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Investigation of hepatitis B virus (HBV) rtS78T/sC69* mutation in a large cohort of chronic HBV-infected patients with nucleoside/nucleotide analogue treatment

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ABSTRACT

This study aimed to investigate clinical occurrence and significance of the rtS78T/sC69* mutation of hepatitis B virus (HBV). A total of 22,009 consecutive chronic HBV-infected patients who underwent resistance testing at the Fifth Medical Center of Chinese PLA General Hospital (Original name Beijing 302 Hospital) from 2007 to 2016 were enrolled. Serum samples were collected for sequence analysis of HBV reverse-transcriptase (RT) and S regions. Phenotypic analysis was performed to evaluate the viral replication capacity and drug susceptibility. The rtS78T mutation was detected in 0.83% (182/22,009) of the patients' samples. All mutations simultaneously created a stop codon at sC69 (sC69*). The prevalence of rtS78T/sC69* did not differ significantly between the patients with and without entecavir/tenofovir treatment. Of the 182 mutation-positive samples, 41 (22.5%) were detected with signature drug-resistance mutations to adefovir (n = 26), lamivudine (n = 11), entecavir (n = 3), and lamivudine plus adefovir (n = 1). The HBV DNA and RNA levels of the rtS78T/sC69* mutant were significantly increased compared to the wild-type; while the mutant had undetectable secreted and intracellular HBsAg, and its half maximal effective concentration to lamivudine, adefovir, entecavir, and tenofovir were 3.73-, 1.61-, 4.76-, and 3.71-fold of the wild-type, respectively. Artificial elimination of the rtS78T mutation had a limited effect on the drug susceptibilities. The data obtained in the present study suggested that the emergence of the rtS78T/sC69* mutation was not closely related to entecavir/tenofovir treatment and itself appeared insufficient to confer drug resistance unless it coexisted with signature drug-resistance mutations.

1. Introduction

Nucleoside/nucleotide analogues (NAs) including lamivudine (LAM), adefovirdipivoxil (ADV), entecavir (ETV), telbivudine (LdT), and tenofovirdisoproxil fumarate (TDF) are the main treatment choices for chronic hepatitis B (CHB) (Lim, 2017). HBV drug-resistance mutations in the viral reverse-transcriptase (RT) region of polymerase remain a concern for long-term NA therapy. Signature or classical resistance mutations include rtM204I/V (LAMr) for LAM (rtM204I is also an LdT-resistant mutation), rtA181V/rtN236T for ADV, LAMr along with at least one of the substitutions at rt184 (A/C/F/G/I/L/M/S), rt202 (C/G/I), and rtM250 (I/L/V) for ETV (Lok et al., 2007; Zoulim and Locarnini, 2013). In addition, some RT mutations may introduce changes in the overlapping S region because of the overlapping reading frames of the RT and S genes in the condensed viral genome, which influence viral activity (Tong and Revill, 2016; Yeh, 2010). HBV rtA181T/sW172* is a typical example (Meldal et al., 2011; Warner and Locarnini, 2008). We have reported the individual virological features and clinical implications of the rtA181T/sW172* and rtA181T/sW172 non-stop mutations (Zhao et al., 2018).

Abbreviations: HBV, hepatitis B virus; NAs, nucleoside/nucleotide analogues; RT, reverse transcriptase; HBsAg, hepatitis B surface antigen; LAM, lamivudine; LdT, telbivudine; ADV, adefovirdipivoxil; ETV, entecavir; TDF, tenofovirdisoproxil fumarate; HCC, hepatocellular carcinoma; CHB, chronic hepatitis B; nt, nucleotide; LLOD, lower limit of detection; ALT, alanine aminotransferase; AST, aspartate aminotransferase; COI, cut off index; EC_{50}, half maximal effective concentration; UDPS, Ultra-deep pyrosequencing

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Recently, three research groups, including ours, identified a few novel mutation patterns that conferred resistance to the first-line NAs, ETV or TDF (Hayashi et al., 2015; Liu et al., 2019a, 2019c; Park et al., 2019). In addition, a case study indicated that a single mutation rtS78T/sC69* enhances viral replication with reduced susceptibility to ETV and TDF, possibly owing to an inadequate virological response to ETV/TDF (Shirvani-Dastgerdi et al., 2017). However, to date, no other investigation has verified this case study-based result. In the present study, we aimed to clarify the clinically prevalent property of the rtS78T/sC69* mutation and its association with the partial resistance to ETV/TDF.

2. Materials and methods

2.1. Patient samples

A total of 29,639 serum samples from 22,009 chronic HBV-infected patients who visited the 5th Medical Center of Chinese PLA General Hospital (originally the Beijing 302 Hospital) and received genotypic resistance testing (direct sequencing) from 2007 to 2016 were enrolled as described previously (Zhao et al., 2018). All patients were treated using NAs. Serum samples for resistance testing were collected and serial samples were collected if available. The illness categories of chronic HBV infection included chronic hepatitis B, HBV-related liver cirrhosis, and hepatocellular carcinoma (HCC). These illnesses were diagnosed according to the 2005 Guideline on Prevention and Treatment of Chronic Hepatitis B in China (Chinese Society of Hepatology et al., 2007). Patients who were co-infected with other hepatitis viruses or human immunodeficiency virus were excluded. Data of the enrolled patients were obtained from the hospital's database. All patients provided their informed consent for the use of the samples for research before enrollment. The study was approved by the Ethics Committee of Beijing 302 Hospital.

2.2. Serological markers and sequencing of HBV RT gene

Biochemical and serological markers and HBV DNA level of the patients were routinely detected in the Center Clinical Laboratory of the hospital as previously described (Zhao et al., 2018). HBV RT region (nucleotide 54–1278) was amplified using an in-house nested PCR with a lower limit of detection (LLOD) of 10 IU/mL using 200 μL serum as previously described (Ren et al., 2010). Clonal sequencing for samples of interest was performed ($\geq$20 clones per sample). Signature resistant mutations in the RT gene and interesting mutations in the S gene were analyzed. Coexisting nucleotides at one position were determined if the sequencing peak of one nucleotide overlapped with at least 25% of the sequencing summit of another dominant nucleotide at the position. Ultra-deep pyrosequencing (UDPS) with detectability of 0.1% mutant frequency was performed by a professional company (OBT Co., Ltd., Shanghai, China).

2.3. Construction of recombinant vectors containing the 1.1 mer HBV genome and site-directed mutagenesis

Recombinant vectors containing either the mutants or wild-type RT gene were constructed for phenotypic analysis based on the pTriEx-mod-1.1 vector that has been used for HBV phenotypic analysis (Ji et al., 2012; Zoulim, 2006). Two mutants were used in the construction, including a clinical isolate and a laboratory (lab)-modified mutant. The rtS78/sC69(lab) mutant was generated by reverse site-directed mutagenesis using rtS78T/sC69* mutant template sequences and the Quick Change Lightning site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. The 5′-TCCTC CAATTTG**T**CCTGGCTATCGC-3' (sense) and 5′-GCGATAGCCAGG**A**CAA ATTGGAGGA-3' (antisense) primers were used to generate the rtS78/sC69 (lab). The wild-type vector was also constructed and the constructs were all verified by DNA sequencing.

2.4. Quantitation and detection of supernatant and intracellular HBV RNA and HBV surface antigen (HBsAg)

The mutants and wild-type HBV genomic vectors were transiently transfected into HepG2 cells. Transfection efficiency was normalized to β-galactosidase with a reporter plasmid (Promega, Madison, WI, USA). After 3 days of cultivation, cell culture supernatants and the cells were collected. HBV RNA was isolated from both fractions using the EasyPure Viral RNA Kit (TransGen Biotech, Beijing, China) and TRIzol Reagent (Ambion, Austin, TX, USA), and treated with DNase I (Thermo Fisher Scientific, Waltham, MA, USA). HBV RNA quantitation was performed as we previously described (Liao et al., 2019). HBsAg levels were detected with a commercial ELISA kit (WANTAI Bio Pharm. Beijing, China) and Roche quantitative Elecsys assay. In addition, a commercial ELISA kit (Keshun Bio., Shanghai, China) was used for preS2 detection. For intracellular HBsAg, cells were lysed by ultrasonication. The experiments were repeated at least three times independently.

2.5. Assessment of viral replication capacity and drug susceptibility

The experiment was performed as previously described (Liu et al., 2015, 2016). Drug susceptibility was determined by comparing the 50% effective concentration of the drug (EC_{50}) of the mutant to the wild-type. This PCR-based assay has been patented in China (ZL 2013103921225) and achieved consistent results compared with classical southern blot-based assay in our previous studies (Liu et al., 2019b, 2019c). The experiments were performed at least three times independently.

2.6. Molecular modeling of HBV RT binding to ETV and TDF

Modeling of the structure of HBV RT was accomplished using the SWISS-MODEL (https://www.swissmodel.expasy.org) based on the crystal structure of the HIV RT (Protein Data Bank accession number, 1rtd) as we previously described (Liu et al., 2019c). In brief, the HBV RT modeling structure was used to compare the binding conformations of wild-type and rtS78T mutant RT proteins with ETV and TDF. Autodock software (version 4.2.6, molecular graphics laboratory) was used to simulate the docking process and evaluate the binding energy of HBV RT with ETV and TDF. Autodock is a component of the MGLTools that is freely available for academic use. More detailed information about Autodock is available at the following website: http://mgltools.scripps.edu/.

2.7. Statistical analyses

Data are presented as mean ± standard deviation or median (interquartile range). Differences between groups were examined by Student's *t*-test (two-tailed) or a chi-square test. Statistical analyses were carried out using the Statistical Program for Social Sciences (SPSS 18.0 for Windows; SPSS Inc., Chicago, IL, USA). A P-value < 0.05 was considered statistically significant.

3. Results

3.1. Clinical prevalence and features of the HBV rtS78T/sC69* mutation

The rtS78T/sC69* mutation was detected in 0.83% (182/22,009) of the patients by direct sequence analysis. Clinical features between rtS78T/sC69*-positive and rtS78T/sC69*-negative patients are shown in Table 1. The rtS78T/sC69* mutation was more frequently detected in older and male patients. No significant differences were observed between the two groups in serum HBV DNA and HBsAg levels, as well as

Table 1
Analysis of clinical features between rtS78T/sC69*-positive and rtS78T/sC69*-negative patients.

Features	rtS78T/sC69*-positive (n = 182)	rtS78T/sC69*-negative (n = 21827)	*P*-value
Age (years)	46.63 ± 10.69	40.80 ± 12.82	< 0.001
Male [n(%)]	158 (86.81%)	17608(80.67%)	0.036
ALT (U/L)	49.50 (29.00–92.00)	42.00 (26.00–83.00)	0.203
AST (U/L)	52.00 (32.00–118.75)	38.00 (26.00–72.00)	0.101
HBV DNA (log_{10} IU/mL)	4.80 ± 2.00	5.05 ± 1.88	0.101
HBsAg (COI)	4735.28 ± 3254.57	4533.94 ± 3505.21	0.435
Genotype (C%/B%)	95.60/2.20	84.51/14.56	< 0.001
LAM/LdT-experienced	80 (43.96%)	10857 (49.74%)	0.137
ADV-experienced	122 (67.03%)	11344 (51.97%)	< 0.001
ETV-experienced	53 (29.12%)	6117 (28.02%)	0.743
TDF-experienced	1 (0.55%)	105 (0.48%)	0.894
Coexistent with LAM-r/LdT-r	11 (6.04%)	4974 (22.79%)	< 0.001
Coexistent with ADV-r	26 (14.29%)	1643 (7.53%)	0.001
Coexistent with ETV-r	3 (1.65%)	1295 (5.93%)	0.015

ALT, alanine aminotransferase; AST, aspartate aminotransferase; COI, cut-off index; LAM, lamivudine; LdT, telbivudine; ADV, adefovirdipivoxil; ETV, entecavir; TDF, tenofovirdisoproxil fumarate. LAM-r, lamivudine-resistance mutation (rtM204V/I); LdT-r, telbivudine-resistance mutation (rtM204I); ADV-r, adefovir-resistance mutation (rtA181V/rtN236T); ETV-r, entecavir-resistance mutation (rtM204V/I plus rt184/202/250 substitution).

the tested biochemical parameters. Compared to rtS78T/sC69*-negative patients, significantly more rtS78T/sC69*-positive patients had been treated with ADV exposure, with similar rates of LAM/LdT, ETV, and TDF treatment. The HBV genotype distribution of the rtS78T/sC69*-positive patients was significantly different from that of the rtS78T/sC69*-negative patients across the studied population (HBV/C 95.60% *vs.* 84.51%, $P < 0.01$).

3.2. Profile of HBV rtS78T/sC69* mutation

As shown in Fig. 1, the serine residue at position 78 was encoded by TCC in the RT region of the wild-type strain. The change of the codon from TCC to ACC led to the change of serine to threonine in the RT region and simultaneously introduced a TGA stop codon in S region at sC69 (sC69*). Coexistence of the rtS78T/sC69* mutation and wild-type was commonly detected (79.1%) in direct sequence analysis. UDPS verified that 99.4% samples had the coexistence and median frequency (inter-quartile range) of rtT78/sC69* mutant in the 170 samples was 42.0% (28.2%, 60.2%) (Supplementary Table 1). In drug-refractory patients, rtS78T/sC69* was usually detected with signature resistance mutations. Table 2 summarizes the rtS78T/sC69* mutation patterns with resistant mutations in the 182 patients with the rtS78T/sC69* mutation. Forty-one (22.5%) rtS78T/sC69*-positive samples were detected along with signature drug-resistant mutations, including the ADV resistance mutation rtA181V/N236T (n = 26), the LAM-resistance mutation rtM204V/I (n = 11), the ETV resistance mutation rtM204V/I plus rt184/250 substitution(s) (n = 3), and the LAM + ADV-resistance mutation rtL180M + A181T + M204V + N236T (n = 1).

3.3. Longitudinal analysis of clinical course of seven representative cases with rtS78T/sC69* mutation during antiviral treatment

Serial serum samples were obtained from seven rtS78T/sC69*-positive patients (A-G) and these patients were subjected to longitudinal analysis.

Patient A initially received ADV monotherapy followed by the addition of LAM. The rtS78T/sC69* mutant was detected in sample A1 (40% of the tested clones) and the rtS78T/sC69* + A181T mutant was detected in sample A2 (87% of the tested clones). Later, the therapy was switched to ETV monotherapy, which effectively suppressed viral load to an undetectable level (Fig. 2A).

Patient B had received ADV monotherapy before treatment with ETV. The rtS78T/sC69*-containing mutants, including rtS78T/sC69*, rtS78T/sC69* + A181T, rtS78T/sC69* + N236T, and/or rtS78T/sC69* + A181T + N236T, were detected in various proportions during ETV therapy in samples B1, B2, and B3, and after the suspension of ETV therapy in sample B4 (Fig. 2B).

Patients C and D successively received sequential LAM and ETV

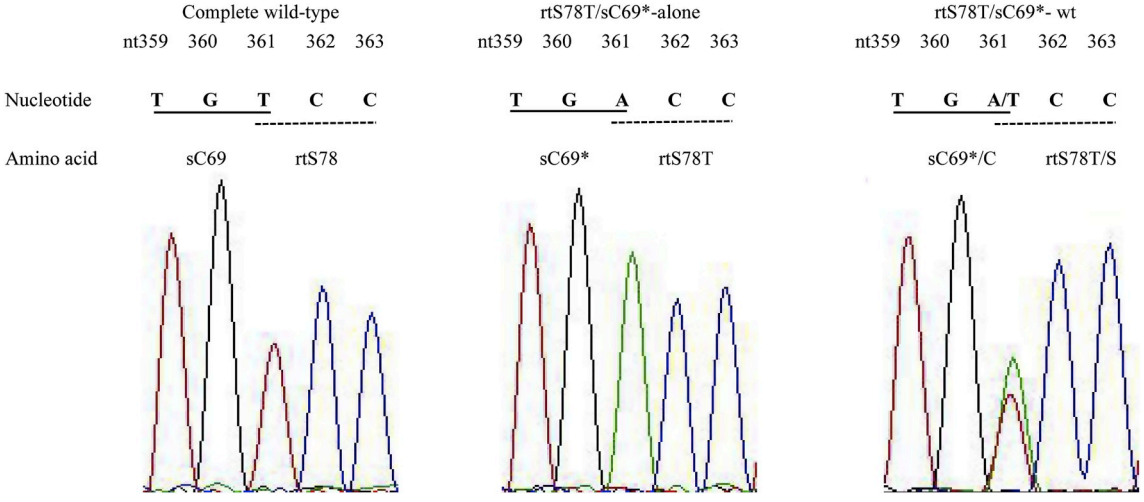

Fig. 1. HBV rtS78T/sC69stop mutation. The black full underline indicates amino acids in the S region and the dashed line indicates amino acids in the reverse-transcriptase region.

Table 2
Direct sequence analysis of the rtS78T/sC69* with NAs resistance mutation patterns across the 182 patients' samples.

Type	Mutation pattern	Number
S78T alone (n = 141)	S78T/sC69*	141
Coexistent with LAM-r (n = 11)	S78T/sC69* + M204I	4
	S78T/sC69* + L180M + M204I	3
	S78T/sC69* + L180M + M204I/V	2
	S78T/sC69* + L180M + M204V	2
Coexistent with ADV-r (n = 26)	S78T/sC69* + N236T ± A181T	20
	S78T/sC69* + A181V + N236T ± A181T	4
	S78T/sC69* + A181V ± A181T	2
Coexistent with ETV-r (n = 3)	S78T/sC69* + L180M + T184A + M204I/V	1
	S78T/sC69* + L180M + T184A + M204V	1
	S78T/sC69* + M204I/V + M250L	1
Coexistent with LAM + ADV-r (n = 1)	S78T/sC69* + L180M + A181T + M204V + N236T	1

NAs, nucleoside/nucleotide analogues; LAM-r, lamivudine-resistance mutation; ADV-r, adefovir-resistance mutation; ETV-r, entecavir-resistance mutation.

monotherapies, and ETV + ADV combination therapy. For patient C, mutants rtL180M + T184A + M204V (48%), rtM204V (14%), rtS78T/sC69* + L180M + T184A (9%), rtS78T/sC69* + L180M + T184A + M204V (9%), rtS78T/sC69* (5%), and rtS78T/sC69* + M204V (5%) were detected in sample C1 at the end of ETV therapy. Furthermore, mutants rtS78T/sC69* (26%), rtA181V (8%), and rtT184A + M204I (4%) were detected in sample C2 during the break of ETV + ADV therapy (Fig. 2C). For patient D, rtS78T/sC69* mutant (17% in D1, 45% in D2, 20% in D3) and rtS78T/sC69* + A181T mutant (15% in D2, 45% in D3) were detected during ETV + ADV therapy (Fig. 2D).

Patient E successively received LAM, ETV, ETV + ADV, ADV, and TDF therapies. The rtS78T/sC69* + A181T + N236T mutant (55%) was detected in concomitance with resistance mutants rtA181T + N236T (35%) and rtA181V + N236T (10%) in sample E2 at the end of ADV therapy (Fig. 2E). Patient F successively received LAM, LAM + ADV, ETV + ADV, and ETV + TDF therapies. The rtS78T/sC69* mutant (5% in F1, 15% in F2) and rtS78T/sC69* + A181T mutant (10% in F1 and F2) were detected during the ETV + ADV therapy (Fig. 2F). Patient G successively received LAM, LAM + ADV, ETV, and ETV + TDF therapies. The mutants rtS78T/sC69* + A181T + M204I (50%) was predominantly detected in concomitance with mutants rtS78T/sC69* + M204I (17%), rtS78T/sC69* + L180M + M204I (6%), and rtS78T/sC69* + S202G + M204I (6%) were detected in sample G2 during ETV therapy (Fig. 2G).

The representative cloned rtS78T/sC69* sequences from each patient, together with other rtS78T/sC69*-containing sequences, have been deposited in GenBank (accession number: MK806447 − MK806464).

3.4. Influence of rtS78T/sC69* on HBV replication, transcription, and HBsAg production

Three replication-competent HBV vectors containing the rtS78T/sC69* mutation (M1) and its revertant rtS78/sC69 (lab), of which rtS78T/sC69* was eliminated artificially (M2), and a natural patient-derived wild-type strain were constructed and subjected to transient transfection of HepG2 cells. Compared to the wild-type, M1 had a similar extracellular HBV DNA level but 1.44-fold higher intracellular HBV DNA level; by contrast, both extracellular and intracellular HBV RNA levels were significantly increased (Fig. 3A and B). Using HBsAg detection reagents (either EILSA or Roche qualitative Elecsys assays), extracellular and intracellular HBsAg were undetectable for M1 but comparable to the levels of the wild-type for M2 (Fig. 3C and D). Using preS2 detection reagent (EILSA), extracellular preS2 levels were similar for the wild-type, M1, and M2; while intracellular preS2 level of M1 had 1.39-fold increase compared to that of the wild-type (Fig. 3E).

3.5. Phenotypic analysis of drug susceptibility

The rtS78T/sC69* mutant and its revertant rtS78/sC69 (lab) were tested for susceptibility to LAM, ADV, ETV, and TDF. The mutant exhibited 3.73-, 1.61-, 4.76-, and 3.71-fold increases in EC_{50} to LAM, ADV, ETV, and TDF compared to the wild-type strain, respectively (Table 3). Artificial elimination of rtS78T/sC69* from the rtS78T/sC69* mutant had a correspondingly limited effect on the susceptibility to the four drugs. The mutants with coexistence of S78T/C69* and signature resistance mutations had very limited change on drug susceptibility compared to counterpart mutants only with the signature resistance mutations (Supplementary Table 2).

3.6. Molecular modeling of the influence of rtS78T/sC69* on viral binding to ETV and TDF

The influence of rtS78T/sC69* mutation on the binding affinity of HBV RT to ETV and TDF were evaluated using Autodock software. The modeling structures of the ligand and receptor are shown in Fig. 4. The amino acid position rtS78 or rtT78 was located away from the drug interaction site. Consistent with the fact that lower binding energy signifies a more stable conformation, the wild-type RT domain had a lower binding energy (−5.54 kcal/mol), binding to ETV by three hydrogen bonds (one O–H:O hydrogen bond and two N–H:O bonds). The rtS78T mutant displayed a changed binding site and slightly increased binding energy to −5.33 kcal/mol (Table 4). The introduction of rtS78T in the three-dimensional model of RT produced a slight decrease in RT binding affinity to TDF from −6.26 kcal/mol of wild-type protein to −6.13 kcal/mol of mutant protein without change in the conformation of the binding site.

4. Discussion

Typical viral resistance mutations have several distinguishing characteristics, including an association with drug therapy resulting in viral rebound or inadequate virological response, appearance in multiple patients exposed to the drug, ability to confer resistance to the virus *in vitro*, and reversion to wild-type sequence in the absence of the selective antiviral pressure (Yang et al., 2002).

The present study provides the first data concerning clinically prevalent properties of the rtS78T/sC69* mutation. Based on the investigation involving 22,009 chronic HBV-infected patients, rtS78T mutation was detected in 182 (0.83%) patients. In all 182 patients, the rtS78T mutation was caused by the nucleotide change of TCC to ACC, which simultaneously introduced a stop codon in the overlapping S region and caused the sC69* mutation. The rtS78T/sC69* mutation appeared unlike a typical ETV/TDF-association-mutation based on the following clinical presentations. First, ETV and TDF exposure had no

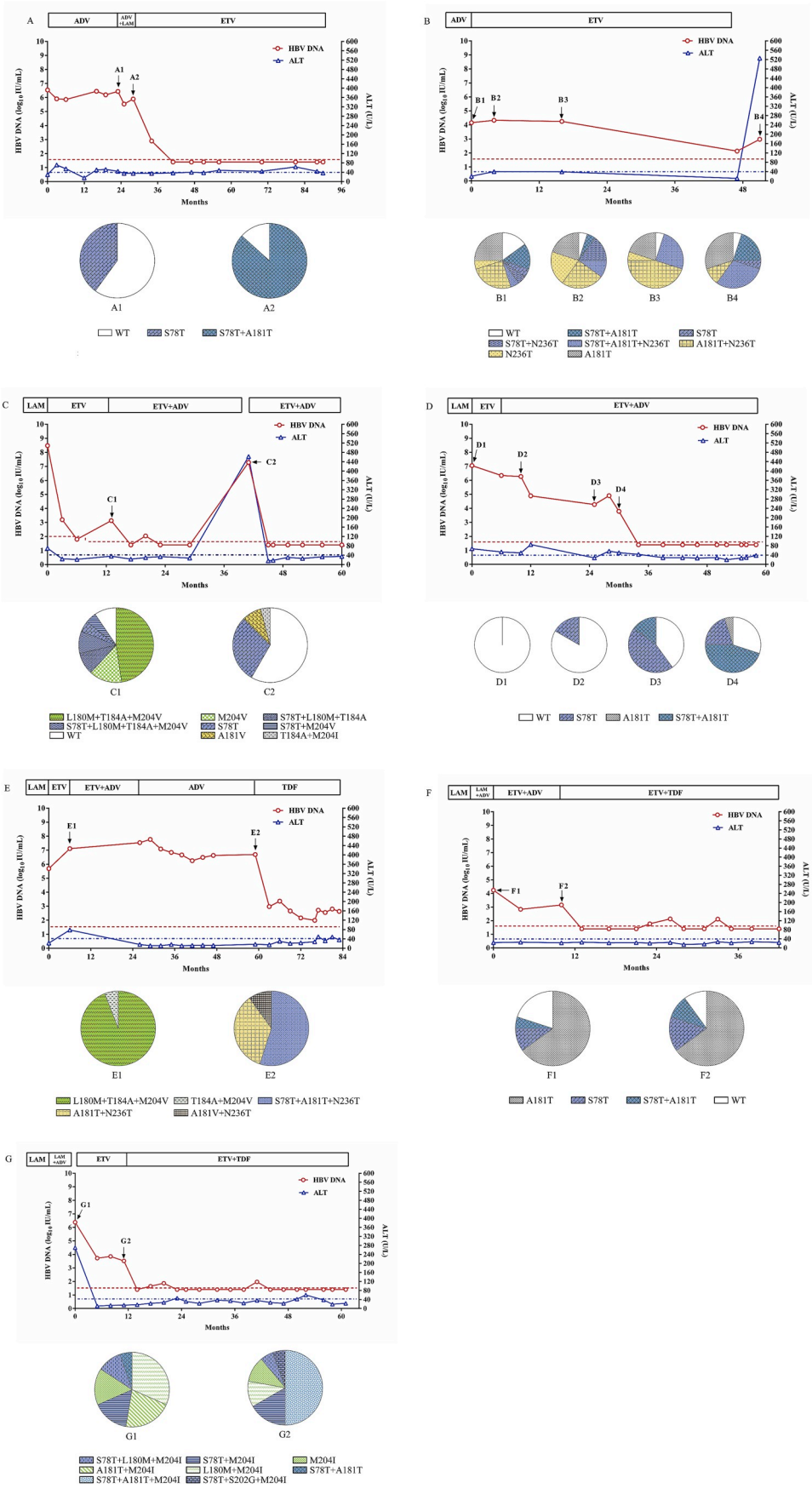

Fig. 2. The evolution of drug-resistance HBV mutants and clinical responses during the antiviral therapies of seven representative patients. The dynamic changes of serum HBV DNA and alanine aminotransferase (ALT) levels are shown along with the antiviral therapies. The duration (months) of the antiviral therapies is indicated by the bars above the graph and serum samples from the patient for cloning are indicated by arrows on the graph. Two dashed lines show the lower detection limit of HBV DNA from two successive periods of clinical (100 IU/mL, 40 IU/mL) and normal ALT levels (40 U/L). Proportions of wild-type (WT) and mutant HBV strains in the viral reverse-transcriptase from each sample are depicted by a series of pie charts. The abbreviations for the drugs are: LAM, lamivudine; ADV, adefovirdipivoxil; ETV, entecavir; TDF, tenofovirdisoproxil fumarate.

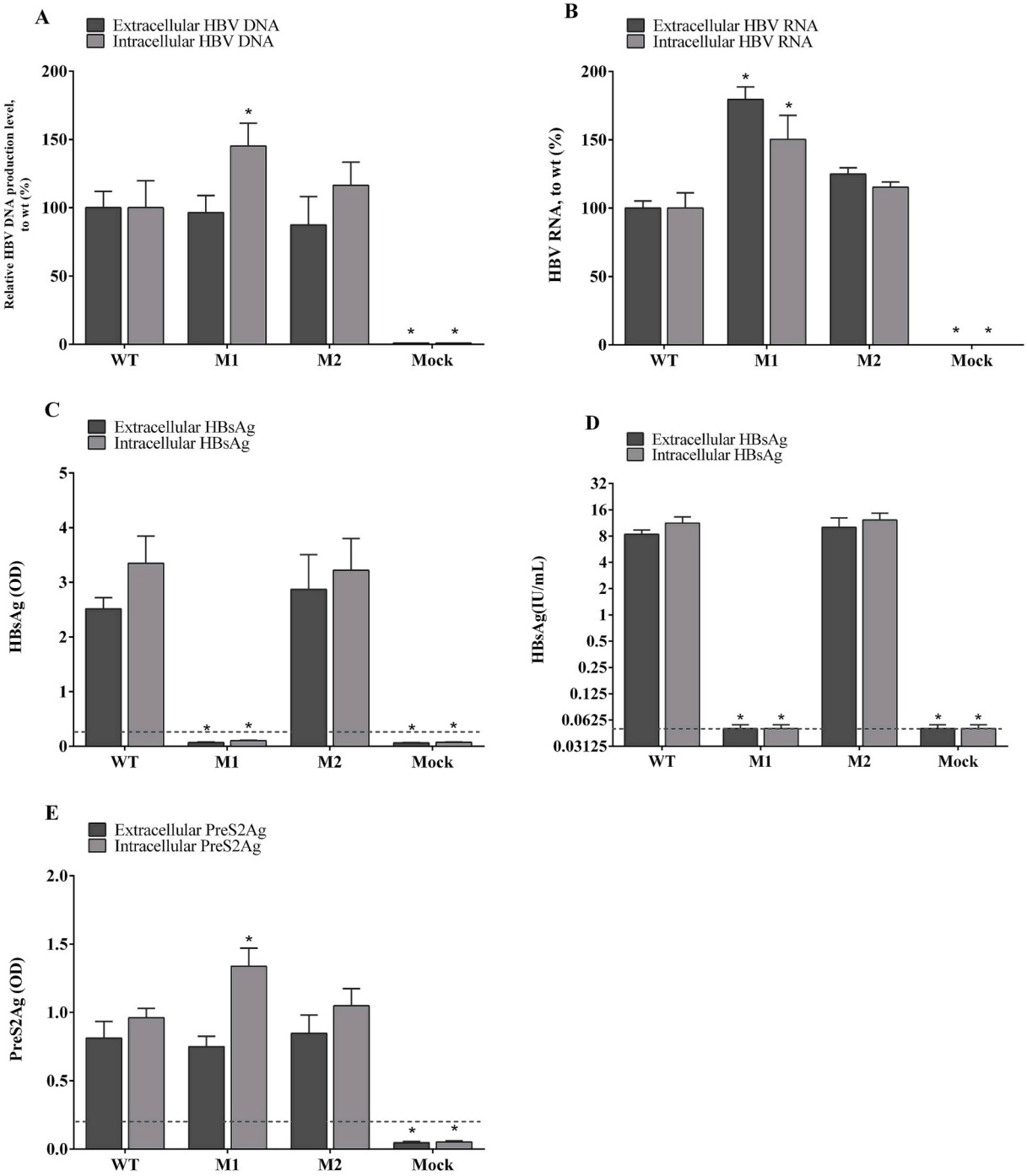

Fig. 3. Measurements of HBV DNA, RNA, HBsAg, and PreS2 levels in individual recombinant vector-transfected HepG2 cells. (A) Relative HBV DNA and (B) HBV RNA levels in cell supernatants (dark grey) and within cells (light grey) of mutants versus wild-type strain. (C) HBsAg levels detected with a commercial ELISA kit. Dashed line represents the cut-off value of the experiments [optical density (OD) 0.22]. (D) HBsAg levels detected with Roche quantitative Elecsys assay. Dashed line represents lower limit of detection for HBsAg (0.05IU/mL). (E) PreS2 levels detected with a commercial ELISA kit. Dashed line represents the cut-off value of the experiments [optical density (OD) 0.20]. Experiments were performed at least three times independently. Abbreviations are: M1, rtS78T/sC69*; M2, rtS78/sC69 (lab); WT, wild-type strain; Mock, empty plasmid vector; OD, optical density. *, $P < 0.05$ compared to wild-type.

significant difference in the rtS78T/sC69*-positive and rtS78T/sC69*-negative patients (Table 1). Second, the rtS78T/sC69* mutation was detected not only in those who underwent the treatment of ETV/TDF (54/182), but also in those who underwent the treatment of LAM (31/182), ADV (67/182), and LAM + ADV (30/182). Third, longitudinal analysis of representative cases showed that the rtS78T/sC69* mutation could be detected without exposure to ETV/TDF in patients for whom ETV monotherapy was efficacious (patient A), its detection at viral rebound during ETV therapy was concomitant with signature ETV resistance mutation rtL180M + T184A + M204V (patient C), and ETV + TDF rescue therapy was effective in suppressing viral replication of rtS78T/sC69*-containing mutants (patients F and G) (Fig. 2).

Although rtS78T/sC69* was not a primary ADV-resistance mutation, the proportion of ADV exposure was higher in rtS78T/sC69*-positive patients than that in rtS78T/sC69*-negative patients. It appeared that ADV exposure might facilitate the development of rtS78T/sC69*

Table 3
Drug susceptible analysis of rtS78T/sC69* mutants.

Viral strain	Lamivudine		Adefovir		Entecavir		Tenofovir	
	EC_{50} (μmol/L)	Fold	EC_{50} (μmol/L)	Fold	EC_{50} (μmol/L)	Fold	EC_{50} (μmol/L)	Fold
Wild-type	0.076 ± 0.008	1.00	2.15 ± 0.04	1.00	0.00071 ± 0.00026	1.00	1.08 ± 0.06	1.00
rtS78T/sC69*	0.28 ± 0.02	3.73	3.47 ± 1.12	1.61	0.0034 ± 0.0021	4.76	4.00 ± 1.18	3.71
rtS78/sC69(lab)	0.12 ± 0.04	1.59	3.50 ± 0.05	1.63	0.0012 ± 0.0001	1.68	2.19 ± 0.02	2.03

EC_{50}, half maximal effective concentration; Fold, EC_{50} of a mutant/EC_{50} of the wild-type; (lab), laboratory-modified mutants by reverse site-directed mutagenesis.

mutation. Similar phenomenon was observed for rtA181T/sW172* mutation which was much more frequently detected in LAM- or ADV-experienced patients while itself was insufficient to cause LAM or ADV resistance (Zhao et al., 2018). Signature ADV-resistance mutation was more frequently detected in rtS78T/sC69*-positive samples than in rtS78T/sC69*-negative samples, suggesting that rtS78T/sC69* mutation might facilitate the development of signature ADV-resistance mutation. In light of our results, HBV genotype C might be a favored factor for the development of rtS78T/sC69* mutation. Likewise, we also observed that rtA181T/sW172* mutation was more frequently detected in genotype C-infected patients than in genotype B-infected patients (Zhao et al., 2018). The mechanism underlying remains classification. The rtS78T/sC69* mutation was commonly concomitant with the wild-type. It has been verified that sC69*mutant unable to produce functional HBsAg could be rescued by wild-type protein expression, which presumably reflected a requirement for their spread and survival *in vivo* (Xiang et al., 2017). Thus, it was explainable that rtS78T/sC69*-positive and rtS78T/sC69*-negative patients had similar HBsAg levels observed in clinical analysis (Table 1).

The rtS78T/sC69* mutant had similar extracellular preS2 level compared to the wild-type (Fig. 3E), suggesting that rtS78T/sC69*-truncated HBsAg was reactive to anti-preS2 and able to be secreted. The results were mutually supportive with the results by Shirvani-Dastgerdi et al., i.e., HBV DNA-containing particles in supernatants of rtS78T/sC69* mutant-transfected Hepatoma cells could be captured by anti-preS1-antibody and protein-A/G Plus-agarose precipitation.

Table 4
Binding energy and hydrogen bonds of viral RT to ETV and TDF.

Ligand	HBV strains	Binding energy (ΔG: kcal/mol)	N–H:O bonds	O–H:O bonds	N–H:N bonds
ETV	wild-type	−5.54	2	1	0
	rtS78T	−5.33	0	2	1
TDF	wild-type	−6.26	1	0	1
	rtS78T	−6.13	1	0	1

RT, reverse-transcriptase; ETV, entecavir; TDF, Tenofovirdisoproxil fumarate.

Phenotypic analysis showed that rtS78T/sC69* mutant exhibited 3.73-, 1.61-, 4.76-, and 3.71-fold increases in EC_{50} to LAM, ADV, ETV, and TDF compared to the wild-type strain, respectively. These slight decreases of ETV and TDF susceptibilities were consistent with the results of a previous study (Shirvani-Dastgerdi et al., 2017). Artificial elimination of rtS78T/sC69* had a correspondingly limited effect on the susceptibility to the drugs. These findings suggest that in phenotypic analyses, a small decrease in ADV susceptibility (2- to 9-fold increase in EC_{50}) might confer clinical resistance. By contrast, LAM mutants had > 500-fold increase, and ETV and TDF mutants had > 10-fold increase (usually > 50-fold) in EC_{50}, which indicated clinical

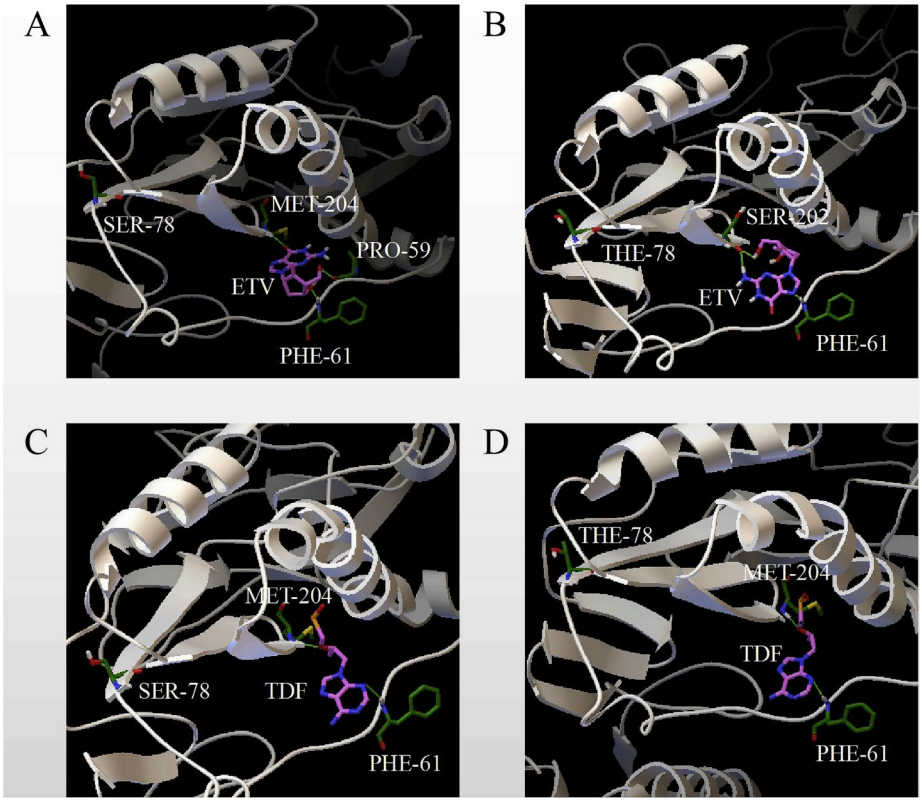

Fig. 4. Three-dimensional structures of ETV- and TDF-binding domains of viral RT. The effects of rtS78T mutation on the binding ability of HBV RT to ETV and TDF were evaluated using a homology model constructed based on the crystal structure of HIV RT. The binding domains of the wild-type with ETV (A), the mutant with ETV (B), the wild-type with TDF (C), and the mutant with TDF (D) are presented. The ligands (ETV and TDF)and interacting residues are shown in stick format. Green lines represent hydrogen bonds. The abbreviations are: ETV, entecavir; TDF, tenofovirdisoproxil fumarate; RT, reverse-transcriptase.

resistance (Lok et al., 2007; Lok and McMahon, 2009; Svarovskaia et al., 2013). Thus, the slight decreases of ETV and TDF susceptibility by the rtS78T/sC69* mutation were not sufficient to independently cause clinical ETV/TDF resistance. Molecular modeling showed that the rtS78T/sC69* mutant RT displayed only a minor change in the binding conformation and only a slight increase in binding energy to ETV and TDF compared to that of the wild-type RT. These observations provide circumstantial evidence that the rtS78T/sC69* mutation had no robust influence on ETV and TDF activity.

HBV has the potential to evolve under environmental pressure through the selection of adaptive mutations and the fittest mutants may emerge depending on their replication capacity, sensitivity to antivirals, and sensitivity to host immune responses (Rajoriya et al., 2017; Xue et al., 2017). Generally, mutant viruses are less fit, but may have a survival advantage in the presence of an antiviral agent (Villet et al., 2009; Zhang et al., 2018). Consistent with published results (Shirvani-Dastgerdi et al., 2017), we also observed that the rtS78T/sC69* mutant enhanced HBV replication and transcription activities, but abrogated regular HBsAg production. These characteristics might increase viral fitness during treatment with antivirals and exposure to immune stresses, and so could contribute to an inadequate virological response upon NA therapy in some cases.

A limitation of our study is its small sample size, as the serial serum samples were only available from seven patients and dynamic clinical information was not comprehensively available for all rtS78T/sC69*-positive patients.

In summary, we present the first data concerning the clinically prevalent properties of HBV rtS78T/sC69* mutation in a large cohort of Chinese patients. We also verified that the mutation changed the viral activities of replication, transcription, expression, and slightly decreased the viral susceptibility to ETV and TDF. However, rtS78T/sC69* mutation was not closely related to ETV/TDF treatment and itself appeared insufficient to confer drug resistance unless it coexisted with signature drug-resistance mutations. The data provide new insights into HBV drug resistance with clinical implications for resistance management.

Authors and contributors

DX and SL contributed substantially to the conception and design of the study. DL, RC, LL, and XL were in charge of the acquisition, analysis, or interpretation of data. QL, BH, MN and JW participated in patient sample collection and clinical information analysis. DX, SL, and YL drafted the manuscript or revised it critically for important intellectual content. All authors read and approved the final version of the manuscript.Acknowledgments

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Conflicts of interest

All authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.104579.

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