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Research Article

In vitro investigation of HBV clinical isolates from Chinese patients reveals that genotype C isolates possess higher infectivity than genotype B isolates

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ABSTRACT

Hepatitis B virus (HBV) genotype B and C are two major genotypes that are prevalent in Asia and differ in natural history and disease progression. The impact of HBV genotypes on viral replication and protein expression has been explored by the transfection of hepatoma cells with replication-competent HBV DNA, which mimics the later stages of the viral life cycle. However, the influence of HBV genotypes on the early events of viral infection remains undetermined, mainly due to the difficulties in obtaining sufficient infectious viral particles for infection assays. Here, we report that a high-titer HBV inoculum can be generated from the transient transfection-based cell model after optimizing transfection conditions and modifying the HBV-expressing construct. By performing *in vitro* infection assays using transiently transfected derived viruses, we found that clinical genotype C isolates possessed higher infectivity than genotype B isolates. Moreover, we identified a naturally occurring mutation sL21S in small hepatitis B surface protein, which markedly decreased the infectivity of HBV genotype C isolates, but not that of genotype B isolates. In summary, using infectious viral particles provided by the optimized transient transfection-based cell model, we have been able to investigate a wide range of HBV variants on viral infectivity, which may contribute to our understanding of the reasons for different clinical outcomes in HBV infections and the development of therapeutic drugs targeting the early stages of HBV life cycle.

1. Introduction

Hepatitis B virus (HBV), the prototype member of the ancient viral family *Hepadnaviridae*, has existed for at least 7000 years (Krause-Kyora et al., 2018). Because the HBV DNA genome is generated by the error-prone reverse transcription of the pregenomic RNA (pgRNA), high level of sequence variability is a feature of HBV (Kramvis, 2014). Currently, based on nucleotide sequence divergence of the entire genome by > 8%, HBV is classified into ten genotypes (A to I, and J), with genotypes A to D most common worldwide (Kim et al., 2011). These genotypes can be subdivided into sub-genotypes with nucleotide sequence divergence between 4% and 7.9% (Kim et al., 2011).

HBV genotype B and C are two major genotypes dominant in Asia. Several clinical studies have provided considerable evidence showing marked differences in natural history and disease progression between these two genotypes (Revill and Tu, 2020). For example, in adulthood, acute hepatitis B patients infected with HBV genotype C are more prone to progress to chronic infection than genotype B infection (Zhang et al., 2008). In chronic hepatitis B patients, HBV genotype C infection is associated with more severe clinical outcomes, with delayed hepatitis B e antigen (HBeAg) seroconversion and higher risk for cirrhosis and hepatocellular carcinoma (Chu et al., 2002; Chan et al., 2004; Yang et al., 2008). To explain the reasons for these genotype-associated clinical outcomes, several *in vitro* studies have explored the impact of HBV genotypes on viral replication and protein expression by transfecting replication-competent HBV DNA into the hepatoma cells, which only mimics the later stages of the viral life cycle (Sugiyama et al., 2006; Qin et al., 2011; Sozzi et al., 2016). However, the impact of HBV genotypes on the early events of viral infection remains unknown, mostly due to the lack of suitable *in vitro* cell culture models supporting viral infection.

To date, several cell culture systems supporting HBV infection have been developed, including primary human hepatocytes, HepaRG,

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induced human hepatocyte-like cells and the sodium taurocholate co-transporting polypeptide (NTCP)-overexpressing hepatoma cell lines (Gripon et al., 1988, 2002; Yan et al., 2012; Xia et al., 2017; Hu et al., 2019). However, a critical limitation of all HBV infection models is the requirement of a high multiplicity of infection (ranging from 100 to 10,000) to achieve acceptable infection levels (Hu et al., 2019). For infection experiments, HBV infectious particles are mostly obtained from the culture supernatants of two HepG2-derived cell lines, HepAD38 and HepG2.2.15, which are stably transfected with a genotype D HBV genome (Verrier et al., 2016). Compared to the stable transfection system, the transient transfection system, which allows viral replication by transient transfecting the hepatoma cells with cloned HBV DNA in over-length constructs, has difficulties in producing sufficient viral particles for infection assays, but it can afford the flexibility of investigating a wide range of HBV clinical variants (Hu et al., 2019). Thus, optimizing the transient transfection system to obtain a high-titer HBV inoculum for infection assays is particularly important to study the infectivity phenotypes of HBV clinical isolates.

The present study showed that a high-titer HBV inoculum could be generated by the transient transfection system through modifying the HBV-expressing construct and optimizing transfection conditions. By performing *in vitro* infection assays using transiently transfected derived viruses, we further investigated the infectivity of twenty HBV clinical isolates cloned from the sera of patients chronically infected with genotype B or C. To our knowledge, this is the first study that directly compares the infectivity phenotypes of HBV genotypes B and C. Moreover, we identified a naturally occurring mutation sL21S in small hepatitis B surface proteins (SHBs), which greatly decreased the infectivity of HBV genotype C isolates, but not that of genotype B isolates.

2. Materials and methods

2.1. Serum samples and construction of HBV-expressing plasmids

Serum samples from chronically infected patients were obtained at the Department of Infectious Diseases, Nanjing Drum Tower Hospital, China. All the patients had not taken antiviral treatment. The study was performed according to the Declaration of Helsinki and approved by the institutional review board of Nanjing Drum Tower Hospital. Written informed consent was obtained from the patients. Viral genomic DNA was extracted from 200 μL of each serum sample described previously (Zhang et al., 2018). Full-length HBV genomes were amplified from the extracted viral DNA by PCR using Phanta® Max Master Mix (Vazyme, Nanjing, China) with the following primers: forward, 5′-CCGGAAAGCTTGAGCTCTTCTTTTTCACC TCTGCCTAATCA-3′; reverse, 5′-CCGGAAAGCTTGAGCTCTTCAAAAAG TTGCATGGTGCTGG-3′ (Günther et al., 1995). The PCR products were purified and sequenced.

The HBV-expressing plasmid containing the 1.3-mer genotype D genome from the HepG2.2.15 cell line was constructed with the procedures described previously (Liu et al., 2019). The HBV-expressing constructs carrying 1.3-mer genotype B or C genome were described previously (Liu et al., 2019). The plasmids containing the 1.1-mer HBV genome were constructed as shown in Supplementary Fig. S1, S2. The homologous recombination was performed using Trelief ® SoSoo Cloning Kit (Tsingke, Beijing, China) according to the manufacturer's instructions. The primers used were listed in Supplementary Table S1. Only the clone with the same sequence as the direct viral PCR product was selected for each patient sample. The sequences of all the constructs are available upon request. The mutation sL21S was introduced into the constructs containing indicated clinical isolates by Fast Site-Directed Mutagenesis Kit (TIANGEN, Beijing, China).

2.2. Cell culture

The human hepatoblastoma cell line HepG2 and HepG2-derived stable cell line HepG2.2.15 were cultured in Eagle's Minimal Essential Medium (EMEM) (WISENT, Nanjing, China) supplemented with 10% fetal bovine serum (FBS) (Gibco, Thornton, Australia). The human hepatocellular carcinoma cell line Huh7 was maintained in Dulbecco's modified Eagle's medium (DMEM) (WISENT, Nanjing, China) with 10% FBS. The stable cell line HepG2-NTCP was generated and cultured as described previously (Liu et al., 2019).

2.3. Detection of HBeAg, HBsAg and HBcAg

HBeAg and hepatitis B surface antigen (HBsAg) in culture supernatants were measured by ELISA kits from Wantai Pharm Inc (Beijing, China) according to manufacturer's instructions. Hepatitis B core antigen (HBcAg) was detected by immunofluorescence staining as described previously (Zhang et al., 2013). Briefly, infected cells were fixed in 4% paraformaldehyde for 30 min and permeabilized in phosphate-buffered saline (PBS) containing 0.2% Triton X-100 for 5 min at room temperature. After three washes with PBS, the cells were blocked with 3% bovine serum albumin for 1 h, followed by incubation with HBcAg antibody (1:200; catalog number GB058629; Gene Tech, Shanghai, China) overnight at 4 °C. The cells were washed for three times with PBS and labeled by Tyramide SuperBoost Kits (Invitrogen, Carlsbad, USA). Finally, the stained cells were imaged using a LEICA DMi8 microscope (LEICA, Wetzlar, Germany).

2.4. Isolation and analysis of HBV DNA

HBV intracellular nucleocapsid-associated DNA was measured as described previously (Liu et al., 2019). Briefly, transfected cells were lysed with the lysis buffer (10 mmol/L Tris, pH7.4, 50 mmol/L NaCl, 1 mmol/L EDTA, 0.5% Nonidet P-40) at 4 °C for 30 min. Cell lysates were centrifuged at 12,000×*g* for 10 min to remove the nuclei, followed by DNase I digestion, proteinase K treatment, and phenol-chloroform extraction.

To measure secreted virion-associated DNA, virions in the supernatants of HepG2.2.15 cells or transiently transfected cells were immunoprecipitated with horse polyclonal anti-HBs antibody (anti-ad/ay; catalog number ab9193; Abcam, Cambridge, UK) to exclude DNA from naked core particles. Briefly, 0.5 mL culture supernatant was incubated with 1 μL anti-HBs antibody for 1 h at 4 °C, followed by adding 30 μL of proteinA/G-Agarose beads (catalog number sc-2003; Santa Cruz, Dallas, USA) and incubating at 4 °C overnight. The beads were washed for three times with PBS and dissolved in the lysis buffer. Then, HBV DNA was extracted with phenol-chloroform after DNase I digestion and proteinase K treatment as described above. The extracted HBV DNA was quantified by quantitative real-time PCR (qPCR) described previously (Liu et al., 2019). All extracted DNA was tested for DNA vector contamination by qPCR with the primers listed in Supplementary Table S1.

To detect HBV covalently closed circular DNA (cccDNA), the total intracellular DNA of infected cells was extracted using TIANamp Genomic DNA Kit (TIANGEN, Beijing, China) according to the manufacturer's instructions. A total of 1 μg extracted DNA was treated with 5 units T5 exonuclease (NEB) in a 10 μL volume for 30 min at 37 °C with subsequent heat inactivation at 99 °C for 5 min. Then, 1 μL of the 10 μL T5 exonuclease treated DNA was added to 20 μL of a qPCR reaction. As reported previously, a pair of specific primers for cccDNA detection was used to analyze HBV cccDNA by qPCR (Yan et al., 2012). The qPCR was performed with the following cycling profile: 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, 62 °C annealing for 15 s, and 72 °C elongations for 45 s.

2.5. Production of HBV particles

A total of 1×10^6 HepG2 cells were plated in each well of a 6-well collagen-coated plate one day before transfection. According to the manufacturer's instructions, the cells were transfected with 2 μg of plasmid DNA using 4 μL Lipofectamine 3000 reagent (Invitrogen). Twenty-four hours post-transfection, the medium was removed, and the cells were maintained in a fresh culture medium after being washed once

with PBS. The culture supernatant, collected every two days, was spun down at 1000×*g* for 5 min to remove any cell debris, and was then concentrated 50-fold via centrifugal filter devices (Biomax 100 kD, Millipore, Darmstadt, Germany).

2.6. HBV infection

A total of 2×10^5 suspended HepG2-NTCP cells were mixed with 50-fold concentrated HBV inoculum in the presence of 4% PEG8000 and 2% DMSO, and plated in each well of a 24-well plate. The volume of HBV inoculum used in each well was 50 μL or less to keep the inoculum < 10% of the total medium volume. Twenty-four hours post-infection, the cells were washed with PBS and cultured with fresh EMEM supplemented with 10% FBS and 2% DMSO. Two days post-infection, the cells were suspended after treatment with 0.25% trypsin and spun down at 300×*g* for 3 min. The cell pellet was re-suspended with fresh EMEM supplemented with 10% FBS and 2% DMSO, and plated in a new well of a 24-well plate. The culture medium of infected cells was refreshed every two days.

In the experiments comparing the infectivity phenotypes of clinical HBV genotype B and C isolates, the HepG2-NTCP cells were infected as described above. From two days post-infection onwards, the re-seeded infected cells were cultured with fresh EMEM supplemented with 10% FBS, 2% DMSO, and 2 μmol/L entecavir (catalog number S5246; Selleck, Houston, USA).

3. Results

3.1. Optimization of transient transfection conditions increased the production of HBV particles

To investigate the infectivity of HBV clinical isolates, we first need to optimize the transient transfection-based cell model to obtain sufficient infectious HBV particles for *in vitro* infection assays. The over-length 1.3-mer HBV DNA construct has been commonly used to study viral replication, and the transfected cells can express all virus gene products and secret enveloped HBV dane particles (Hu et al., 2019). Thus, we constructed an HBV-expressing plasmid containing 1.3-mer genotype D genome from HepG2.2.15 cells and used this construct to optimize transient transfection conditions. After testing several types of transfection reagents, we concluded that using transfection reagent Lipofectamine 3000 to transfect over 90% confluent HepG2 cells, not Huh7 cells, allowed the highest efficiency of virion production, reaching about 3×10^5 IU/mL (Fig. 1A). Previously, it was reported that the virion production of HepG2.2.15 cells could be enhanced when cultured on collagen-coated dishes with reduced concentration of serum and addition of DMSO to the culture medium (Glebe et al., 2001). Our data showed that treatment of collagen also increased the virion production of transiently transfected HepG2 cells by about three folds (Fig. 1B), while no effect and opposite effect on virion production was observed when the cells were cultured with DMSO and lower concentrations of FBS, respectively (Supplementary Fig. S3). Next, we determined the virion production as a function of time. We found that HBV DNA titer was highest at day 7 post-transfection (Fig. 1C), reaching about 1.6×10^6 IU/mL, close to HepG2.2.15 cells (~ 3×10^6 IU/mL) cultured in our lab.

HepG2-NTCP cells were successfully infected using infectious virions concentrated from the supernatant of transfected HepG2 cells as evidenced by continuous secretion of HBeAg during the testing period and staining infected cells for HBcAg (Fig. 1D), although the infection level was not high. However, when we applied the same method to determine the infectivity of HBV clinical isolates we previously cloned from the sera of patients (Liu et al., 2019), we found that the infection levels of the clinical genotype B and C isolates were much lower than that of the genotype D isolate from HepG2.2.15 cells (Supplementary Fig. S4). It is probably because HBV genotype B and C isolates have a longer preS1 domain than genotype D, which can dramatically decrease viral replication and infectivity *in vitro* (Li et al., 2020). Thus, a much higher titer of virus inoculum is required to achieve acceptable infection levels with HBV genotype B and C isolates.

3.2. Modification of the HBV-expressing construct increased the production of HBV particles

We focused on modifying the 1.3-mer HBV-expressing construct to increase virion production further. We introduced the strong cytomegalovirus (CMV) immediate-early promoter to drive the expression of HBV pgRNA as reported previously (Fallows and Goff, 1995). Besides, we

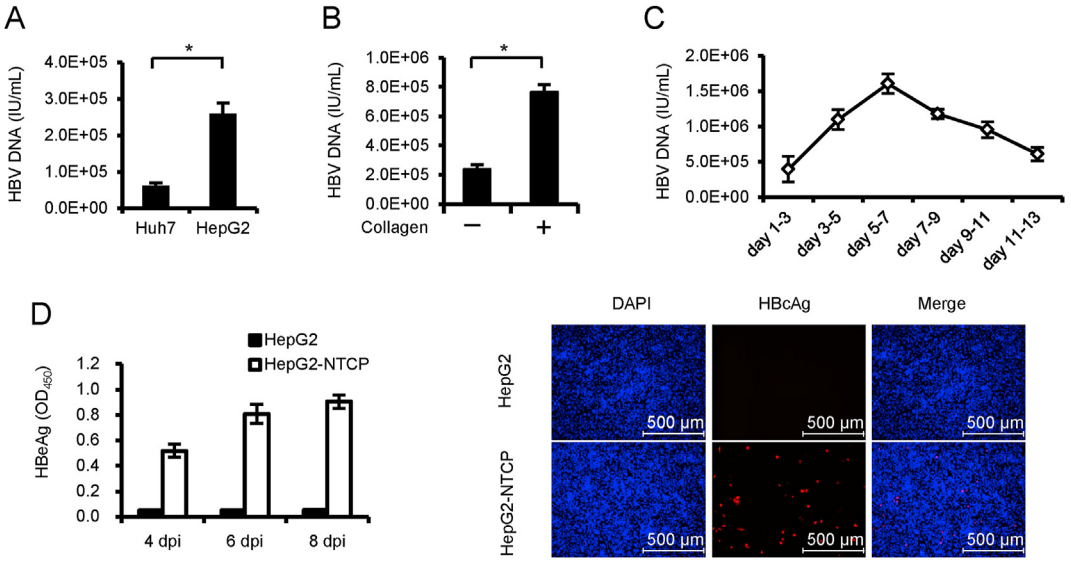

Fig. 1. Optimization of transient transfection conditions increased the production of HBV particles. **A** Huh7 or HepG2 cells were transiently transfected with 1.3-mer HBV-expressing construct cloned from HepG2.2.15 cells. The culture medium was collected at days 1–3 and days 3–5 post-transfection. The virion DNA in mixed supernatants was quantified by qPCR. **B** Effect of collagen treatment on virion DNA production. **C** Kinetics of the virion DNA production in the culture supernatant of HepG2 cells transfected with 1.3-mer HBV-expressing construct. **D** HepG2 or HepG2-NTCP cells were infected with HBV at 100 MOI using a 50-fold concentrated culture medium of transfected HepG2 cells collected at days 5–7. Secreted HBeAg in the supernatants of infected cells and HBcAg in the infected cells were detected by ELISA and immunofluorescence, respectively. Results are shown as mean values ± standard deviation. Statistical analysis was performed by the Student's *t*-test (two-tailed). *$P < 0.05$. HBeAg, hepatitis B e antigen; HBcAg, hepatitis B core antigen; NTCP, sodium taurocholate cotransporting polypeptide.

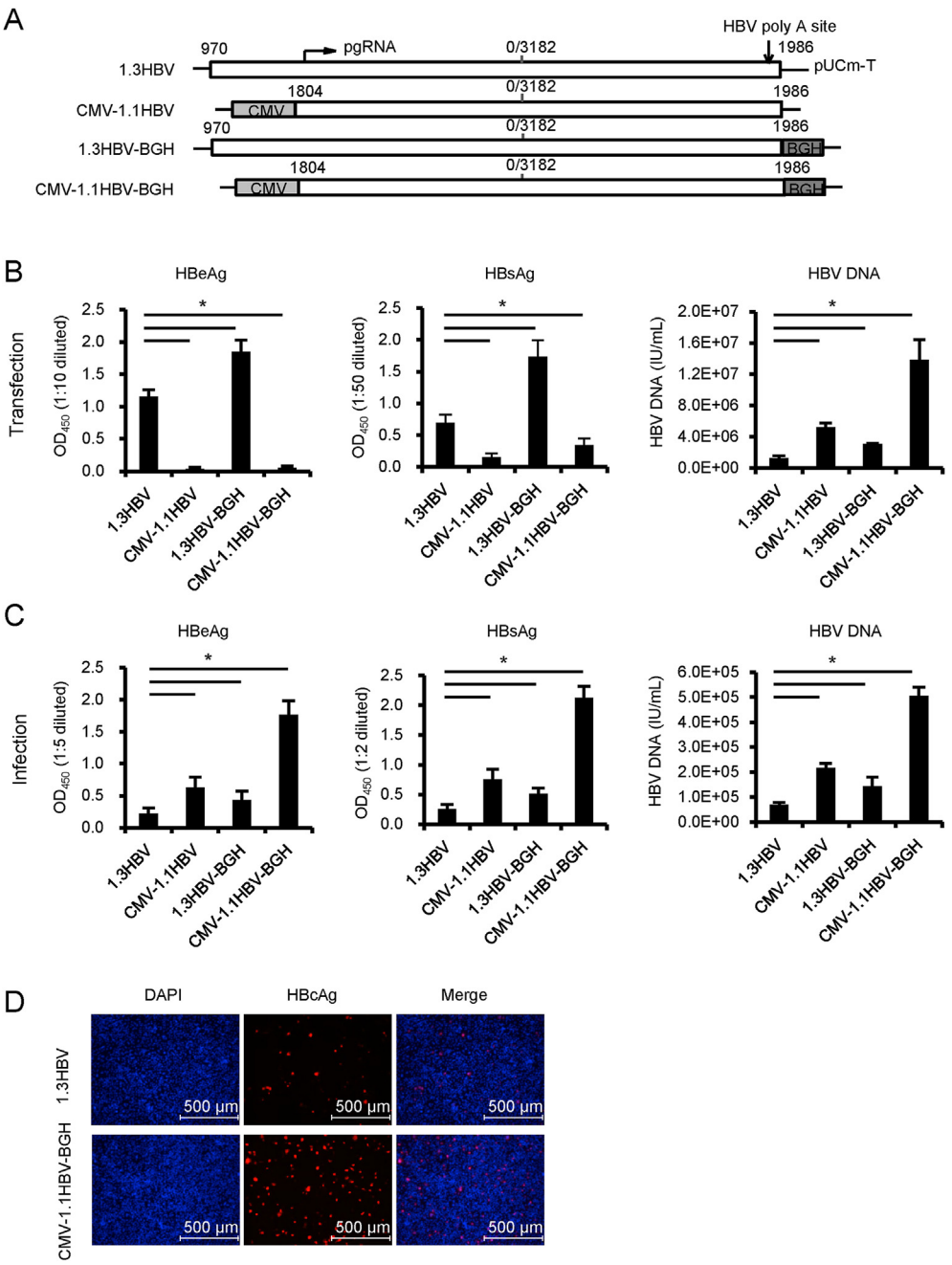

Fig. 2. Comparison of virion production of different HBV-expressing constructs. **A** Schematic illustration of different HBV-expressing constructs. **B** HepG2 cells were transiently transfected with indicated HBV-expressing constructs. The culture medium was collected at days 5–7 post-transfection. Secreted HBeAg and HBsAg were measured by ELISA. Virion DNA was quantified by qPCR. **C** HepG2-NTCP cells were infected with 50 μL 50-fold concentrated culture medium of HepG2 cells transfected with indicated HBV-expressing constructs (the MOI is ranging from 100 to 1000). On day 8 post-infection, HBeAg, HBsAg and HBV DNA in the supernatants of infected cells were measured. **D** On day 8 post-infection, infected cells were fixed and HBcAg expression was detected by immunofluorescence. Results are shown as mean values ± standard deviation. Statistical analysis was performed by the Student's *t*-test (two-tailed). $^{*}P < 0.05$. CMV, cytomegalovirus; BGH, bovine growth hormone; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBcAg, hepatitis B core antigen; NTCP, sodium taurocholate cotransporting polypeptide.

employed the bovine growth hormone (BGH) polyadenylation signal for efficient transcription termination and polyadenylation of viral mRNA (Fig. 2A), given that the polyadenylation site of HBV works inefficiently (Sheets et al., 1990). Our data showed that the introduction of the CMV promoter increased HBV DNA production by about three folds, while HBeAg and HBsAg expressions were decreased by about one hundred and three folds, respectively (Fig. 2B). In contrast, the application of the BGH polyadenylation signal increased all HBV replication markers by over two folds (Fig. 2B). By combining the CMV promoter and the BGH polyadenylation signal, the HBV DNA titer in the supernatant can reach around 1.4×10^7 IU/mL, about ten-fold higher than that of the original 1.3-mer HBV-expressing construct (Fig. 2B).

Next, we used the same volume of concentrated culture medium from different HBV-expressing constructs to infect HepG2-NTCP cells. As expected, infection levels strongly correlated with levels of virion DNA, but not levels of HBeAg and HBsAg, in the culture supernatants of transfected cells. Compared to the original 1.3-mer HBV-expressing construct, about ten-fold more HBeAg, HBsAg and HBV DNA were produced by the cells infected with the virus derived from modified 1.1-mer HBV-expressing construct (Fig. 2C). Moreover, we confirmed the result by staining infected cells for HBcAg (Fig. 2D).

3.3. *Transfection of the 1.1-mer HBV-expressing constructs containing clinical genotype B and C isolates produced high-titer viral particles*

Next, we were interested in comparing the infectivity phenotypes of clinical HBV genotype B and C isolates. To this end, we collected the sera with high HBV DNA levels from twenty chronically infected patients (Table 1), ten of which were infected with genotype B (subgenotype B2) and ten with genotype C (subgenotype C2). There was no significant difference in the age (32.1 ± 7.81 vs. 32.4 ± 7.06 years) and HBV titer ($1.4 \times 10^8 \pm 1.6 \times 10^8$ vs. $0.77 \times 10^8 \pm 0.61 \times 10^8$ IU/mL) between the

Table 1
Demographic and virological characteristics of patients from whom HBV isolates were cloned.

Patients infected with genotype B				Patients infected with genotype C			
Isolate	Age (y)	Sex	HBV (IU/mL)	Isolate	Age (y)	Sex	HBV (IU/mL)
B-1	25	Female	5.3×10^8	C-1	45	Male	1.5×10^7
B-2	28	Female	2.7×10^8	C-2	24	Female	1.3×10^8
B-3	29	Male	2.6×10^7	C-3	32	Male	8.7×10^7
B-4	43	Male	2.8×10^7	C-4	42	Male	2.8×10^7
B-5	44	Male	1.3×10^7	C-5	31	Female	1.2×10^8
B-6	24	Male	7.7×10^7	C-6	36	Male	7.6×10^7
B-7	38	Male	2.5×10^8	C-7	25	Male	1.9×10^8
B-8	27	Male	6.3×10^7	C-8	33	Male	1.1×10^7
B-9	25	Male	3.4×10^7	C-9	25	Male	1.1×10^7
B-10	38	Male	1.4×10^8	C-10	31	Female	1.0×10^8

two groups of patients. We constructed one 1.1-mer HBV-expressing construct carrying the genome of the dominant strain by selecting the clone with the same sequence as the direct viral PCR product for each patient. Sequencing analysis found that two genotype B isolates (B-7, B-10) and two genotype C isolates (C-3 and C-4) harbored the classic G1896A mutation, which can increase the HBV genome replication (Scaglioni et al., 1997; Qin et al., 2011).

We then transfected these HBV-expressing constructs into HepG2 cells to produce viral particles. On day 7 post-transfection, the levels of virion DNA in culture supernatants were analyzed by qPCR. Our data showed that high virion productions were achieved in all twenty clinical isolates, ranging between 5×10^6 and 2×10^7 IU/mL, suggesting that the optimized transient transfection system can be universally used for obtaining high virion production. Compared to genotype B isolates, we found that genotype C isolates often produced more virion DNA (Fig. 3A). Consistent with previous studies (Qin et al., 2011; Sozzi et al., 2016), all four isolates with the G1896A mutation had high virion production (Fig. 3A). To exclude the possibility that observed differences in HBV DNA levels might result from bias in qPCR analyses, we performed qPCR using the same amount of 1.1-mer HBV-expression constructs and observed similar detection sensitivity for all clinical isolates (Fig. 3B). Contrasting the HBV DNA findings, marked differences were observed in the levels of secreted HBsAg across HBV isolates, and genotype B isolates often expressed more HBsAg than genotype C isolates (Fig. 3C).

3.4. *In vitro infection experiments revealed that clinical genotype C isolates possessed higher infectivity than genotype B isolates*

Next, we infected HepG2-NTCP cells with concentrated culture medium containing the same number of viral particles to determine the infectivity of these clinical isolates. Since different HBV isolates have distinct protein expression patterns, it is difficult to directly compare viral infectivity by comparing the expression levels of viral proteins, e.g. HBeAg and HBsAg. Thus, instead, we measured the levels of cccDNA in infected cells. To only detect cccDNA generated from incoming virions, we treated infected cells with entecavir to block the replenishment of cccDNA from newly synthesized relaxed circular DNA. On day 8 post-infection, we first measured extracellular HBeAg and HBsAg, confirming that all clinical isolates successfully infected HepG2-NTCP cells (Fig. 4A and B). As expected, the isolates with the G1896A mutation secreted no HBeAg (Fig. 4A). qPCR analysis of cccDNA in the infected cells revealed great variability in viral infectivity across HBV isolates, and the infectivity of genotype C isolates was significantly higher than that of

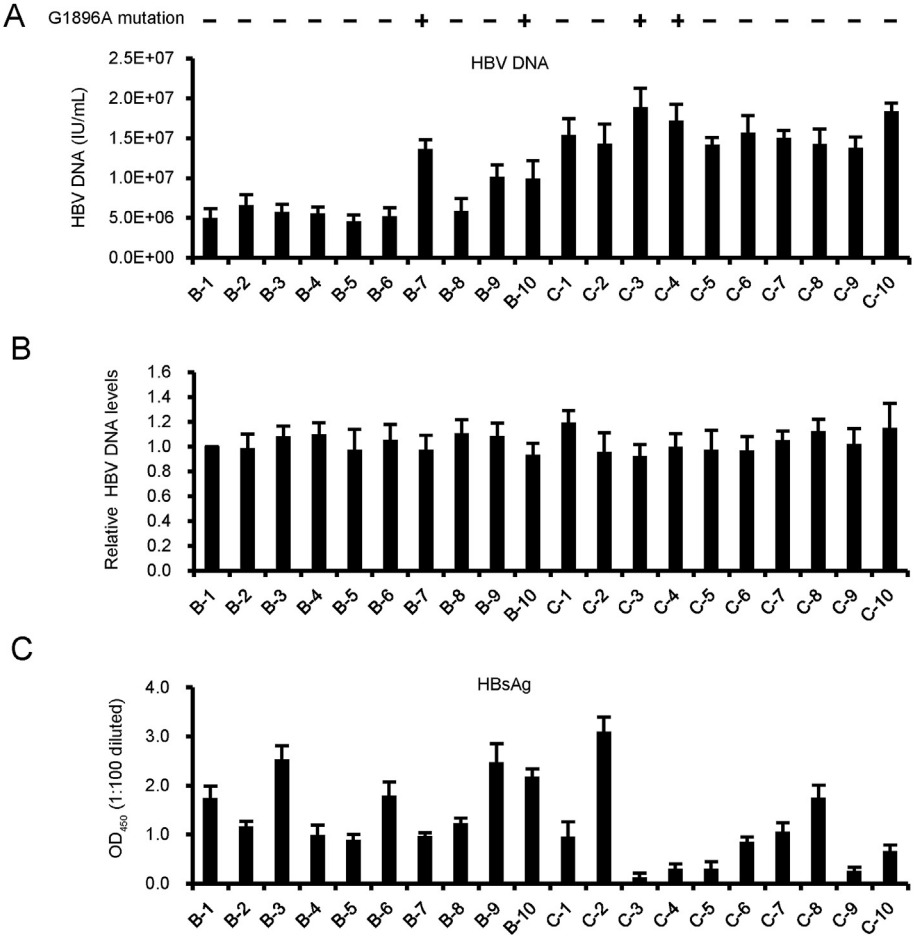

Fig. 3. Comparison of virion production and HBsAg secretion among ten genotype B isolates and ten genotype C isolates. HepG2 cells were transiently transfected with CMV-directed 1.1-mer HBV-expressing constructs cloned from patients chronically infected with HBV. The culture supernatants were collected at days 5–7 post-transfection. **A** HBV virion DNA in the supernatants was quantified by qPCR. **B** The detection efficiency of qPCR on HBV total DNA was determined by using the same amount of CMV-directed 1.1-mer constructs containing indicated HBV isolate genome. **C** Secreted HBsAg was measured by ELISA. Results are shown as mean values ± standard deviation. HBsAg, hepatitis B surface antigen; CMV, cytomegalovirus.

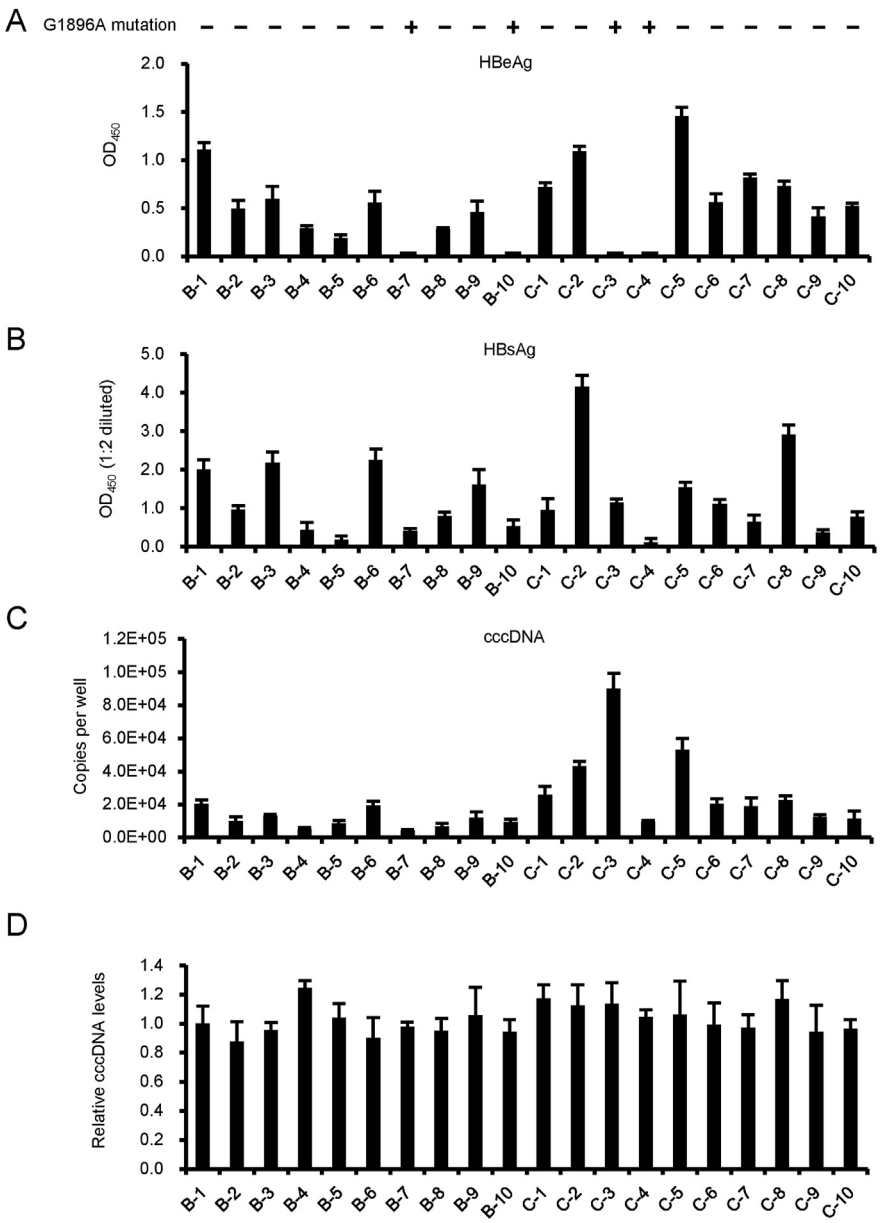

Fig. 4. Comparison of the infectivity phenotypes of HBV genotype B and C isolates. HepG2-NTCP cells were infected with the same number of indicated HBV particles concentrated from the culture supernatants of transfected HepG2 cells at 500 MOI. Two days post-infection, the infected cells were cultured with addition of 2 μmol/L entecavir. On day 8 post-infection, HBeAg (**A**) and HBsAg (**B**) in the supernatants of infected cells were measured by ELISA. **C** The levels of cccDNA in infected cells were quantified by qPCR. Results are shown as mean values ± standard deviation. **D** The detection efficiency of qPCR on HBV cccDNA was determined by using the same amount of viral genome fragment (nucleotides 970 to 3215/1 to 262) of each isolate constructed by fusion PCR. Statistical analysis was performed by the Student's *t*-test (two-tailed). HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; cccDNA, covalently closed circular DNA.

genotype B isolates ($P = 0.025$) (Fig. 4C). Similar to HBV DNA detection, we also checked the detection sensitivity of cccDNA across HBV isolates. Since cccDNA primers targeting nucleotides 1582 to 2316 region of HBV genome cannot detect 1.1-mer HBV-expression constructs (nucleotides 1804 to 3215/1 to 1986), we constructed viral genome fragment (nucleotides 970 to 3215/1 to 262) of each isolate by fusion PCR, and then performed qPCR analysis using the same amount of these fragments. Our data revealed that observed differences in cccDNA levels were not due to bias in qPCR analyses (Fig. 4D).

3.5. *Identification of the sL21S mutation reducing the infectivity of genotype C isolates*

Among the ten genotype C isolates, the infectivity of isolate C-4 was much lower than other genotype C isolates (Fig. 4C). Next, we tried to identify the naturally occurring mutation(s) responsible for the observed difference. Until now, three determinants of virus infectivity have been identified in HBV envelope proteins (Fig. 5A) (Lepère-Douard et al., 2009; Tu and Urban, 2018). Based on that, we made three chimeric viruses with isolate C-4 and high infectious isolate C-5 (Fig. 5A). By measuring these viruses' infectivity, we found that the mutation(s) affects the infectivity of isolate C-4 located in nucleotides 155 to 262 regions of the viral genome (Fig. 5B). Two mutations, sN3S and sL21S, in SHBs were identified (Fig. 5C). Among the ten genotype C isolates, the sN3S mutation also existed in another two isolates (C-6 and C-9), while only isolate C-4 harbored the sL21S mutation. We first introduced the two mutations into high infectious isolate C-5. We found that both mutations did not affect virion production. Still, the sL21S mutation affected secreted HBsAg levels (Fig. 5D), which is probably by reducing the antigenicity of HBsAg (Xiang et al., 2017). *In vitro* infection experiments revealed that the sL21S mutation also markedly decreased viral infectivity, as measured by cccDNA levels in infected cells (Fig. 5E). Conversely, secreted HBsAg levels and the infectivity of isolate C-4 were significantly increased when the sL21S mutation was reverted to wild type (Fig. 5F and G).

Interestingly, one genotype B isolate B-10 also harbored the sL21S mutation. However, its infectivity was similar to other genotype B isolates (Fig. 4C). Therefore, we wondered whether the sL21S mutation affected the infectivity of genotype B isolates. To this end, we reverted the sL21S mutation to wild type in isolate B-10 and observed no changes

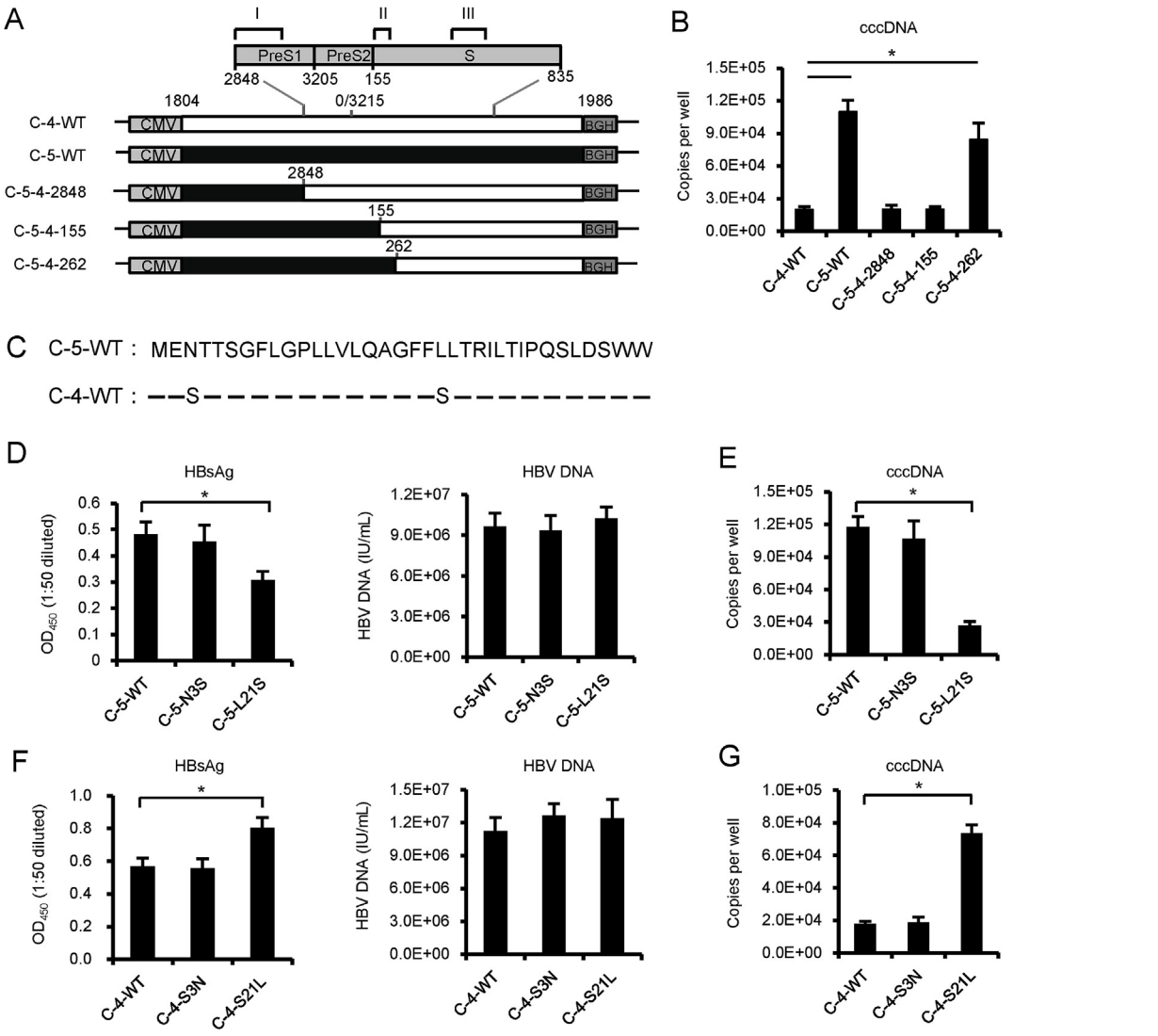

Fig. 5. Identification of the sL21S mutation in SHBs. **A** (I-III) Three identified determinants of virus infectivity in HBV envelope protein. **B** HepG2-NTCP cells were infected with the same number of indicated HBV particles concentrated from the culture supernatant of transfected HepG2 cells at 1000 MOI. At day 8 post-infection, the levels of cccDNA in infected cells were quantified by qPCR. **C** The mutations were identified in nucleotides 155 to 262 regions of the viral genome. **D** HepG2 cells were transiently transfected with the HBV-expressing plasmids containing wild-type or mutated isolate C-5. The effects of the sN3S and sL21S mutations on secreted HBsAg levels and virion DNA production in the supernatants of transfected cells were measured by ELISA and qPCR, respectively. **E** HepG2-NTCP cells were infected with the same number of indicated HBV particles concentrated from the culture supernatants of transfected HepG2 cells at 1000 MOI. On day 8 post-infection, the effects of the sN3S and sL21S mutations on viral infectivity were determined by quantifying cccDNA in infected cells. **F** The sN3S and sL21S mutations in the isolate C-4 were reverted to wild type. The constructed HBV-expressing plasmids were transfected into HepG2 cells, and secreted HBsAg levels and virion DNA production in the supernatants of transfected cells were measure by ELISA and qPCR, respectively. **G** HepG2-NTCP cells were infected with the same number of indicated HBV particles concentrated from the culture supernatants of transfected HepG2 cells at 1000 MOI. On day 8 post-infection, the levels of cccDNA in the cells infected with indicated viruses were measured by qPCR. Results are shown as mean values ± standard deviation. Statistical analysis was performed by the Student's *t*-test (two-tailed). $^*P < 0.05$. SHBs, small hepatitis B surface protein; HBsAg, hepatitis B surface antigen; cccDNA, covalently closed circular DNA.

in secreted HBsAg levels, virion production and viral infectivity (Fig. 6A and B). Furthermore, we introduced the sL21S mutation into three more HBV isolates (each strain for genotype B, C, and D) to confirm our observations. Consistently, our data showed that the sL21S mutation only affected the HBsAg levels and the infectivity of genotype C isolate (Fig. 6C and D).

4. Discussion

In the present work, to study the infectivity of HBV clinical isolates, we optimized the transient transfection-based cell model, aiming for conveniently producing high-titer infectious viral particles of HBV variants. Our data show that the optimized transient transfection system allows much higher efficiency of HBV replication and can generate about five-fold more infectious HBV particles than the stably transduced cell line HepG2.2.15. Thus, it enables the investigation of a wide range of HBV variants on viral infectivity.

In contrast to high virion DNA production, the levels of HBeAg and HBsAg were markedly reduced in the supernatant of the cells transfected with the modified 1.1-mer HBV-expressing construct. HBeAg, the processed shortest p17 form of the p25 precore protein, is produced from the precore RNA, which is longer than pgRNA (Tong and Revill, 2016). Because the precore RNA transcript is initiated at the position upstream from nucleotides 1804 of the HBV genome, the 1.1-mer HBV-expressing construct under the control of the CMV promoter does not transcribe the precore RNA. However, we can still detect very low levels of HBeAg

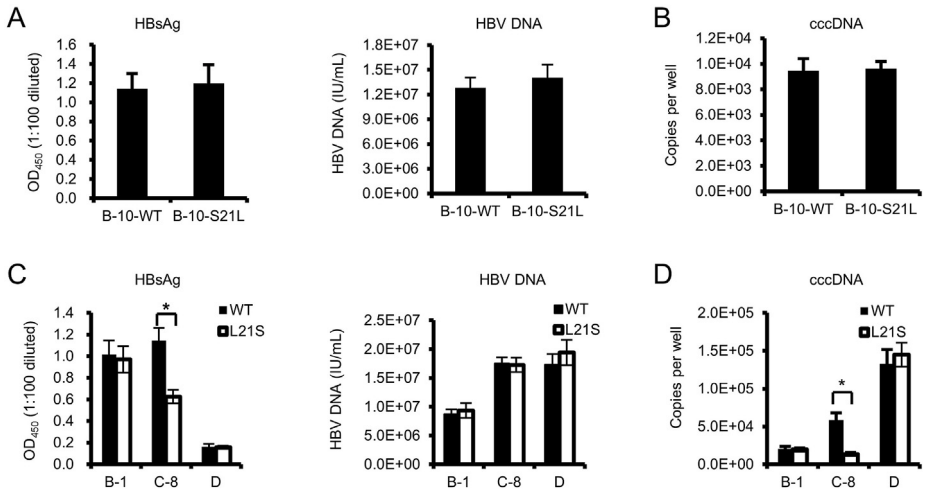

Fig. 6. Investigation of the effects of the sL21S mutation on the viral infectivity of genotype B, C, and D isolates. **A** The sL21S mutation in isolate B-10 was reverted. The constructed HBV-expressing plasmids were transfected into HepG2 cells, and secreted HBsAg levels and virion DNA production in the supernatants of transfected cells were measured by ELISA and qPCR, respectively. **B** HepG2-NTCP cells were infected with the same number of indicated HBV particles concentrated from the culture supernatants of transfected HepG2 cells at 1000 MOI. On day 8 post-infection, the effect on viral infectivity was determined by quantifying cccDNA in infected cells. **C** The sL21S mutation was introduced into wide-type isolates B-1, C-8 and D. The constructed HBV-expressing plasmids were transiently transfected into HepG2 cells. The effects of the mutations on secreted HBsAg levels and virion DNA production in the supernatants of transfected cells were measure by ELISA and qPCR, respectively. **D** HepG2-NTCP cells were infected with the same number of indicated HBV particles concentrated from the culture supernatants of transfected HepG2 cells at 1000 MOI. On day 8 post-infection, the effect of the sL21S mutation on viral infectivity was determined by quantification of cccDNA in infected cells. Results are shown as mean values ± standard deviation. Statistical analysis was performed by the Student's *t*-test (two-tailed). $^*P < 0.05$. HBsAg, hepatitis B surface antigen; cccDNA, covalently closed circular DNA.

(Fig. 2B). This signal might be from the cccDNA formed by intracellular trafficking of the newly synthesized relaxed circular DNA to the nucleus. The expression of HBsAg was reduced might due to transcriptional interference from the CMV promoter (Chen et al., 2016). Fortunately, our data revealed that reduced HBeAg and HBsAg in the supernatant did not affect the infectivity of secreted viral particles, which probably is because HBeAg is not required for HBV infection *in vitro* and HBsAg is abundant. Thus, using the HBV inoculum produced from CMV-directed 1.1-mer HBV-expressing construct increases infection levels and greatly reduces signal interference from carryover HBeAg and HBsAg in the inoculum. To be noted, even with the high-titer HBV inoculum obtained from the optimized transient transfection system, the infection levels of HBV genotype B and C isolates were still not high in our study due to much lower infectivity of these two genotypes compared to that of genotype D. In the future study, a highly permissive HepG2-NTCP cell clone could be selected as some researchers did to further increase the robustness of viral infection assay (Ko et al., 2018).

It has been well documented that there are marked differences in clinical outcomes between the patients infected with HBV genotype B and C (Qin et al., 2011; Revill and Tu, 2020), with the reasons remaining to be clarified. Due to similar geographic distributions of these two genotypes, it is reasonable to assume that viral factors might play a more important role than host and environmental factors. Previous studies suggested genotype C isolates either have the higher replicative capacity (Sugiyama et al., 2006; Sozzi et al., 2016), or secrete more virions than genotype B isolates (Qin et al., 2011). Unlike previous studies, which all only study the later stages of the HBV life cycle, we took advantage of high virion production from the optimized transient transfection system to study the infectivity phenotypes of HBV genotype B and C. To exclude differences derived from clonal variability, we constructed ten clinical isolates for each genotype. Analysis of the full genome sequence revealed that these HBV isolates belong to the B2 and C2 subgenotypes, the most common subtypes of genotype B and C in China (Zhang et al., 2008), which is consistent with the previous findings of Qin et al., who also cloned multiple HBV genotype B and C isolates from Chinese carriers (Qin et al., 2011). Qin et al. suggested that genotype C isolates secrete more virions, consistent with our result (Fig. 3A). Sugiyama et al. and Qin et al. observed marked variability in replication capacity among HBV clones of the same genotype (Sugiyama et al., 2006; Qin et al., 2011), which is not surprising given that sequence variability in clinical HBV isolates of the same genotype is very high common. Xiang et al. analyzed SHBs sequences cloned from chronic hepatitis B patients infected with genotype C and found that amino acid substitutions occurred in 191 of 230 (83.04%) patients (Xiang et al., 2017). The mutations in hepatitis B surface proteins may affect viral entry, resulting in reduced or increased viral infectivity. As expected, great variability in viral infectivity within the same genotype was observed in our study (Fig. 4C). Interestingly, statistical analysis reveals that genotype C isolates possessed significantly higher infectivity than genotype B isolates (Fig. 4C). Higher infectivity, combined with more secreted virions, will make genotype C isolates spread faster than genotype B isolates, which might partially explain the worse clinical outcomes of genotype C than that of genotype B.

Our study also identified a naturally occurring sL21S mutation located in the first transmembrane (TM1) domain of HBV envelope proteins. Although the TM1 domain has been proven to be essential for viral infectivity, the residue sL21 in the TM1 domain was shown to be not crucial by extensive mutational analysis using an HBV genotype D strain (Lepère-Douard et al., 2009). This finding is consistent with our result that the sL21S mutation only affected the infectivity of genotype C isolates, but not that of genotype B and D isolates (Figs. 5 and 6), indicating another amino acid residue(s) is(are) involved in regulating the effect of the sL21S mutation on viral infectivity. Our data also reminds us that it should be cautious about drawing a conclusion based on the experimental results obtained from one particular genotype strain.

The naturally occurring sL21S mutation was identified in about 5% of chronic hepatitis B patients infected with genotype C isolates by Xiang et al. (2017). Xiang et al. also proved that the sL21S mutation could affect HBsAg levels of genotype C isolates by reducing the antigenicity of HBsAg to impair the binding capacity of anti-HBs antibodies (Xiang et al., 2017), indicating that the emergence of the sL21S mutation might attribute to the pressure of host immune response. Intestinally, in our study, the sL21S mutation was identified only in the isolates with the

G1896A mutation, which disrupts HBeAg expression due to host immune response (Kramvis et al., 2018). This observation indicates that the sL21S mutation is selected because of the host immune attack. Although it was both identified in clinical genotype B and genotype C isolate, our data suggested that the sL21S mutation only affected HBsAg levels of genotype C isolates, but not that of genotype B isolates (Fig. 6). Thus, besides the role in reducing the antigenicity of HBsAg, the sL21S mutation probably has other unknown biological functions, which require further investigation.

5. Conclusions

In conclusion, the optimized transient transfection-based cell model can serve as a simple and efficient tool for providing high-titer infectious HBV particles. We have compared the infectivity of clinical HBV genotype B and C isolates using transiently transfected derived viruses. The limitation of our study is that we only studied subgenotypes B2 and C2 from Chinese patients. The infectivity phenotypes of other subgenotypes of genotype B and C, as well as other HBV genotypes, remain to be determined.

Data availability

The datasets used and/or analyzed in this study are obtained and available from the corresponding authors upon a reasonable request.

Ethics statement

The study was performed according to the Declaration of Helsinki and approved by the institutional review board of Nanjing Drum Tower Hospital. Written informed consent was obtained from the patients.

Author contributions

Tingting Liu: investigation, methodology, funding acquisition. Anlei Liu: formal analysis, resources. Yong Liu: resources. Shan Cen: writing - review & editing, visualization. Quan Zhang: conceptualization, writing - original draft, project administration, funding acquisition.

Conflict of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2022.03.008.

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