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Prevalence of the entecavir-resistance-inducing mutation rtA186T in a large cohort of Chinese hepatitis B virus patients

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ABSTRACT

This study aimed to clarify whether rtA186T and rtI163V substitutions of hepatitis B virus (HBV) contributed to entecavir (ETV) resistance. A total of 22,009 Chinese patients with chronic HBV infection who received resistance testing at Beijing 302 Hospital from 2007 to 2016 were enrolled. Among them, 6170 patients had been treated with ETV. The HBV reverse transcriptase gene was screened by direct sequencing and verified by clonal sequencing. Phenotypic analysis was performed for evaluating replication capacity and drug susceptibility. Classical ETV-resistance mutations rtT184/S202/M250substitution + rtM204V/I ± L180M (LAM-r), rtA186T, and rtI163V were detected in 1252 (5.69%), 14 (0.06%), and 230 (1.05%) of the 22,009 patients, respectively. The rtA186T mutation always coexisted with LAM-r, but not with rtI163V. The 14 rtA186T-positive patients were all treated with LAM and ETV, and the emergence of the rtA186T + LAM-r was closely associated with virological breakthrough or inadequate virological response to ETV. By contrast, the emergence of rtI163V was not related to ETV treatment. Six rtA186T-positive patients were followed up longitudinally, showing that these patients all had received sequential adefovir and LAM monotherapies prior to ETV treatment. Compared to wild-type strain, two patient-derived mutants' rtL180M + A186T + M204V and rtL180M + T184S + A186T + M204V had 86.7% and 89.2% decreased replication capacity, 210- and 555-fold increased ETV resistance, respectively; and artificial elimination of rtA186T largely restored their ETV sensitivity. The rtA186T mutants remained sensitive to tenofovir. In conclusion, our study confirmed that rtA186T plus LAM-r is a novel ETV-resistance mutation pattern which conferred ETV resistance in multiple Chinese patients.

1. Introduction

Hepatitis B virus (HBV) is a partially double-stranded DNA virus that causes severe diseases such as acute, chronic, and fulminant hepatitis, cirrhosis, and hepatocellular carcinoma (Tang et al., 2018). It has been estimated that more than 240 million people are chronically infected with HBV around the world and 93 million of them are in China (Hou et al., 2017; Lampertico et al., 2017). Treatment of chronic HBV infection is aimed at persistently suppressing viral replication to prevent disease progression (Zoulim and Mason, 2012). Currently, five nucleos(t)ide analogues (NAs) are licensed for treating HBV infection in China: lamivudine (LAM), adefovir dipivoxil (ADV), entecavir (ETV), telbivudine (LdT), and tenofovir disoproxil fumarate (TDF), which was recently approved in 2014. As major anti-HBV agents, NAs may effectively inhibit HBV replication through targeting the reverse-transcriptase (RT) region of HBV polymerase. However, NAs have no direct effect on the covalently closed circular DNA of the original HBV replication template and thus patients require long-term treatment that may increase the risk of drug resistance (Menéndez-Arias et al., 2014).

Classical primary resistance mutations include rtM204V/I ± L180M (LAM-r) for LAM (rtM204I was also an LdT-resistance mutation), rtA181V/rtN236T for ADV, LAM-r along with substitutions at rtT184

Abbreviations: HBV, hepatitis B virus; NAs, nucleos(t)ide analogues; LAM, lamivudine; ADV, adefovir dipivoxil; ETV, entecavir; LdT, telbivudine; TDF, tenofovir disoproxil fumarate; RT, reverse transcriptase; nt, nucleotide; HBsAg, hepatitis B surface antigen; LLOD, lower limit of detection; ALT, alanine aminotransferase; EC_{50}, half maximal effective concentration; VBT, virological breakthrough

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(A/C/F/G/I/L/M/S), rtS202 (C/G/I), and/or rtM250 (I/L/V) for ETV (Lok et al., 2007; Zoulim and Locarnini, 2009). Although ETV resistance rarely occurred in NAs-naïve patients, the rates of resistance increased to 51% in LAM-refractory patients (Colonno et al., 2006; Tenney et al., 2009). The reason lies in LAM-r can favor rapid selection of additional mutants with overt resistance against ETV (Geipel et al., 2015). Recently, rtA186T and rtI163V mutations were detected in an ETV-refractory patient and the two mutations were reported to confer ETV resistance in concomitance with LAM-r (Hayashi et al., 2015). So far, no other investigation has verified this case study-based result. In recent years, we have identified several unusual HBV RT mutations associated with LAM-, ADV-, and multidrug-resistance of HBV on the basis of genotypic analysis of large numbers of NA-experienced chronic HBV-infected patients in combination with phenotypic analysis (Ji et al., 2012; Liu et al., 2010, 2011, 2014a, 2014b, 2015, 2016). In this study, we aimed to clarify whether rtA186T and rtI163V mutations really contribute to ETV resistance mutations.

2. Materials and methods

2.1. Patient samples

A total of 29,639 serum samples from 22,009 chronic HBV-infected patients who visited 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 received NAs treatment, and among them, 28.03% (6170/22,009) received either ETV treatment only or in combination with other NAs treatment. 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. The standards for diagnosing these illnesses categories were according to Guideline on prevention and treatment of chronic hepatitis B in China (2005) (Chinese Society of Hepatology et al., 2007). Patients who were co-infected with other hepatitis viruses or human immunodeficiency virus were excluded. The enrolled patients were from the Database of Beijing 302 Hospital and all 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, quantitation of HBV DNA

Biochemical and serological markers and HBV DNA levels from the patients were routinely detected in the Central Clinical Laboratory of Beijing 302 Hospital. HBV DNA levels were determined using a real-time quantitative PCR kit (Fosun Pharmaceutical Co., Ltd., Shanghai, China) with a lower detection limit of 100 IU/ml before April 2012 and 40 IU/ml afterward.

2.3. HBV genotypic resistance analysis and phylogenetic tree analysis

The methods used for HBV genotypic resistance analysis and phylogenetic tree analysis were performed as previously described (Li et al., 2010; Liu et al., 2011). A 1225 bp long viral gene fragment (nucleotides (nt) 54–1278) that covers the full-length RT genetic region (nt 130–1161) and overlaps the S genetic region (nt 155–835) was amplified using a one-tube nested PCR assay with a 10 IU/ml LLOD with an input of 200 μL serum, which was more sensitive than the regular HBV DNA quantitation assay used in the clinic. Clonal sequencing of the RT gene was performed using the TA cloning strategy.

2.4. Construction of viral amplicons containing 1.1 mer HBV genome

The amplicons containing either the various 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 elsewhere (Ji et al., 2012; Zoulim, 2006). Four rtA186T-related mutants were used in the construction, including two clinical isolates and two laboratory-modified ones (lab). The rtL180M + M204V(lab) and rtL180M + T184S + M204V(lab) mutants were generated by reverse site-directed mutagenesis using rtL180M + A186T + M204V and rtL180M + T184S + A186T + M204V mutant template sequences, respectively. A Quick Change Lightning site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) was applied according to the manufacturer's instructions. The primers (senses) were 5′-CAGTTTACTAGT **G**CCATTTGTTCAG-3′ for generating the rtL180M + M204V(lab), and 5′-CAGTTTTCTAGT**G**CCATTTGTTCAG-3′ for generating the rtL180M + T184S + M204V(lab). In addition, three rtI163V-related mutant strains were constructed in the same way, including rtI163V, rtI163V + rtL180M + M204V, and rtL180M + M204V(lab). The laboratory strain was generated using rtI163V + L180M + M204V mutant template sequences. The primers (senses) were 5′-GTATTCCCATCCCATC**A**TCC TGG-3'. The wild-type amplicon was also constructed and the constructs were all verified by DNA sequencing.

2.5. Assessment of viral replication capacity and drug susceptibility

The phenotypic analysis was performed as previously described (Liu et al., 2015, 2016). Briefly, the mutants and wild-type HBV genomic amplicons were transiently transfected into HepG2 cells and cultured in the presence or absence of serially diluted NAs. Relative replication capacity of a mutant strain was compared to wild-type in the absence of NAs. Relative replication capacities of mutant strains *vs.* the wild-type strain from the same patient were together analyzed to minimize the influence brought by patient difference. Drug susceptibility was determined by comparing the 50% effective concentration of the drug (EC_{50}) of the mutant to the wild-type. The serial concentrations were 0, 0.001, 0.01, 0.1, 1.0, and 10 μmol/L for ETV, and 0, 0.78, 3.125, 12.5, 50, and 200 μmol/L for TDF. The experiments were performed at least three times independently.

2.6. Statistical analysis

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 analysis was carried out using the Statistical Program for Social Sciences (SPSS 18.0 for Windows; SPSS Inc., Chicago, IL, USA). A *P*-value of < 0.05 was considered statistically significant.

3. Results

3.1. Clinically prevalent profile of classical ETV-resistance mutations

HBV ETV-resistance mutations were detected in 5.69% (1252/22,009) of total patients or 20.29% (1252/6170) patients that had received ETV treatment. Among the 1252 patients, HBV genotypes B, C, and D (HBV/B, HBV/C, and HBV/D) were determined for 143 (11.42%), 1096 (87.54%), and 13 (1.04%) patients, respectively. The mutation patterns were LAM-r + rtT184 (41.29%), rtS202 (35.86%), rtM250 (14.38%), rtT184 + S202 (7.35%), rtT184 + M250 (0.72%), and rtS202 + M250 (0.40%).

3.2. Clinical prevalence and features of patients with the HBV rtI163V mutation

The rtI163V mutation was detected in 1.05% (230/22,009) of the patients. Clinical and virological features between rtI163V-positive and rtI163V-negative patients are shown in Table 1. No significant difference was observed in either ETV exposure or in ETV mutation coexistence between the rtI163V-negative and rtI163-positive patients. No

Table 1
Analysis of clinical features between rtI163V-positive and rtI163V-negative patients.

Features	rtI163V-positive (n = 230)	rtI163V-negative (n = 21,779)	*P* value
Age (year)	42.81 ± 11.88	40.97 ± 13.31	0.021
Gender (male)	193 (83.91%)	17,637 (80.98%)	0.260
HBV DNA (log_{10} IU/mL)	5.08 (3.54–6.48)	4.87 (3.25–6.38)	0.166
Genotype (C %/B %)	89.43%/10.57%	84.76%/15.00%	0.052
LAM/LdT-experienced	150 (65.22%)	10,787 (49.53%)	< 0.001
ADV-experienced	148 (64.35%)	11,318 (51.97%)	< 0.001
ETV-experienced	67 (29.13%)	6103 (28.02%)	0.710
TDF-experienced	0 (0.00%)	106 (0.49%)	0.289
Coexistent with LAM/LdT-resistance mutation	55 (23.91%)	4930 (22.64%)	0.645
Coexistent with ADV-resistance mutation	30 (13.04%)	1639 (7.53%)	0.002
Coexistent with ETV-resistance mutation	14 (6.10%)	1238 (5.68%)	0.793

LAM, lamivudine; ADV, adefovir dipivoxil; ETV, entecavir; LdT, telbivudine; TDF, tenofovir disoproxil fumarate.

rtI163V was detected in combination with rtA186T.

3.3. Clinical prevalence and features of patients with the HBV rtA186T mutation

The rtA186T mutation was detected in 0.06% (14/22,009) of total patients or 0.23% (14/6170) of patients who had received ETV treatment. Clinical and virological features between rtA186T-positive and rtA186T-negative patients are shown in Table 2. Compared to rtA186T-negative patients, rtA186T-positive patients had significantly higher rates of ETV exposure and ETV resistance mutation coexistence.

Clinical information with the mutation patterns of the 14 rtA186T-positive patients is presented in Table 3. Coexistence of LAM-resistance mutations was detected in all rtA186T-positive patients. Clonal sequencing verified that LAM-r + rtA186T mutations were present in the same viral genomes from samples of the 14 patients and coexistence of classical ETV-resistance mutations (rtT184I, rtT184S, and rtM250V) was detected in some samples.

Table 2
Analysis of clinical features between rtA186T-positive and rtA186T-negative patients.

Features	rtA186T-positive (n = 14)	rtA186T-negative (n = 21,995)	*P* value
Age (year)	47.29 ± 6.34	40.99 ± 13.30	0.004
Gender (male)	13 (92.86%)	17,817 (81.00%)	0.258
HBV DNA (log_{10} IU/mL)	4.76 (3.43–5.45)	4.70 (3.08–6.23)	0.111
Genotype (C %/B %)	85.71%/14.29%	84.80%/14.95%	0.942
LAM/LdT-experienced	14 (100.00%)	10,923(49.66%)	< 0.001
ADV-experienced	11 (78.57%)	11,455 (52.08%)	0.047
ETV-experienced	14 (100.00%)	6156 (27.99%)	< 0.001
TDF-experienced	0 (0.00%)	106 (0.48%)	0.795
Coexistent with LAM/LdT-resistance mutation	14 (100.00%)	4971 (22.60%)	< 0.001
Coexistent with ADV-resistance mutation	1 (7.14%)	1669 (7.59%)	0.950
Coexistent with ETV-resistance mutation	3 (31.43%)	1250 (5.68%)	0.011

LAM, lamivudine; ADV, adefovir dipivoxil; ETV, entecavir; LdT, telbivudine; TDF, tenofovir disoproxil fumarate.

3.4. Longitudinal analysis of clinical course with HBV mutations during NA therapy

Serial serum samples were obtained from six rtA186T-positive patients (patient 1–6 in Table 3) and these patients were subjected to longitudinal analysis.

Patient 1 received IFN, LAM, ADV, and ETV sequential monotherapies for 24, 8, 2, and 64 months, respectively. At the time of virological breakthrough (VBT) in response to ETV (sample A1), rtL180M + A186T + M204V, rtL180M + M204V, rtL180M + A181C + M204V, and rtL180M + T184S + A186T + M204V mutants and the wild-type strain were detected in 58%, 19%, 13%, 5%, and 5% of the tested viral clones, respectively. Afterward, ADV was added on to ETV for 55 months and the viral load fluctuated between $2.1 - 3.5$ log_{10} IU/mL. In samples A2 and A3, the rtL180M + A186T + M204V mutant was predominantly detected (41% in A2, 100% in A3) in concomitance with rtL180M + M204V mutants (45% in A2), rtL180M + T184S + A186T + M204V mutants (9% in A2), and rtL180M + A181C + M204V mutants (5% in A2). In sample A4, the rtL180M + A181C + M204V mutant became predominant (Fig. 1A).

Patient 2 had received sequential LAM and ADV monotherapies for 22 and 51 months, respectively, before treatment with ETV. VBT occurred at month 36 with HBV DNA rebound to 4.5 log_{10} IU/mL. In sample B1 at this time-point, rtL180M + A186T + M204V and rtL180M + M204V mutants were detected, occurring in 83% and 17% of tested viral clones, respectively. At month 49, HBV DNA increased to 5.1 log_{10} IU/mL and the dominant mutation pattern was changed to rtL180M + T184L + M204V (Fig. 1B).

Patient 3 had received sequential LAM and ADV monotherapies for 36 and 9 months, respectively, before treatment with ETV. Viral rebound occurred due to the suspension of ETV treatment. Afterward, the patient was again treated with ETV and the rtL180M + A186T + M204V mutant was detected in sample C1 (25% of tested clones) and sample C2 (95% of tested clones) (Fig. 1C).

Patient 4 had received sequential LAM and ADV monotherapies for 18 and 30 months, respectively, before treatment with ETV. In sample D1 at baseline and sample D2 at month 15, the rtA181V mutation was predominantly detected. VBT occurred at month 49 and two rtL180M + A186T + M204V mutants, rtL180M + A186T + M204V (95%) and rtL180M + A186T + M204V + M250V (5%), were detected in sample D3. Afterward, ADV was added on to ETV which effectively suppressed HBV DNA levels and the wild-type strain became predominant in sample D4 at month 92 (Fig. 1D).

Patient 5 had received sequential LAM and ADV monotherapies for 48 and 12 months, respectively, before treatment with ETV + ADV. Viral rebound occurred due to the suspension of ETV + ADV treatment with an increased HBV DNA of 6.7 log_{10} IU/mL. In the E1 time-point sample, the rtT184I + A186T + M204I (33%) and rtT184I + M204I (67%) mutants were detected in the viral pool. At the subsequent E2 time-point, the rtT184I + A186T + M204V (55%), rtT184I + M204I (40%), and rtT184I + A186T + M204I + M250V (5%) mutants were detected in the viral pool. Afterward, TDF was added on to the ETV which effectively suppressed HBV DNA levels and the wild-type strain became predominant in sample E3 at month 73 of the combination therapy (Fig. 1E).

Patient 6 had received sequential LAM and ADV monotherapies for 33 and 18 months, respectively, before treatment with ETV. HBV DNA fluctuated between $2.6 - 5.8$ log_{10} IU/mL during the 60-month ETV treatment. In samples F1 (at month 10), F2 (at month 21), and F3 (at month 54), the rtL180M + A186T + M204V mutant was predominantly detected at 91%, 83%, and 100%, respectively (Fig. 1F).

3.5. Phylogenetic tree analysis

Phylogenetic tree analysis for the 18 cloned HBV RT gene sequences from the 14 rtA186T-positive patients is presented in Fig. 2A and shows

Table 3
Clinical information of the patients with rtA186T-containing mutations.

Patient	Age	Gender	Genotype (subtype)	HBeAg	HBsAg (IU/ml)	HBVDNA (log_{10} IU/ml)	ALT (U/L)	Antiviral schedule (month)	Direct sequencing	Clonal sequencing
P1	38	M	C (C2)	(+)	6081	7.26	130	IFN24→LAM8→ADV2→ETV64	rtL180M + A186T + M204V	rtL180M + A186T + M204V (12/21), rtL180M + M204V (4/21) rtL180M + A181C + M204V (3/21), WT (1/21) rtL180M + T184S + A186T + M204V (1/21)
P2	54	M	C (C2)	(−)	4364	4.48	21	LAM22→ADV51→ETV36	rtL180M + A186T + M204V	rtL180M + A186T + M204V (19/23), rtL180M + M204V (4/23)
P3	41	M	B (B2)	(−)	273	3.54	22	LAM36→ADV9→ETV18→NT2→ETV13	rtL180M + A186T + M204V	rtL180M + A186T + M204V (19/20), WT (1/20)
P4	38	M	C (C1)	(+)	6346	2.87	27	LAM18→ADV30→ETV49	rtL180M + A186T + M204V	rtL180M + A186T + M204V (21/22), rtL180M + A186T + M204V + M250V (1/22)
P5	50	M	C (C1)	(+)	2974	3.06	13	LAM48→ADV12→ETV + ADV36→NT5→ETV + ADV11	rtT184I + A186 T/A + M204I	rtT184I + A186T + M204I (11/20), rtT184I + M204I (8/20) rtT184I + A186T + M204I + M250V (1/20)
P6	57	F	C (C2)	(+)	5786	5.34	22	LAM33→ADV18→ETV30	rtL180M + A186T + M204V	rtL180M + A186T + M204V (21/23), rtL180M + M204V (2/23)
P7	47	M	C (C2)	(+)	3939	4.07	59	LAM48→ADV14→ETV10→ETV + ADV6	rtL180M + A186 T/A + M204V	rtL180M + A186T + M204V (14/23), rtL180M + M204V (8/23) rtL180M + T184A + M204V (1/23)
P8	52	M	B (B2)	(−)	5898	7.71	126	LAM36→ADV18→ETV58→ETV + ADV6	rtL180M + A186T + M204V	rtL180M + A186T + M204V (22/22)
P9	44	M	C (C2)	(−)	2150	3.39	32	LAM29→ETV4	rtL180M + A186T + M204V	rtL180M + A186T + M204V (20/21), rtM204I (1/21)
P10	52	M	C (C2)	(+)	3050	4.22	56	IFN12→LAM33→NT1→ADV36→ETV39	rtL180M + A186T + M204V	rtL180M + A186T + M204V (19/23), rtL180M + M204V (1/23), rtA181V (3/23)
P11	54	M	C (C2)	(+)	2247	2.96	38	LAM48→ADV36→ETV24	rtL180M + A186T + M204V	rtL180M + A186T + M204V (14/20), rtL180M + M204V (4/20), rtA181V + A186T + M204V (1/20), rtA181V (1/20)
P12	40	M	C (C2)	(+)	17,280	7.72	105	LAM48→ETV37	rtL180M + T184L + A186T + M204V	rtL180M + A186T + M204V (18/23), rtL180M + T184L + M204V (4/23) rtL180M + T184I + M204V (1/23)
P13	42	M	C (C2)	(+)	562	5.49	85	LAM36→ETV57	rtL180M + A186T + M204V	rtL180M + A186T + M204V (20/21), rtL180M + M204V (1/21)
P14	53	M	C (C2)	(+)	6259	4.55	74	LAM36→ADV27→ETV24	rtL180M + A186T + M204V	rtL180M + A186T + M204V (15/20), rtL180M + T184L + M204V (2/20) rtL180M + M204V (3/20)

IFN, interferon-a; LAM, lamivudine; ADV, adefovir dipivoxil; ETV, entecavir; NT, not treated with antivirals.

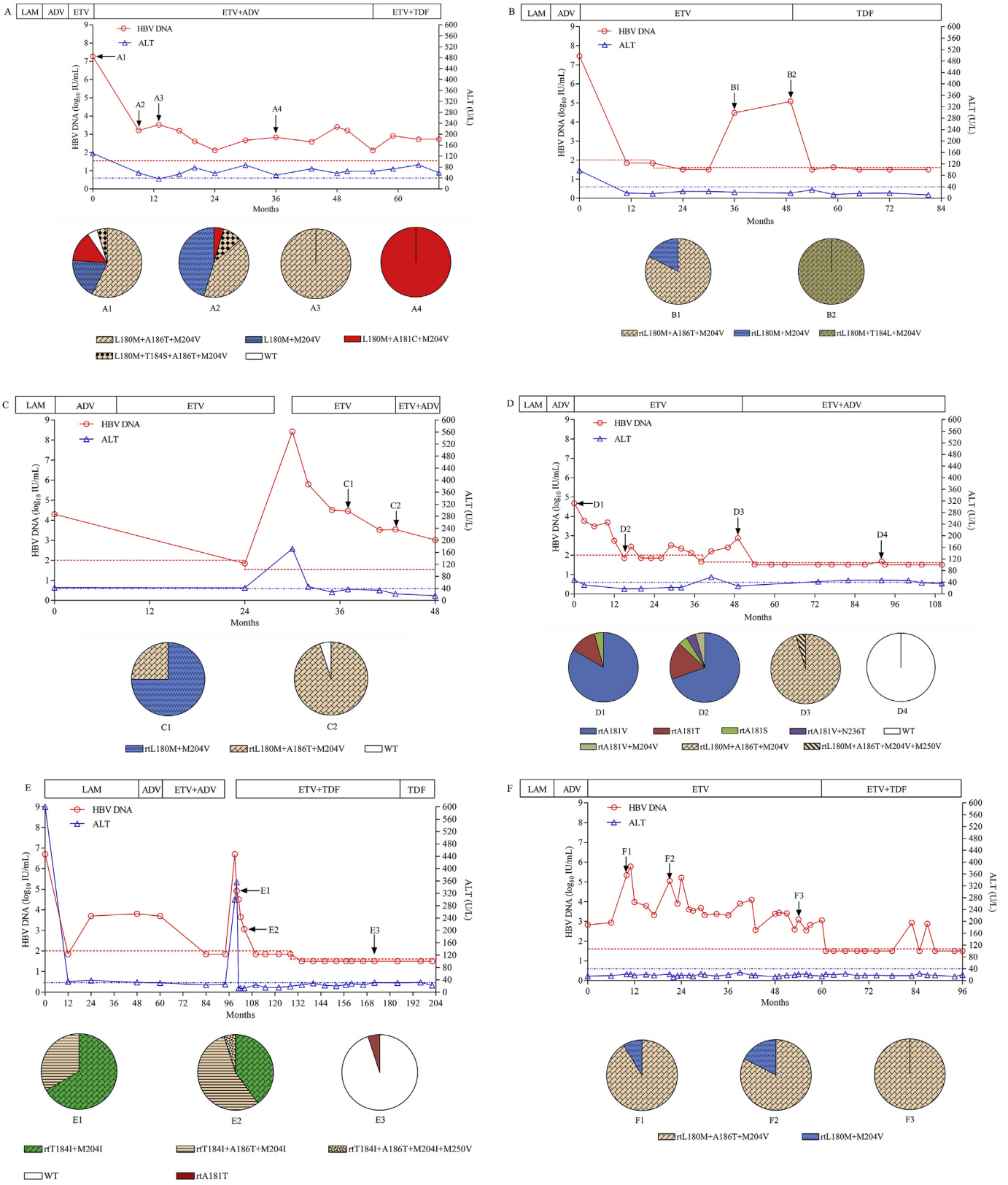

Fig. 1. The evolution of drug-resistant HBV and clinical responses during the antiviral therapies for representative patients (n = 6). The dynamic changes of serum HBV DNA and alanine aminotransferase (ALT) levels are shown along with the antiviral therapies. The duration (months) of the antiviral therapies 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. IFN, interferon-a; LAM, lamivudine; ADV, adefovir dipivoxil; ETV, entecavir; and TDF, tenofovir disoproxil fumarate.

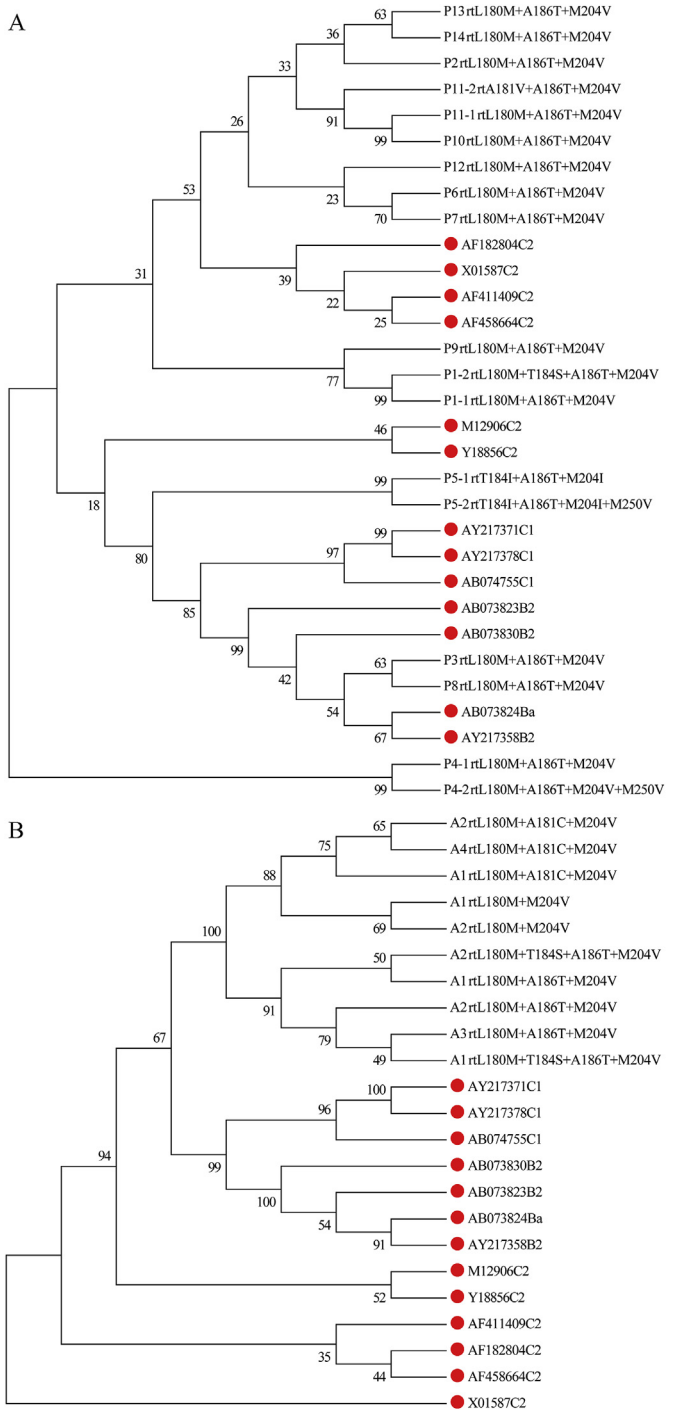

Fig. 2. Phylogenetic tree analysis for HBV RT sequences from rtA186T-positive patients. (A) Analysis of 19 RT sequences containing rtL180M + A186T + M204V mutations from 14 patients. P represents patient. (B) Analysis of 11 RT sequences from serial serum samples of a representative patient (patient 1). The reference sequences are marked with red solid circles. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

that 12 patients were infected with genotype C HBV and two patients (P3 and P8) were infected with genotype B HBV. A phylogenetic tree analysis for 10 cloned RT gene sequences from serial serum samples of patient 1 is presented in Fig. 2B, showing that the viral sequences tended to cluster by drug-resistance type.

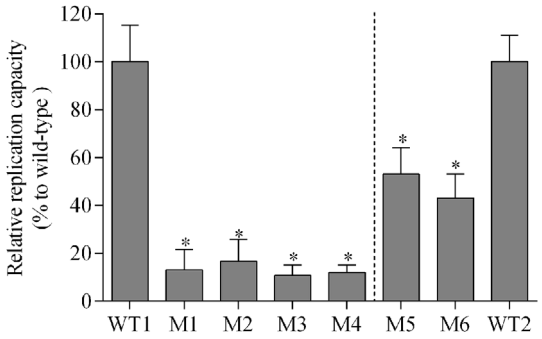

Fig. 3. Assessment of natural replication capacity of HBV. Relative replication capacities of one wild-type (WT1), two mutant strains isolated from serial samples from a representative patient (patient 1), and two laboratory-modified strains generated by reverse site-directed mutagenesis from two patient-derived mutants were analyzed and compared to the wild-type strain (taken as 100%) in the absence of drug treatment (right). Two classical entecavir resistance mutants and one wild-type strain (WT2) from another entecavir-refractory patient were taken as references for the analysis (left). Data are presented as the mean ± standard deviation. Experiments were performed at least three times independently. WT, wild-type; M1, rtL180M + A186T + M204V; M2, rtL180M + M204V(lab); M3, rtL180M + T184S + A186T + M204V; M4, rtL180M + T184S + M204V(lab); M5, rtL180M + S202G + M204V; M6, rtL180M + T184A + M204V. *$P < 0.05$ compared to wild-type.

3.6. Phenotypic analysis of replication capacity and drug susceptibility

For rtA186T mutation, phenotypic analysis was performed for two interesting representative mutants (rtL180M + A186T + M204V and rtL180M + T184S + A186T + M204V), and wild-type strain derived from patient 1, and two laboratory-modified viral strains, rtL180M + M204V (lab) and rtL180M + T184S + M204V(lab). In addition, two classical ETV-resistance mutants and one wild-type strain of genotype C HBV (GenBank accession numbers: GQ402156, GQ402157, and GQ402151, respectively) derived from another ETV-refractory patient were analyzed as the references. The mutation patterns were as follows: M1, rtL180M + A186T + M204V; M2, rtL180M + M204V(lab); M3, rtL180M + T184S + A186T + M204V; M4, rtL180M + T184S + M204V(lab); M5, rtL180M + S202G + M204V; M6, rtL180M + T184A + M204V. Compared to the wild-type, the replication capacity of the six mutants (M1 − M6) was significantly decreased ($P < 0.05$). M1 and M3 had a larger decrease (13.3% and 10.8% of wild-type 1, respectively) than M5 and M6 (53.0% and 43.0% of the wild-type 2, respectively). Change of rtT186 to A186 in M1 and M3 did not restore the replication capacity exhibited by M2 and M4, respectively (Fig. 3).

As shown in Table 4, the M1 and M3 mutants exhibited 210.45- and 555.22-fold resistance to ETV compared to wild-type 1, respectively. As references, the two classical ETV-resistance mutants exhibited 137.67- and 110.33-fold resistance to ETV compared to wild-type 2, respectively. The change of rtT186 to A186 in M1 and M3 largely restored their ETV sensitivity. The two rtA186T mutants had a slightly increased EC_{50} to TDF, 1.76- and 8.11-fold of wild-type 1, respectively, which was insufficient to cause TDF resistance.

For rtI163V mutation, three representative mutants were tested, i.e., rtI163V, rtI163V + L180M + M204V, and rtL180M + M204V(lab). The three mutants had 98.6%, 60.0% and 65.0% replication capacities of the wild-type, and 1.25-fold, 23.75-fold and 13.50-fold ETV EC_{50} values of the wild-type, respectively.

4. Discussion

Two types of HBV resistance mutations are defined, namely, primary resistance mutations and secondary (compensatory) mutations. Primary resistance mutations directly reduce susceptibility to NAs, but compensatory mutations can restore impaired viral replication capacity

Table 4
Drug susceptible analysis of representative HBV strains.

Viral strain	Entecavir		Tenofovir	
	EC_{50} (μmol/L)	Fold	EC_{50} (μmol/L)	Fold
Wild-type 1	0.00067 ± 0.00015	1.00	2.170 ± 0.234	1.00
rtL180M + A186T + M204V	0.141 ± 0.020	210.45	3.830 ± 0.607	1.76
rtL180M + T184S + A186T + M204V	0.372 ± 0.050	555.22	17.590 ± 2.128	8.11
rtL180M + M204V(lab)	0.020 ± 0.002	29.85	3.015 ± 0.064	1.39
rtL180M + T184S + M204V(lab)	0.056 ± 0.011	83.58	3.390 ± 0.190	1.56
Wild-type 2	0.003 ± 0.001	1.00	0.710 ± 0.110	1.00
rtL180M + S202G + M204V	0.413 ± 0.050	137.67	1.548 ± 0.290	2.18
rtL180M + T184A + M204V	0.331 ± 0.040	110.33	1.426 ± 0.250	2.01

EC_{50}, the 50% effective concentration of drug. Fold, the EC_{50} of mutant/the EC_{50} of wild-type. Wild-type 1 and two subsequent mutant strains were derived from serial serum samples from an rtA186T-positive patient. rtL180M + M204V(lab) and rtL180M + T184S + M204V(lab) were laboratory-modified strains created by eliminating the rtA186T mutation from the two rtA186T-positive mutants. Wild-type 2 and two subsequent classical entecavir-resistance mutant strains were derived from another entecavir-refractory patient.

of viral isolates with primary mutations to improve the fitness of the mutant virus (Zoulim and Locarnini, 2009). Primary resistance mutations have several distinguishing characteristics including an association with drug therapy, a rebound in viremia or an inadequate virological response, an ability to confer phenotypic resistance *in vitro*, and are found in multiple patients exposed to the drug (Yang et al., 2002). In light of this, the evidence that rtA186T and rtI163V are ETV-resistance mutations is not comprehensive enough because the mutations were only detected in one ETV-refractory patient (Hayashi et al., 2015). In this study, we provided the relevant evidence confirming that rtA186T + LAM-r was an ETV-resistance mutation pattern and clarified their clinical profile in Chinese patients. In addition, our results suggested that rtI163V was unlikely an ETV-resistance-associated mutation.

HBV has the potential to evolve under environmental pressure through the selection of adaptive mutations, with the fittest mutants emerging depending on their replication capacity, their sensitivity to antivirals, and to the host immune responses (Rajoriya et al., 2017). Generally, mutant viruses are less fit, meaning they do not replicate as well as wild-type virus, but may have a survival advantage in the presence of an antiviral agent (Villet et al., 2009; Zhang et al., 2018). Compared to classical ETV mutations, the occurrence of the rtA186T mutation was much lower for ETV resistance in the clinic. This could be explained by the low rtA186T replication capacity of the rtA186T mutants. Intriguingly, replacing rtT186 with rtA186 did not restore the replication capacity of the two tested rtA186T mutants, suggesting that another amino acid change in addition to A→T at position rt186 might contribute to decreased replication capacity of the rtA186 mutants. Determining whether viral immune adaptation had an influence on the development of the rtA186T mutants will require further study to clarify.

Regarding phenotypic analyses, a small decrease in *in vitro* ADV susceptibility (2–9-fold increase in EC_{50}) may confer clinical resistance. By contrast, a > 50-fold increase in EC_{50} for ETV and TDF, and a > 500-fold increase in EC_{50} for LAM are usually needed for clinical resistance (Lok and McMahon, 2009; Lok et al., 2007; Svarovskaia et al., 2013). Single LAM-r had decreased susceptibility to ETV but was not sufficient to cause clinical ETV resistance. Different ETV-resistance mutation patterns had various ETV resistance to some extent. As typical examples, one investigation group reported that LAM-r, rtT184L + LAM-r, rtS202G + LAM-r, rtM250V + LAM-r, and rtT184G + rtS202I + LAM-r mutants had 27-, 246-, 402-, 1028-, and > 1333-fold decreases of ETV susceptibility, respectively, compared to the wild-type (Walsh et al., 2010). Our study identified that LAM-r, rtA186T + LAM-r, and rtA186T + rtT184S + LAM-r mutants had an approximately 30-, 210-, and 555-fold decrease of ETV susceptibility, respectively, compared to the wild-type, which supports the direct contribution of the rtA186T mutation to ETV resistance. Consistent with Walsh's study, we also found that viral strains that harbored multiple primary resistance mutations had greater ETV resistance, as exhibited by rtA186T + rtT184S + LAM-r mutants.

Out of 14 rtA186T-positive patients, serial serum samples were only available from six patients, and these patients were subjected to longitudinal analysis. All six patients had a history of treatment failure with sequential LAM and ADV monotherapies. Therefore, the presence of both LAM- and ADV-resistance mutants in the viral pool was likely a driving factor for the emergence of the rtA186T mutants. The rtA186T + LAM-r strain was the predominant mutant strain at the time of poor virological response to ETV in three patients (patient 1, 3, and 6), indicating that the rtA186T + LAM-r strain itself could be completely responsible for ETV resistance in the clinic. Interestingly, the coexistence of the rtA186T + LAM-r and rtA181C + LAM-r mutants was observed in samples A1 and A2 in patient 1, with a conversion of the dominant mutant from rtA186T + LAM-r to rtA181C + LAM-r in sample A4 during ETV + ADV treatment (Fig. 1A). In fact, the rtA181C mutant was detected in 18 patients with a virological breakthrough or inadequate virological response to ETV in this cohort of patients (data are not shown), which suggests that rtA181C + LAM-r is another non-classical ETV-resistance mutation. Consistent with our observation, a recent analytic study proposed that the rtA181C + LAM-r mutation was associated with ETV resistance (Rose et al., 2018).

Switching to TDF is currently the preferred rescue therapy for ETV resistance (Lim et al., 2016). As TDF was not licensed for treating HBV infection until 2014 in China, the combination of ETV + ADV was recommended as an alternative and preferential rescue therapy for ETV resistance in successively issued guidelines (Chinese Society of Hepatology, et al., 2011; Hou et al., 2017). An investigation showed that virological response rates of ETV + ADV for ETV-refractory patients were 35%, 43%, 65%, and 76% at 6, 12, 24, and 36 months of treatment, respectively (Kim et al., 2015). There is still a need to improve the rate of virological response for ETV-resistance patients. Our study showed that two patient-derived rtA186T mutants, as well as two classical ETV-resistance mutants, all remained sensitive to TDF, supporting the use of TDF-based rescue therapy as a priority treatment for ETV-refractory patients regardless of which ETV-resistance mutation pattern they harbor.

In summary, for the first time, we clarified the clinical prevalence of HBV rtA186T and demonstrated that rtA186T may confer ETV resistance in multiple Chinese patients. This study provided new insights into HBV drug resistance with clinical implications for resistance management.

Conflicts of interest

All authors declare that they have no competing interests.

Authors and contributors

DX and YL participated with substantial contributions to the conception or design of the work. LL, RC, XL, and DL were in charge of the acquisition, analysis, or interpretation of data for the work. YZ, QL, and BH participated in the work related to patient sample collection and clinical information analysis. DX, YL, and XL contributed to drafting the work or revising it critically for important intellectual content. All authors read and approved the final version to be published.

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Appendix A. Supplementary data

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