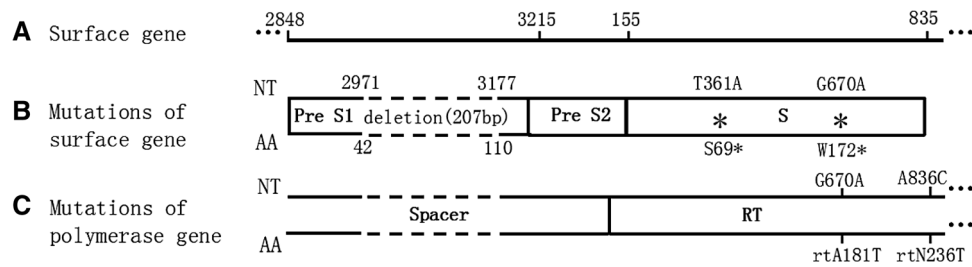
A Surface gene

2848   3215   155   835

B Mutations of surface gene

NT   2971   3177   T361A   G670A
Pre S1 deletion(207bp)   Pre S2   *   S   *
AA   42   110   S69*   W172*

C Mutations of polymerase gene

NT   G670A   A836C
Spacer   RT
AA   rtA181T rtN236T

Fig. 1 Mutations in the surface gene and overlapping P gene in all or most PCR clones from a clinical HBV isolate associated with adefovir resistance. **a** Nucleotide sequence of the surface gene. **b** Mutations in the surface gene at the nucleotide (NT) and amino acid (AA) levels. The dashed line indicates deletion, while stars indicate nonsense mutations. **c** Mutations in the P gene at the NT and AA levels

mutations (found in the PCR product and clones c1, c2, c3, and c4) introduced A181T and N236T mutations in the RT domain of the P protein, respectively, which are responsible for ADV resistance [17]. Due to the overlap of the P gene with the surface gene, the G670A mutation caused the W172* nonsense mutation in the S gene (Fig. 1). Since all the clones harbored an additional nonsense mutation (C69*) further upstream, translation of L, M, and S proteins would all terminate after residue 68 in the S domain. This should abolish HBsAg production and virion formation.

Functional characterization of the PCR clones

The six PCR clones as 1.1mer construct were transiently transfected to Huh7 cells. No HBsAg could be detected from culture supernatant of cells transfected with any clone. Southern blot analysis of cell lysate revealed high replication capacity of clones c1, c3, and c4 and lack of replication for clone c2, c6, and c8 (Fig. 2a). Sequence analysis revealed insertion of a T between nt1956 and 1957 in the core gene for clone c2, although the reason for the lack of replication of clones c6 and c8 remained unclear. Supplementary Table shows mutations in the core and P proteins as well as in the

pregenome encapsidation signal epsilon in clones c6 and c8, which are absent in c1, c3, and c4 clones.

Next, clone c4 was transfected in parallel with wild-type clones of subgenotype B2 (WTB) and subgenotype C2 (WTC). Clone c4 produced more intracellular capsid-associated replication intermediate than the wild-type construct of the same genotype (WTC) or a different genotype (WTB) (Fig. 2b). No virions could be immunoprecipitated from culture supernatant of cells transfected with clone c4 (data not shown).

Role of surface gene mutations in the high replication phenotype of clone C4

The most striking features of the six clones were two nonsense mutations in the S gene and a large in-frame deletion in the preS1 region. Site-directed mutagenesis was used to reinsert the 207nt to clone c4, and parallel transfection experiments in Huh7 cells revealed much reduced intracellular level of replicative HBV DNA for c4+207nt than the original c4 (Fig. 2c). To examine whether nonsense mutations in the S gene increased intracellular level of replicative DNA by blocking virion formation and release, a trans-complementation assay was performed. Clone WTC, c4,
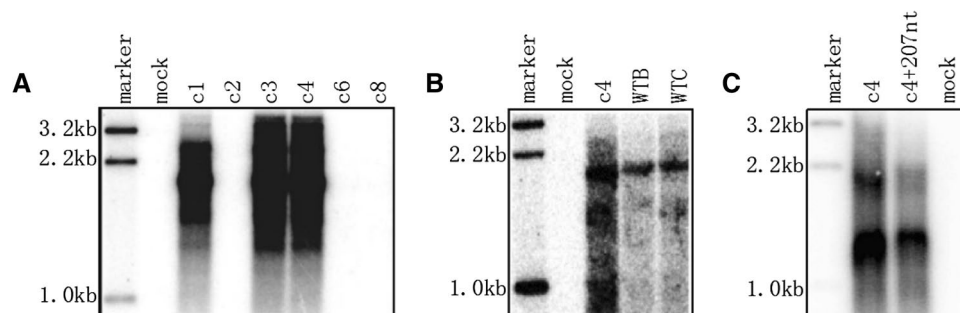

Fig. 2 High replication capacity of PCR clones of a clinical HBV isolate in association with the 207nt deletion in the preS1 region. The six PCR clones and a wild-type clone of subgenotype B2 (WTB) and subgenotype C2 (WTC) were transfected to Huh7 cells as 1.1mer construct. In addition, the deleted 207nt in clone c4 was put back to restore a full-length genome of 3215nt (c4+207nt). Cells were harvested 72 h later for Southern blot analysis of intracellular replicative intermediates. **a** Comparison among the 6 PCR clones. **b** Comparison between clone c4 and WTB as well as WTC. **c** Comparison between original clone c4 and c4+207nt

and c4+207nt were co-transfected with increasing amount of the 0.7mer expression construct for L, M, and S proteins. In contrast to WTC, both c4 and c4+207nt failed to express surface proteins or to secrete HBsAg when transfected alone (Fig. 3d, e). Co-transfecting with 0.7mer construct dose-dependent increased intracellular surface proteins and extracellular HBsAg for all the three clones. Southern blot analysis revealed increased intracellular replicative DNA and extracellular virion DNA for all the clones (WTC, c4, c4+207nt) when co-transfected with 0.1 μg or 0.3 μg of 0.7mer construct (Fig. 3b, c), but further increasing the expression construct to 0.9 μg rather reduced both replicative DNA and virion DNA. A similar trend was observed for intracellular core protein (Fig. 3a). Nevertheless, restoring HBsAg and virion secretion did not alter the higher intracellular level of replicative DNA for clone c4 than WTC or c4+207nt (Fig. 3b).

Differences of subcellular distribution of HBcAg and the impact of coexpression of HBsAg on the distribution of core

In this study, we compared the intracellular expression of HBcAg and HBsAg. In contrast to WTC, the HBcAg expression in c4 and c4+207nt was mainly in the nucleus, while in WTC it was mainly in the cytoplasmic space. Co-transfection with the 0.7mer expression construct for L, M, and S proteins did not alter the high level of HBcAg in nucleus (Fig. 4). Both c4 and c4+207nt failed to express HBsAg. Co-transfection with 0.7mer construct could restore the intracellular HBsAg which was mainly expressed in the cytoplasm similar to WTC (Fig. 5).

In vitro sensitivity of clone C4 to commonly used nucleos(t)ide analogs

WTC was sensitive to all the four nucleos(t)ide analogs (Supplementary Figure). Clone c4 displayed a mild reduction in sensitivity to LAM and TDF (Fig. 6a, d) and significantly decreased sensitivity to ADV (Fig. 6b), but remained sensitive to ETV (Fig. 6c). The calculated IC50 was 3.85 μmol/L for LAM, 29.29 μmol/L for ADV, 0.18 μmol/L for ETV, and 1.09 μmol/L for TDF, respectively. A similar trend was observed in clone c4+207nt (Supplementary Figure). Clone c4 had approximately 25.6-fold reduced susceptibility to ADV, and was slightly resistant to LAM and TDF, with 6.4-fold and 3.4-fold increase in IC50 compared with WTC.

Discussion

The current study focused on a clinical HBV isolate from a patient in whom ADV therapy failed. Sequence analysis of full-length HBV clones from the serum sample revealed an isolate of the C2 subgenotype. The drug-resistant rtA181T and rtN236T mutations were found in all or most clones. The rtA181T mutation was accompanied by W172* nonsense mutation in the S gene. However, another nonsense mutation further upstream, C69*, would truncate all the three surface proteins after residue 68 in the S domain. The same C69* mutation has been found in a Korean HCC patient [18]. All the six clones also harbored an in-frame deletion of 207nt in the preS1 region. Many studies from East Asia indicated that preS deletions are associated with progressive liver diseases [18–21]. The frequency of preS deletions tends to increase
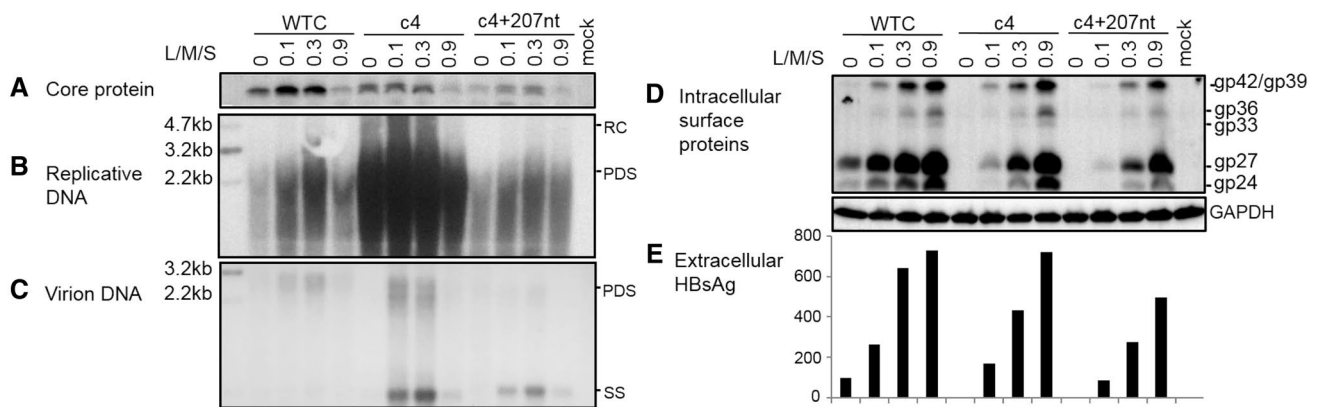

Fig. 3 Supplementing surface proteins to rescue virion secretion failed to abrogate the higher replication phenotype of clone c4 relative to c4+207nt and WTC. The 1.1mer construct (1 μg) of clone c4, c4+207nt, or WTC was co-transfected with indicated amount of the 0.7mer expression construct for surface proteins, and transfected Huh7 cells were harvested 4 days later. a, d Western blot analysis of intracellular core protein (a) and surface proteins (d), with GAPDH serving as a loading control. b, c Southern blot analysis of intracellular replicative DNA (b) and extracellular virion-associated DNA (c). HBV DNA size markers were loaded at 200 pg (b) and 20 pg (c), respectively. e ELISA for extracellular HBsAg, with the value for WTC set at 100%
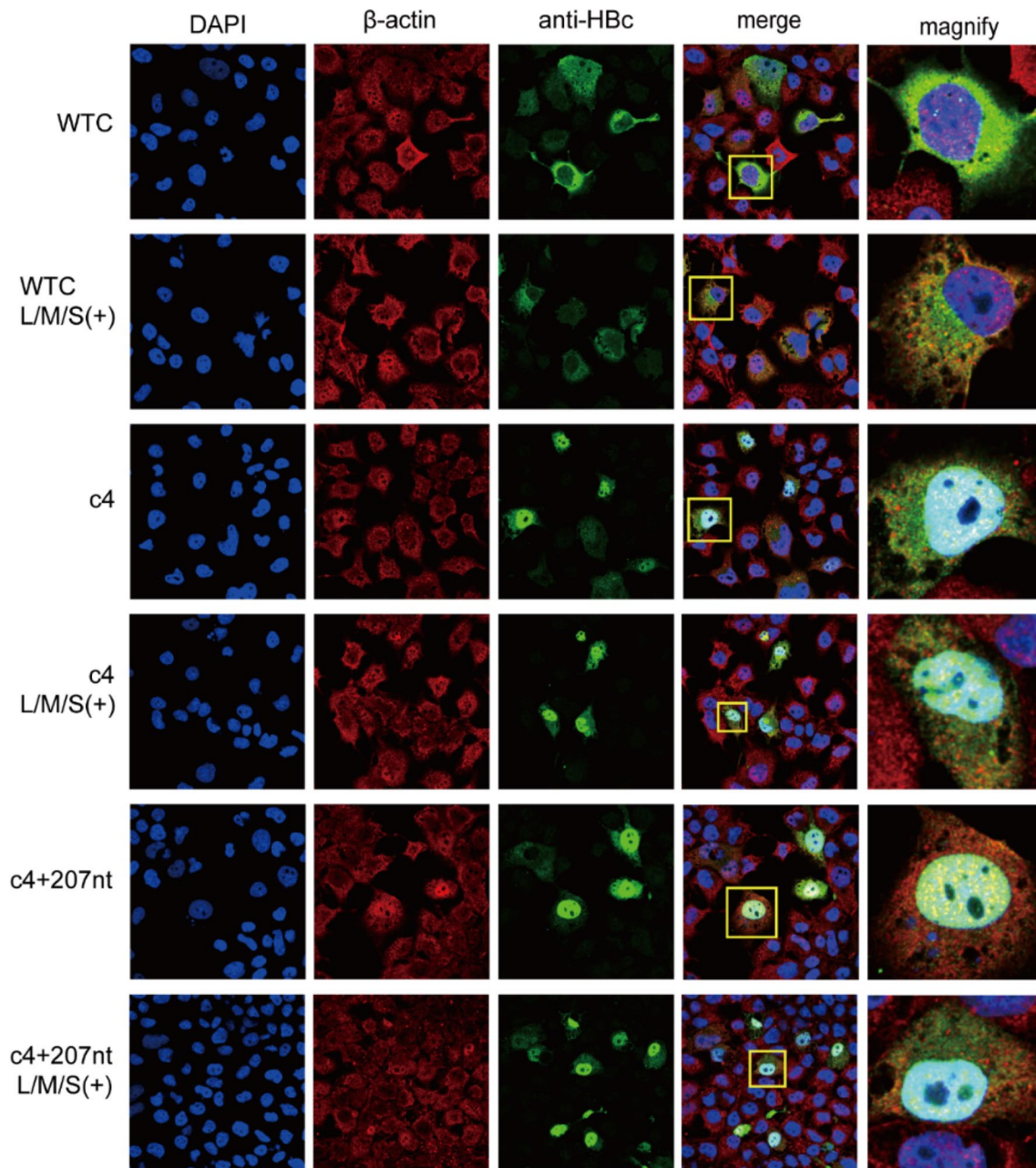
Fig. 4 The nuclear HBcAg expression in the c4 and c4+207nt is much higher than in WTC, co-transfection of HBsAg did not alter the subcellular distribution of core. Huh7 cells transfected with 1 μg of 1.1mer construct of clone c4, c4+207nt, or WTC alone, or together with 0.3 μg of the 0.7mer L/M/S expression construct for surface proteins. 2 days post transfection, cells were fixed onto the coverslips and immunofluorescently stained with DAPI (blue), β-actin (red), and anti-HBc (green) and visualized by a laser confocal scanning microscope

according to the severity of liver diseases and is higher in genotype C than genotype B [22]. Accumulation of deletion variants in the nucleus may result in increased synthesis of progeny DNA. If this is accompanied by an increase in the cccDNA pool and viral protein synthesis, these cells could be more susceptible to T-cell or cytokine-mediated killing. Therefore, a higher immune response could result in severe

liver disease and even hepatic failure [23]. Nucleotides 2971–3177 deleted from this HBV isolate cover the entire SPII promoter, which could abolish M and S protein expression even without the nonsense mutations in the S gene.

We converted the six PCR clones to a 1.1mer construct, which would ensue efficient pgRNA transcription and genome replication driven by the CMV promoter. All the
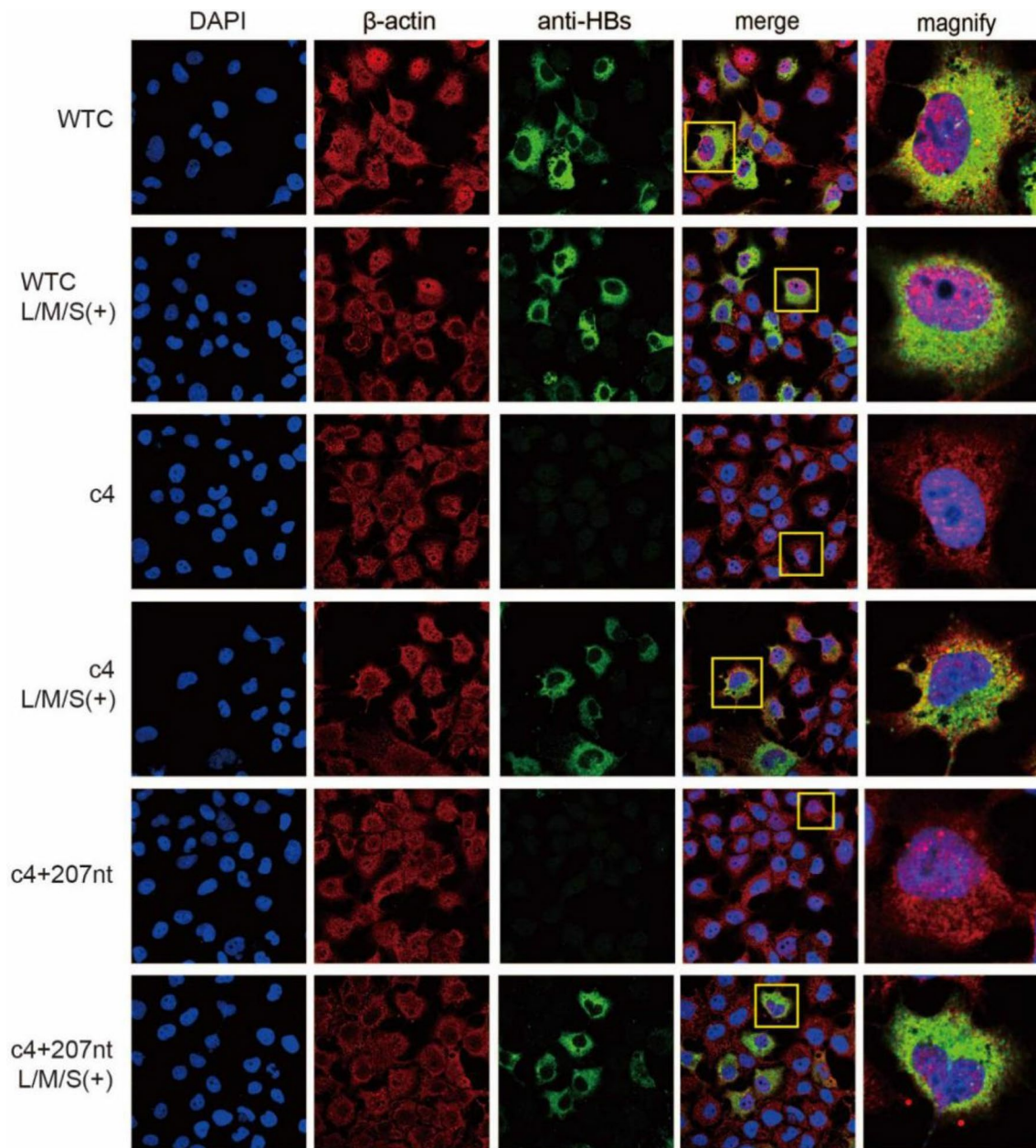
Fig. 5 The intracellular expression of HBsAg. There is no HBsAg expression in c4 and c4+207nt clones; after supplementing surface proteins, the expression of HBsAg was similar to WTC. 1 μg of the 1.1mer construct of c4, c4+207nt, and WTC was transfected alone or together with 0.3 μg of the 0.7mer L/M/S construct using Lipofectamine 3000 reagent into Huh7 cells. 2 days post transfection, cells were fixed onto the coverslips and immunofluorescently stained with DAPI (blue), β-actin (red), and anti-HBs (green) and visualized by a laser confocal scanning microscope

six clones were deficient in HBsAg production as expected. Clones c1, c3, and c4 had similar level of replicative intermediate (Fig. 2a). Clone c4 was subject to further functional characterization. It was deficient in virion production as expected (Fig. 3c), but produced more replicative DNA than the wild-type clones of subgenotype C2 and subgenotype B2 (Fig. 2b). In this regard a previous report found that a 183nt deletion covering nt 2984–3166 in genotype D

(preS1 residues 47–107) abolished HBsAg production due to deleted SPII promoter, but rather increased intracellular level of replicative DNA [24]. Co-transfection of an expression plasmid for the three surface proteins rescued virion secretion but abolished the "high replication" phenotype [24]. Our findings with clone c4 were different. Adding low amounts (0.1 μg or 0.3 μg) of the L/M/S expression construct rescued virion secretion for clone c4 (and c4+207nt)

Fig. 6 Sensitivity of clone c4 to commonly used nucleos(t)ide analogs. Huh7 cells transfected with clone c4 were treated with indicated concentrations of lamivudine (LAM), adefovir (ADV), entecavir (ETV), or tenofovir (TDF) for three days. Intracellular replicative intermediates were analyzed by Southern blotting. Data were quantified using Image J software. The numbers above each panel are the concentrations of each drug (μmol/L), and below each panel are signal intensities as percentile of the untreated well (set at 100)
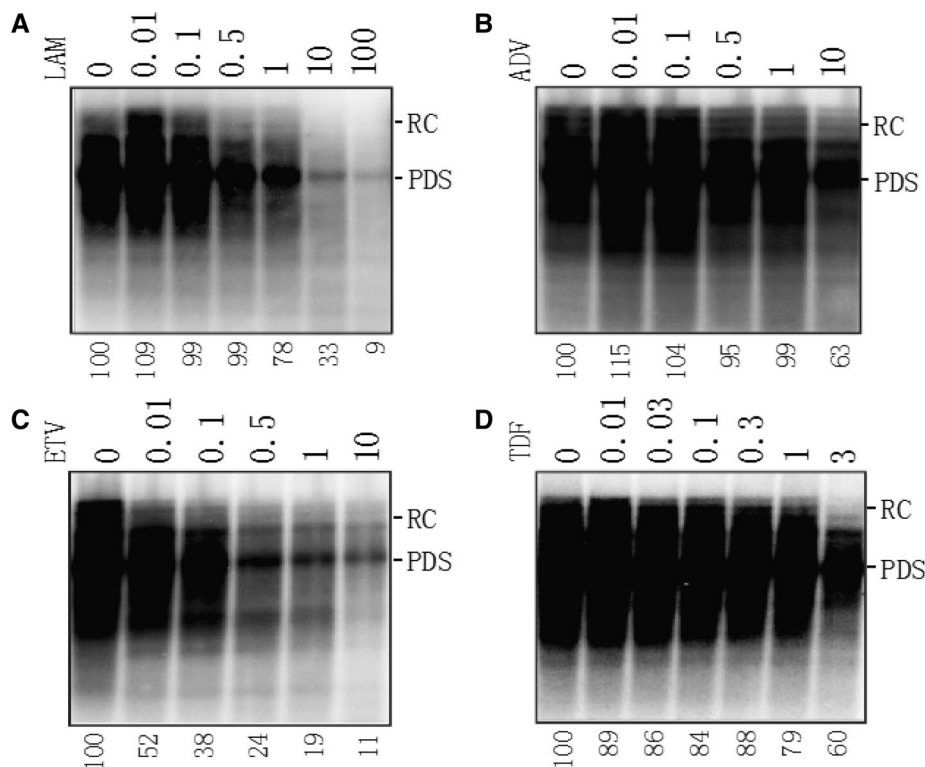
but did not change the high replication phenotype of clone c4 relative to WTC (Fig. 3b). On the other hand, adding back the deleted 207nt markedly reduced intracellular levels of replicative DNA (Figs. 2c, 3b) and core protein (Fig. 3a). Therefore, the large in-frame deletion in the preS1 region rather than virion secretion defect was mostly responsible for the high replication phenotype.

HBcAg plays a role as the major antigen for viral immune responses. It can be histologically classified into cHBcAg (cytoplasmic expression), nHBcAg (nuclear expression) and c-nHBcAg (both cytoplasmic and nuclear expression) [25]. The degree of viral replication in nHBcAg expression was higher than in cHBcAg expression [26]. Our finding indicated that c4 and c4+207nt have a high level of nHBcAg, while WTC showed mainly cHBcAg (Fig. 4). That may be part of the molecular mechanisms of the high replication phenotype of c4.

One may wonder whether the high replication phenotype observed in cell culture reflects situations in the patient, and how can HBV genomes with nonfunctional surface gene be released to the blood. We suspect that surface encoding transcripts are expressed from integrated HBV DNA. Virus DNA integration into the host chromosome is a common feature of the Hepadnaviridae Family [27]. The structural arrangement of the integrated HBV DNA form necessarily affects the expression of all viral open reading frames except the HBsAg ORF, which can stay intact and maintain its position under its native promoter [28]. Next-generation

sequencing (NGS) of liver tissue will further clarify whether HBsAg ORF integration has occurred. A drawback of the 1.1mer construct is that pgRNA transcription is not driven by the endogenous core promoter. Thus, it will be important in the future to repeat the transfection experiments using dimer or 1.5mer constructs. That will also allow us to check for the impact of A1762T/G1764A core promoter mutations found in this HBV isolate, which are known to augment pgRNA transcription to increase genome replication [29–31].

ADV is an adenine nucleotide analog, which has been shown to be effective against both wild-type and LAM-resistant HBV both in vitro and in vivo. ADV resistance occurred commonly at the surface antigen or RT domain and can also affect the changes of preS deletions. The frequency of preS deletions between nucleotide 3037-56 was higher at the emergence of ADV resistance compared to that at the emergence of LAM resistance [32]. We speculate that the 207nt deletion in the preS1 region of this patient may also be related to the application of ADV.

The most important ADV-resistant HBV mutants have rtN236T and rtA181V/T [33]. Development of the HBV rtA181T/sW172* mutant could occur during prolonged LAM therapy, conferring cross resistance to ADV [34]. Previous studies have shown that the incidence of ADV resistance was high in LAM-resistant patients treated with ADV for a short term or without overlapping LAM [35, 36]. Thus, ADV overlapping with LAM therapy rather than ADV

monotherapy is necessary to reduce the incidence of ADV resistance in patients with LAM resistance. Clinical isolates containing the rtA181T + rtN236T double mutant exhibited reduced susceptibility to TDF in genotypes B and C [37], which is also true for the HBV isolate studied here.

HBV has a small and compact genome of 3.2 kb, which made it difficult to develop HBV-based recombinant plasmids for liver targeting and therapy. In this regard, the 207nt deletion enhances rather than diminishing HBV genome replication, and virion secretion of this mutant could be rescued by trans-complementation through another plasmid. That has enabled insertion of foreign sequence into this deletion mutant for diagnostic or therapeutic purposes [38]. Based on c4, we previously generated another highly replicative HBV vector (named 5c3c) by expanding the deletion region to 308 bp for the cargo gene insertion. Recently, we showed 5c3c with an IL-21 gene insertion could secrete infective recombinant HBV virions when in *trans* provided with surface protein, which could be used for liver-targeting gene delivery [39]. In a summary, c4 clone provides a platform for recombinant vector construction.

Author contributions Jiming Zhang and Weifeng Zhao designed experiments. Ting Wang, Yanli Qin, Jing Zhang, and Xinyan Li performed experiments and analysis. Ting Wang, Yanli Qin, Shuping Tong, and Jiming Zhang wrote the manuscript. All authors reviewed the manuscript.

Compliance with ethical standards

Conflict of interests The authors declare no conflict of interests.

Ethical approval The study reporting research involving Human Participants was conducted in accordance with the ethics principles of the Declaration of Helsinki.

Informed consent The study was approved by our institutional review board and written informed consent was obtained from all subjects.

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