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Original Article

Multiple drug-resistant HBV mutation may contribute to poor response of adefovir + entecavir in entecavir-resistant patients

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Abstract

Introduction: Adefovir plus entecavir (ADV+ETV) rescue therapy in ETV-resistant patients with chronic hepatitis B virus (HBV) infection is suboptimal in some patients. This study aims to elucidate the evolutionary characteristics of drug-resistant HBV mutants and their association with clinical responses in such patients.

Methodology: Thirty-seven ETV-resistant patients were enrolled, among whom twelve had an inadequate virological response to ADV+ETV rescue therapy. The clonal sequence (≥ 20 clones/sample) of HBV reverse transcriptase gene was performed to identify the resistance mutations. Phenotypic analysis was performed to evaluate the replication capacity and drug susceptibility of the mutants.

Results: ETV-resistant mutants were continuously detected in 10 of the 12 patients, and multidrug-resistant (MDR) mutants, including a novel strain (rtL180M+A181V+T184A+S202G+M204V), were detected in two patients. Seven of the 12 patients who subsequently received tenofovir (TDF)-based therapy for 38 (23−60) months all achieved undetectable HBV DNA after treatment, and ETV-resistant mutants converted to wild-type in the four patients' samples. In contrast, the other five patients who did not achieve an adequate virological response had remaining of ETV-resistant mutants. The novel MDR strain exhibited multiple resistances to LAM, ADV, and ETV, and 11.2-fold lower susceptibility to TDF.

Conclusions: This study is the first to demonstrate that MDR HBV mutations may contribute to the poor efficacy of ADV+ETV combination therapy in ETV-resistant patients. Moreover, a novel MDR HBV strain was identified. Our results indicate that a TDF-based rescue therapy would be effective for the treatment of the refractory cases.

Key words: Hepatitis B virus; entecavir-resistant; multidrug-resistant; mutation; rescue therapy.

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Introduction

Over 240 million people are estimated to have chronic hepatitis B virus (HBV) infection worldwide, with around 78 million in China [1,2]. Treatment of chronic hepatitis B (CHB) is aimed at suppressing viral replication to the lowest possible level, thereby halting the progression of liver disease [3]. Nucleoside and nucleotide analogues (NAs) are known to be major anti-HBV agents that can effectively inhibit HBV replication. However, NAs have no direct effect on HBV original replication template covalently closed circular DNA. As a result, patients would require long-term NA treatment, which could increase the risk of drug resistance [4]. Currently, five NAs are licensed for use in the treatment of HBV infection in China, i.e., lamivudine (LAM), adefovir dipivoxil (ADV), entecavir (ETV), telbivudine (LdT), and tenofovir disoproxil fumarate (TDF). Among these, LAM, ADV, and ETV had longer term of usage and were still widely used, while TDF was recently approved in 2014 in China with relative higher selling price. Signature resistance mutations include rtM204I/V±L180M (LAM-r) for LAM (rtM204I was also a LdT-resistant mutation), rtA181V/rtN236T for ADV, LAM-r plus one of the substitutions (sub) at rtT184 (A/C/F/G/I/L/M/S), rtS202 (C/G/I), and/or rtM250 (I/L/V) for ETV [5–7]. In addition to these signature resistance-mutations, several novel resistance mutations were identified recently. For example, two novel mutations rtA186T and rtI163V from an ETV-refractory patient were reported to account for ETV resistant in concomitance with LAM-r [8,9]. We found

that rtA181C+LAMr conferred resistance to ETV [10]. We also identified several mutations which might contribute to LAM or ADV resistance [11–14]. Multidrug-resistant (MDR) HBV has multiple mutations that are co-localized on a single viral genome. These confer resistance to antiviral agents with a favorable cross-resistance profile, that is, resistance to both nucleoside analogues (LAM/LdT ± ETV) and nucleotide analogues (ADV) [15]. MDR HBV infection has been reported in a few studies [15–18]. and MDR strain was proved to be more endurance to combined drugs compared to individual drug-resistant HBV mixture [19].

ETV is a potent antiviral that has a high barrier to resistance and is recommended as a first-line anti-HBV agent. Although ETV resistance rarely occurs in NAs-naive patients, the rates of resistance can reach up to 51% in LAM-refractory patients [20]. For ETV-resistant patients, ADV+ETV used to be recommended as a preferential rescue therapy [21], and continues to be one of the rescue therapies used [22]. However, in clinical practice, its efficacy is suboptimal in several patients. The factors influencing this lower efficacy are yet to be elucidated.

This study aimed to clarify the evolutionary characteristics of drug-resistant HBV mutants and their association with clinical responses in ETV-resistant patients with inadequate virological response to ADV+ETV rescue therapy.

Methodology

Study Population

This is a retrospective study. A total of 22,009 HBV chronic patients who received genotypic resistance testing from 2007 to 2016 were initially taken into account as study candidates. ETV-resistant mutations were detected in 5.69% (1,252/22,009) patients, as described in our previous study [10]. The conditions for patient enrollment were as follows: (1) the patients had chronic HBV infection, received medical care and genotypic resistance testing (direct sequencing) in Fifth Medical Center of PLA General Hospital (Beijing 302 Hospital), and were detected with ETV-resistant mutation before being added-on ADV to ETV therapy; (2) the patients were followed up for at least five years from beginning of NA therapy, and their serum samples were availably taken at multiple time-points, including during their resistance to ETV and during the subsequent rescue therapy. As a result of these conditions, a total of 37 patients were enrolled in the study. The illness categories of chronic HBV infection included CHB and CHB-related liver cirrhosis. The standards for the diagnosis of these illnesses were based on the Guideline of Prevention and Treatment for Chronic Hepatitis B issued by Chinese Society of Infectious Diseases and Parasitology and Chinese Society of Hepatology [23], as detailed in our previous study [24]. An inadequate or partial virologic response (VR) was defined as a decrease in HBV DNA level of more than 1 $\log_{10}$ IU/mL, but detectable HBV DNA levels after at least 12 months of therapy in compliant patients [1]. Patients who were co-infected with other hepatitis viruses (HAV, HCV, HDV, and HEV) or HIV were excluded. These patients were obtained from the Database of Fifth Medical Center of PLA General Hospital (Beijing 302 Hospital) and all provided their informed consent for the use of their samples before enrollment in the Database of Fifth Medical Center of PLA General Hospital (Beijing 302 Hospital). The study was approved by the Ethics Committee of Fifth Medical Center of PLA General Hospital (Beijing 302 Hospital) (approval no. 2013052D).

Serological Markers and Quantitation of HBV DNA

The biochemical and serological markers, as well as the HBV DNA levels of the patients, were routinely measured and recorded in the Central Clinical Laboratory of Fifth Medical Center of PLA General Hospital (Beijing 302 Hospital). The HBV DNA level was determined using a real-time quantitative PCR kit (Fosun Pharmaceutical Co., Ltd., Shanghai, China). The lower limit of detection (LLOD) of the assay was 40 IU/ml.

Sequencing of HBV RT Gene

HBV DNA was extracted from the patient's serum by DNAout (Tianenze, Beijing, China) as previously described [17,18]. One-tube nested PCR was used for the amplification of HBV RT gene (nucleotides (nt) 54−1278) (Chinese patent ZL 200910092331.1) with 10 IU/mL LLOD at input of 200 μL serum, which was more sensitive than HBV DNA quantification in clinical practice. Clonal sequencing was performed using TA cloning.

Construction of Viral Amplicons Containing 1.1 mer HBV Genome

Amplicons containing 1.1 mer genotype C HBV genome harboring the patient-derived RT genes of five resistance mutants (including two MDR mutants) were constructed for phenotypic analysis based on pTriEx-mod-1.1 vector provided by Prof. Fabien Zoulim, University Lyon, France [25]. These amplicons would be used later specifically for the study of patient 5. The

wild-type RT gene from the same patient was constructed into the pTriEx-mod-1.1 vector for use as a reference.

Assessment of Viral Replication Capacity and Drug Susceptibility

The phenotypic analysis was performed as previously described [11−14]. The mutant and wild-type HBV genomic amplicons were transiently transfected into HepG2 cells and cultured in the presence or absence of NAs. Transfection was carried out using the X-tremeGENE HP DNA transfection reagent (Roche, Mannheim, Germany) and transfection efficiency was normalized using a β-galactosidase reporter plasmid (Promega, Madison, WI, USA). Five hours post-transfection, new medium containing serially diluted NAs was added, and the medium was replaced every other day. Relative replication capacity of a mutant to wild-type strain was determined in the absence of NAs. Drug susceptibility was determined by comparing the 50% effective concentration of the drug (EC_{50}) of a mutant to wild-type strain. The serially diluted concentrations of the drugs used to determine the EC_{50} were as follows: 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. To determine the inhibitory rate of ADV+ETV, TDF, and TDF+ETV, 10 μmol/l of ETV, 50 μmol/l of ADV, and 200 μmol/l of TDF were used. After four days of culture, the cells were harvested and lysed. Viral core particles were immunoprecipitated using an anti-HBc/protein A+G complex, and HBV replicative intermediates in the core particle were released and quantitated by real-time PCR. Drug susceptibility was determined by comparing EC50 of the drug for the mutants to that for the wild-type. The experiments were performed at least three times, independently.

Statistical Analysis

Data were presented as mean ± standard deviation (SD) or median (range). The differences between variables were examined using Student's *t*-test, Chi-square test, and Fisher's exact test. Statistical analysis was performed in Statistical Program for Social Sciences (SPSS 18.0 for Windows; SPSS Inc., Chicago, IL, USA). A *P*-value of < 0.05 (two-tailed) was considered statistically significant.

Results

Clinical and Viral Mutational Characteristics of Patients Before ADV+ETV Rescue Therapy

The clinical characteristics of the 37 ETV-resistant patients before ADV+ETV rescue therapy are shown in Table 1. None of the patients was found to show incompliance to antiviral therapies. Among the 37 patients, twelve had an inadequate VR and twenty-five had an adequate VR to ADV+ETV rescue therapy. The clinical characteristics and HBV resistance mutations of the 12 patients with inadequate VR to ADV+ETV rescue therapy are summarized in Table 2. These patients all had detectable levels of HBV DNA in their serum and ETV-resistant mutations before initiating the ADV+ETV rescue therapy. Clonal sequence analysis (≥ 20 clones/sample) was performed for the serum

Table 1. Clinical characteristics of the 37 entecavir-resistant patients before ADV+ETV rescue therapy.

Group	Inadequate VR (n = 12)	Adequate VR (n = 25)	P value
Age	44 (35−56)	44 (32−69)	0.204
Sex (male)	12 (100.0%)	24 (96.0%)	0.676
HBV DNA (log_{10} IU/mL)	6.90 (2.29–8.91)	4.70 (2.55−6.93)	0.046
HBV genotype (B/C/D)	0/12/0	5/19/1	0.076
Cirrhosis	4 (33.3%)	5 (20.0%)	0.311
ALT (U/l)	38 (16−356)	34 (9−232)	0.323
HBsAg (COI)	5611 (813.5−6560)	6558 (2176-10389)	0.204
HBeAg (+)	10 (83.3%)	13 (52.0%)	0.067
Anti-HBe (+)	2 (16.7%)	10 (40.0%)	0.148
LAM resistance	12 (100.0%)	15 (60.0%)	0.411
ADV resistance	10 (83.3%)	21 (84.0%)	0.650
ETV-resistant mutation pattern			
LAM-r+rtT184^{sub}	9 (75.0%)	10 (40.0%)	0.049
LAM-r+rtS202^{sub}	0	10 (40.0%)	0.009
LAM-r+rtM250^{sub}	2 (16.7%)	2 (8.0%)	0.391
LAM-r+rtT184^{sub}+rtS202^{sub}	1 (8.3%)	3 (12.0%)	0.609

VR: virological response; COI: cut off index; LAM-r: rtM204I/V ± L180M; sub: substitution. The resistance mutations shown here were obtained using direct sequence analysis.

Table 2. Characteristics and HBV mutation patterns of the 12 patients with an inadequate virological response before ADV+ETV rescue therapy.

Patient	HBV genotype	Sex/age	HBeAg status	LAM resistance	ADV resistance	ETV resistance	Resistant mutations	HBV DNA (Log_{10} IU/mL)	ALT (U/l)
1	C	Male/49	(–)	Yes	Yes	Yes	rtL180M, rtT184L, rtM204V	7.45	51
2	C	Male/50	(+)	Yes	Yes	Yes	rtL180M, rtT184A, rtM204V	2.93	40
3	C	Male/44	(+)	Yes	Yes	Yes	rtL180M, rtM204I, rtM250L	7.78	56
4	C	Male/56	(+)	Yes	Yes	Yes	rtL180M, rtT184L, rtM204V	3.85	45
5	C	Male/38	(+)	Yes	Yes	Yes	rtL180M, rtT184A, rtS202G, rtM204V	8.91	101
6	C	Male/37	(+)	Yes	Yes	Yes	rtL180M, rtT184L, rtM204V	2.92	36
7	C	Male/49	(+)	Yes	Yes	Yes	rtT184I, rtM204I	4.92	356
8	C	Male/48	(+)	Yes	Yes	Yes	rtL180M, rtM204V, rtM250V	8.05	176
9	C	Male/44	(+)	Yes	No	Yes	rtL180M, rtT184L, rtM204V	7.66	24
10	C	Male/35	(–)	Yes	Yes	Yes	rtL180M, rtT184L, rtM204V	6.38	16
11	C	Male/36	(+)	Yes	Yes	Yes	rtT184L, rtM204I	8.11	28
12	C	Male/40	(+)	Yes	No	Yes	rtL180M, rtT184L, rtM204V	7.41	33

LAM: lamivudine; ADV: adefovir dipivoxil; ETV: entecavir; TDF: tenofovir disoproxil fumarate; ALT: alanine aminotransferase.

samples of the 12 patients to determine the occupation of drug-resistant HBV mutants in the viral pool. The results showed that the ETV-resistant mutants were dominant in all patient samples at the tested time-points (Figure 1; refer to the first pie of each patient in the figure, except patient 5, for whom the second pie should be referred to). In particular, the ETV-resistant mutants rtL180M+T184L+M204V (occupied 100% of tested viral clones), rtL180M+T184A+M204V (100%), rtL180M+M204I+M250L (100%), and rtL180M+T184L+M204V (95%) were detected in the samples of patients 1 to 4, respectively. For patient 5, the resistance mutants rtL180M+S202G+M204V, rtL180M+T184A+M204V, rtS202G+M204V, rtA181V, rtL180M+A181V+M204V, rtL180M+M204V, and rtL180M+A181V occupied 57%, 14%, 5%, 9%, 5%, 5%, and 5% of the tested viral clones, respectively; rtL180M+A181V+M204V was a previously-reported MDR mutant. For patient 6, the resistance mutants rtL180M+M204V and rtL180M+T184L+M204V occupied 94% and 6% of the tested viral clones, respectively. For patients 7 to 12, the ETV-resistant mutants rtT184I+M204I±L180M (100%), rtL180M+M204V+M250V (100%), rtL180M+T184L+M204V (85%), rtL180M+T184L+M204V (100%), rtT184I+M204I (100%), and rtL180M+T184L+M204V (90%) were found to be dominant in the samples, respectively.

Clinical Response and Viral Mutant Evolution upon ADV+ETV Rescue Therapy

The 12 patients were subsequently switched to ADV+ETV therapy for 17 (12–63) months. None of these patients achieved adequate VR, with a median serum HBV DNA level of 3.46 (2.36–8.04) log10 IU/mL at the observation time-point.

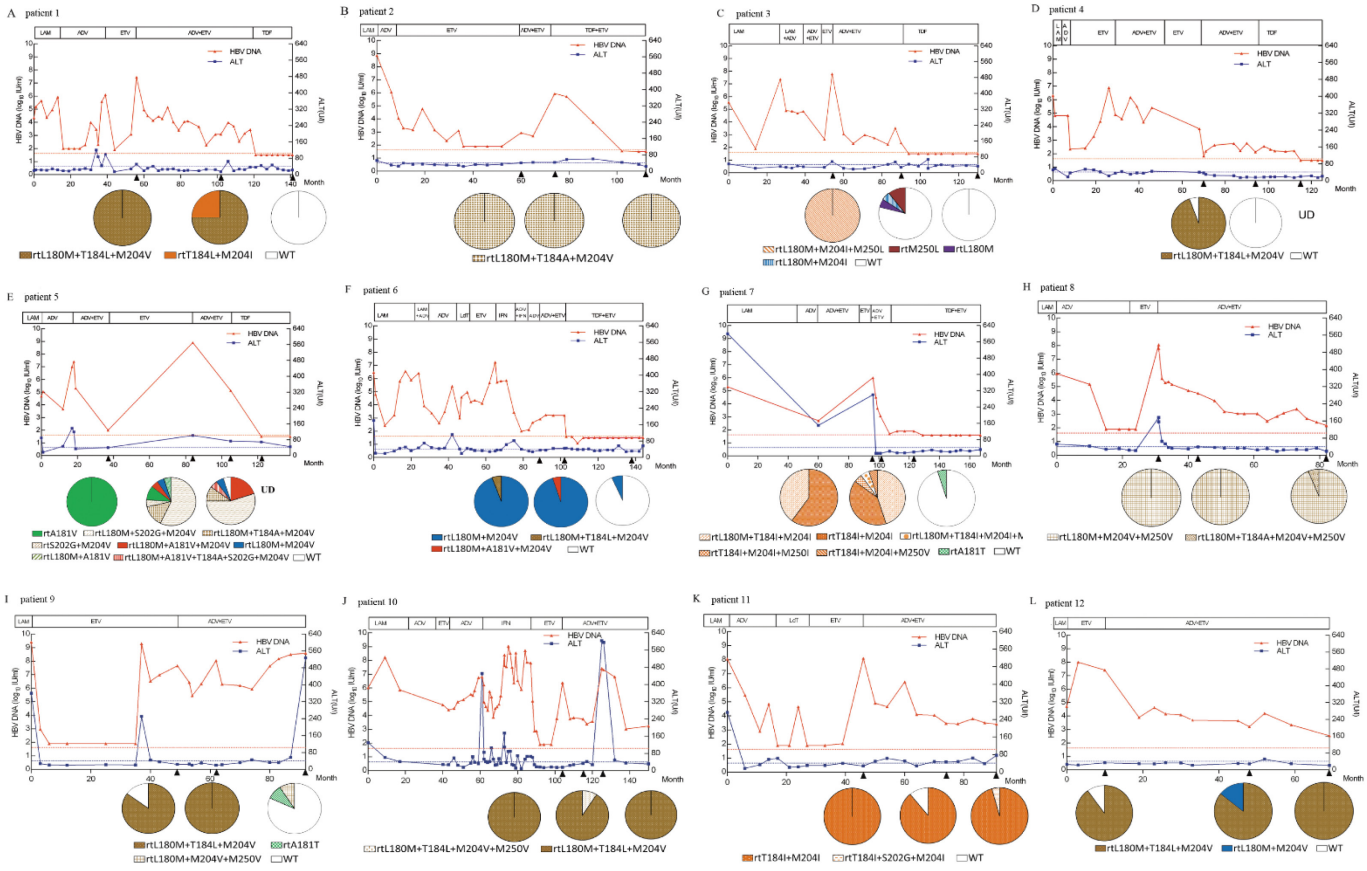
The evolution of the drug-resistant mutants in the viral pool of the 12 patients during the antiviral therapies was analyzed by clonal sequencing. As shown in Figure 1 (refer to the second pie of each patient in the figure, except patient 5, for who the third pie should be was referred to), ETV-resistant mutants were detected in 9 of the 12 patients' samples as follows:, the mutants rtL180M+T184L+M204V (75%) and rtT184L+M204I (25%) for patient 1; rtL180M+T184A+M204V (100%) for patient 2; rtL180M+S202G+M204V (55%) and rtL180M+T184A+M204V (10%) for patient 5; rtL180M+T184I+M204I (45%), rtT184I+M204I (40%), rtT184I+M204I+M250I (5%), rtL180M+T184I+M204I+M250V (5%), and rtT184I+M204I+M250V (5%) for patient 7; rtL180M+M204V+M250V (100%) for patient 8; rtL180M+T184L+M204V (100%) for patient 9; rtL180M+T184L+M204V (90%) and rtL180M+T184L+M204V+M250V (10%) for patient 10; rtT184I+M204V (89%) for patient 11; and rtL180M+T184L+M204V (86%) and rtL180M+M204V (14%) for patient 12.

Table 3. Phenotypic analysis of ETV and TDF susceptibility for representative viral strains.

	Entecavir		Tenofovir	
	EC_{50} (μmol/l)	**Fold**	**EC_{50} (μmol/l)**	**Fold**
Wild-type	0.004 ± 0.006	1.0	1.828 ± 0.926	1.0
rtL180M+M204V	0.080 ± 0.013	20.1	3.473 ± 3.553	1.9
rtL180M+S202G+M204V	0.459 ± 0.504	114.8	3.948± 2.548	2.2
rtL180M+T184A+M204V	0.368 ± 0.827	92.0	3.583± 3.406	2.0
rtL180M+A181V+M204V	0.060 ± 0.035	15.0	13.002 ± 3.584	7.1
rtL180M+A181V+T184A +S202G+M204V	0.798 ± 0.863	199.5	20.474 ± 8.422	11.2

EC_{50}: 50% effective concentration of the drug. The fold-changes in susceptibility represent the relative decrease compared to the wild-type.

Figure 1. Evolution of drug-resistant HBV and clinical response during antiviral therapies of 12 patients with an inadequate virological response to ADV+ETV.



The duration of the antiviral therapies in months are indicated by bars. Individual patients are indicated by A–L. The two dashed lines denote the lower detection limit of HBV DNA (40 IU/ml) and normal ALT level. The proportions of the wild-type (WT) and drug-resistant mutants in each sample are depicted by a series of pie charts. IFN, interferon-α; LAM, lamivudine; ADV, adefovir dipivoxil; ETV, entecavir; TDF, tenofovir disoproxil fumarate; UD, undetectable.

The MDR strains rtL180M+A181V+M204V (20%) (GenBank accession no. MK374374) and rtL180M+A181V+T184A+S202G+M204V (5%) (MK374373), which has not been documented before, were detected for patient 5. rtL180M+A181V+M204V (5%) was detected for patient 6, who had a dominant LAM-resistant mutant rtL180M+M204V in concomitance. By contrast, the wild-type strain was detected in the samples of patients 3 (79%) and 4 (100%).

Clinical Response and Viral Mutant Evolution upon TDF-rescue Therapy post-ADV+ETV Therapy

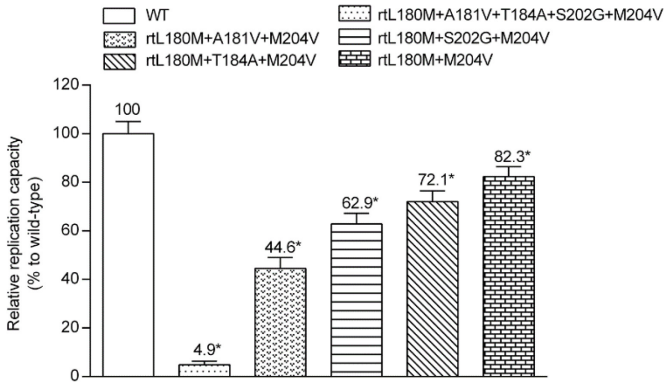
After the ADV+ETV rescue therapy, 7 of the 12 patients (patients 1 to 7) were switched to TDF (n = 4) or TDF+ETV (n = 3) therapy for 38 (23–60) months. The other five patients (patients 8 to 12) were continued on ADV+ETV treatment for an additional 25 (17–39) months. As a result, the seven TDF-treated patients all achieved undetectable levels of HBV DNA, with no virological breakthrough during the 20 (10–51) months of continuous follow-up. The virological, biochemical, and serological responses of the 7 patients treated with TDF-based rescue therapy are summarized in Supplementary Table 1. By contrast, none of the other five patients who underwent extended ADV+ETV therapy achieved an adequate VR, with a median serum HBV DNA level of 3.23 (2.16–8.59) $\log_{10}$ IU/ml. None of the twelve patients showed an increase in serum creatinine until the end of the observation period. At 9 months after the start of the TDF-based rescue therapy, two patients (patients 1 and 2) showed a transient decrease of the serum phosphorus levels (normal range: 0.9–1.34 mmol/l). No occurrence of hypophosphatemic osteomalacia was observed in either of these two patients.

As shown in Figure 1 (refer to the third pie of each patient in the figure, except for patient 5, for who the fourth pie should be referred to), at 23, 39, 36, and 16 months after initiating TDF-based rescue therapy, the conversion of ETV-resistant mutants into the wild-type strain as the dominant strain in the viral pool was observed in 4 of the patients (patients 1, 3, 6, and 7), with efficient viral suppression. In addition, two patients in this group (patients 4 and 5) had undetectable levels of HBV DNA, even when using the sensitive viral RT gene amplification technique. ETV-resistant mutants were found to be the dominant strain only in patient 2. In the patients who received the extended ADV+ETV therapy, ETV-resistant mutants were detectable for in all of the patients, with the mutants being dominant in the viral pool of four of these patients (patients 8, 10, 11, and 12). For patient 9, the wild-type strain, rtA181T mutant, and rtL180M+M204V+M250V mutant accounted for 82%, 9%, and 9% of the tested viral clones, respectively.

Replication Capacity and Drug Susceptibility of Representative Mutants

Phenotypic analysis was performed for the five resistance mutants and the wild-type strains derived from the serial serum samples of patient 5. Compared to the wild-type strains, the LAM- and ETV-resistant mutants showed a slight decrease in their viral replication capacity (82.3%, 72.1%, and 62.9% of the wild-type for rtL180M+M204V, rtL180M+T184A+M204V, and rtL180M+S202G+M204V, respectively). By contrast, the replication capacity decreased further with statistically significance for two of the MDR strains: rtL180M+A181V+M204V and rtL180M+A181V+T184A+S202G+M204V (44.6% and 4.9% of the wild-type, respectively) (Figure 2). The results of the drug susceptibility analysis for the 5 mutants and wild-type strain are summarized in Table 3. Specifically, the MDR strains rtL180M+A181V+T184A+S202G+M204V (with ETV-resistant mutations) and rtL180M+A181V+M204V (without ETV-resistant mutation) showed a decrease in their susceptibility to ETV of around 200- and 15-fold, respectively; by contrast, two MDR strains showed an intermediate

Figure 2. Assessment of HBV natural replication capacity.



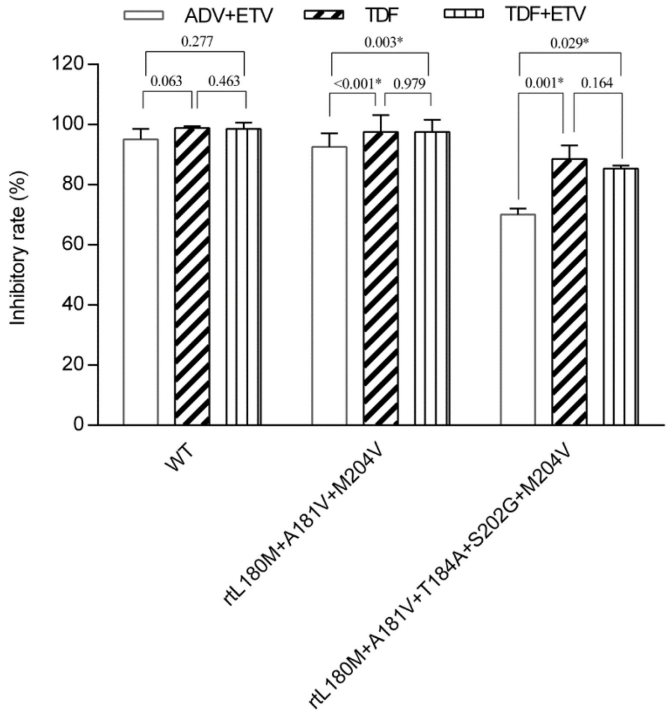
The relative replication capacities of the mutants isolated from serial samples of a representative patient (Patient 5) were analyzed and compared to those of the wild-type strain (100%) in the absence of drugs. The experiments were performed at least three times, independently. *P < 0.05 (mutant *vs.* wild-type). P < 0.001 (rtL180M+A181V+T184A+S202G+M204V, rtL180M+A181V+M204V, rtL180M+S202G+M204V *vs.* wild-type). P = 0.002, 0.009 (rtL180M+T184A+M204, rtL180M+M204V *vs.* wild-type).

decreased susceptibility to TDF (11.2- and 7.1-fold). The comparison of the inhibitory rate between the wild-type strain and two MDR strains against ADV+ETV, TDF, and TDF+ETV treatment is shown in Figure 3. For an additional perspective, a comparison of the inhibitory effects of the different drugs on the wild-type strain and the two MDR strains is shown in Supplementary Figure 1.

Discussion

The selection of an appropriate treatment strategy is critical for patients with chronic hepatitis B. The management of patients with HBV infection should involve a treatment that consistently reduces the viral load and prevents the development of mutations, which may result in drug resistance [26]. A number of studies have demonstrated that ADV+ETV rescue therapy is efficient for HBV patients resistant to ETV [27–30]. As a nucleoside analogue, ETV has complementary cross-resistance profile with the nucleotide analogue ADV. This provides a basis for the use of the two drugs in combination as a rescue therapy for these patients. Compared to ETV, ADV has a relatively weak suppressive effect on ETV-resistant HBV strains, whereby a lower dosage (10 mg/day) is required, limiting the number of side effects [31]. This was verified in our study, where 10 of the 12 patients with an inadequate VR to ADV+ETV showed a persistent existence of ETV-resistant mutants. The antiviral activity of ADV was reported to be reversely related to the baseline viral load [28]. In this study, the 12 patients with an inadequate VR also showed higher baseline levels of HBV DNA than that the 25 patients with an adequate VR (Table 1). In addition, genotype C HBV infection comprised 100% (12/12) and 76% (19/25) in inadequate VR and adequate VR patients upon ADV+ETV rescue therapy in our study, respectively. The genotype C HBV-infected patients have been reported to have relatively poor clinical outcomes [32]. In our study, genotype C HBV infection was more frequently observed in patients with an inadequate VR than those with an adequate VR, indicating that the HBV genotype may be another influencing factor for the efficacy of ADV+ETV rescue therapy.

Figure 3. Comparison of the inhibitory rate between the wild-type strain and two MDR strains against ADV+ETV, TDF, and TDF+ETV treatment.



MDR: multidrug-resistant; ETV: entecavir; ADV: adefovir dipivoxil; TDF: tenofovir disoproxil fumarate. Data are presented as mean ± standard deviation. Experiments were performed at least three times, independently. *P < 0.05.

One interesting finding of our study was the emergence of MDR HBV strains in two patients during ADV+ETV treatment (Figure 1E, 1F), including a novel strain. The replication competence of a HBV resistance mutant is essentially dependent on two factors: natural replication capacity and degree of resistance to antivirals [32]. We speculated that the relatively weak inhibition of ADV in ETV-resistant mutants provided an opportunity for the selection of ADV-resistant mutations in ETV-resistant strains, allowing for viral strain fitness under the pressure of ADV+ETV treatment. It is believed that MDR HBV is difficult to treat and poses a potential transmission risk. In light of our results, however, additional primary resistance mutations would result in a defective replication capacity of the virus (Figure 2), which restricted the viability and reduced the transmission of MDR strains. In addition, although MDR experienced an increase of about 10-fold in EC_{50} to TDF, it was insufficient to overcome a high-dose (300 mg/day) of TDF in treatment.

Another interesting finding was that, in 4 patients who subsequently underwent TDF-based rescue therapy (patients 1, 3, 6, and 7), the ETV-resistant HBV mutants were converted into wild-type strains in their viral pools. This suggested that ETV-resistant mutants lost their advantage in replication competence over the wild-type under newly-switched drug pressure, indicating the effectiveness of the treatment. Among the 4 patients, patient 6 were detected with MDR strain and with restoration of wild-type after switching to

TDF+ETV. Because increased number of primary resistant mutations would increase impairment of viral replication capacity (as shown in Figure 2), MDR viral strains only have relative replication competence advantage under inadequate antiviral treatment. When antiviral treatment is suspended or alternative to more efficacious antiviral treatment, MDR strains will lose their replication competence advantage upon wild-type strain.

TDF and TDF+ETV have been previously reported to be efficient for the treatment of ETV-resistant patients [33−36], including Chinese patients [31,37,38]. Our results reinforced the fact that TDF-based rescue therapy was efficient in ETV-resistant patients who had an inadequate VR upon ADV+ETV rescue therapy. Consistently, phenotypic analysis verified that TDF and TDF+ETV treatment had a stronger inhibitory effect on the two MDR mutants compared to ADV+ETV treatment. In a multicenter, real-world cohort study, the long-term use of TDF monotherapy showed a non-inferior antiviral efficacy compared to TDF-based combination therapy in patients with MDR [39]. In another multicenter cohort study, the efficacy of TDF monotherapy was not different from that of the TDF-based combination therapy [40]. Consistently, the results of the phenotypic analysis performed in our study verified that TDF and TDF+ETV were able to effectively inhibit the two MDR mutants (Figure 3). However, TDF-based rescue therapy had minor impact on the serological response, as none of the seven patients acquired HBeAg seroconversion and HBsAg loss. We recently analyzed the dynamic changes of HBV drug-resistance mutants with clinical responses to various antiviral therapies for a unique patient. This patient was followed-up over 189 months and LAM-ADV-, ETV-, and multidrug-resistance strains were successively detected. The results suggested that viral drug susceptibility, replication capacity, and perhaps immunological adaptation may play coordinated roles in the fitness of drug-resistance mutants [14]. A newly publication reported that rtS106C+rtH126Y+rtD134E+rtL269I mutations conferred TDF resistance in multiple patients [41]. The TDF-associated mutations were not detected in the HBV clones from the 12 patients enrolled in this study.

Our study was based on real-life clinical practices. Fifth Medical Center of PLA General Hospital (Beijing 302 Hospital) is one of the largest hospitals for infectious and liver diseases in China. The majority of patients who visited the hospital came from different regions of China, which restricted us to collect serial samples for many interesting patients and enrolled more patients in this study. In addition, some drug-resistant mutations in HBV RT region may influence the fitness of the mutants studied by introducing mutations in the overlapping S region, as we recently reported [42]. Further study is needed to clarify the potential influence of the passive S mutations on the efficacy of anti-HBV treatment.

In conclusion, in addition to demonstrating that an inadequate VR of ADV+ETV in ETV-resistant patients was closely associated with the insufficient inhibition of ETV-resistant mutants and the development of MDR mutants, we identified a novel MDR mutant with multidrug-resistant HBV mutants. Switching to TDF-based therapy was an effective strategy, wherein the conversion of ETV-resistant mutants into the wild-type strain indicated the efficacy of this rescue therapy. This study provides new insights into virological characteristics of HBV drug resistance.

Conclusions

This study is the first to demonstrate that MDR HBV mutations may contribute to the poor efficacy of ADV+ETV combination therapy in ETV-resistant patients. Moreover, a novel MDR HBV strain was identified. Our results indicate that a TDF-based rescue therapy would be effective for the treatment of the refractory cases.

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Authors' contributions

DX and JL designed and organized the experiments. JS, YL, RC, LZ, YZ, LL, and XL performed the experiments and collected patients' samples. JS, YL, LML, and RC analyzed the data. DX and JS wrote the paper.

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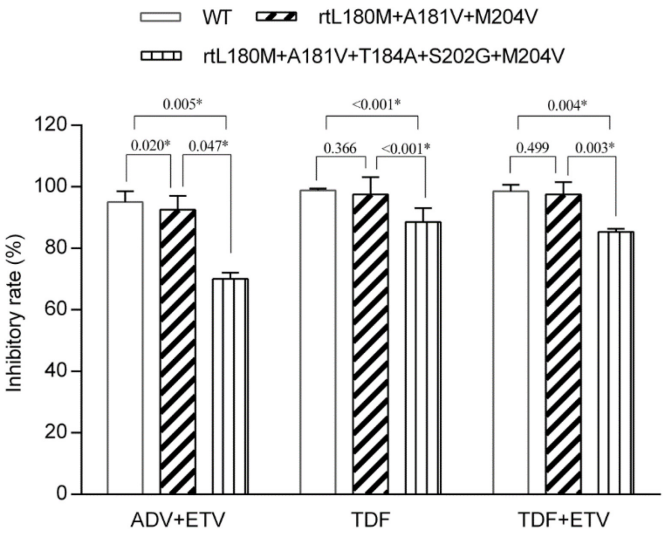
Annex – Supplementary Items

Supplementary Table 1. Virological, biochemical, and serological responses of the 7 patients following TDF-based rescue therapy.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7
Follow-up (months)	23	38	40	27	33	42	60
At initiation							
HBV DNA (log_{10} IU/mL)	3.45	5.93	2.36	2.54	5.13	3.20	1.90
ALT (U/l)	37	43	27	17	73	45	15
CRE (μmol/l)	80	86	60	92	93	75	75
P (mmol/l)	0.89	1.18	1.21	1.19	1.26	1.01	1.24
HBsAg (COI)	6,254	4,077	10,199	5,730	4,125	6,009	2,410
HBeAg status	Negative	Positive	Positive	Positive	Positive	Positive	Positive
anti-HBe (COI)	1.51	6.81	1.95	1.63	1.08	1.00	1.38
In 3 months							
HBV DNA (log_{10} IU/mL)	1.60	–	1.60	2.32	–	1.60	1.90
ALT (U/l)	36	–	43	18	–	42	–
CRE (μmol/l)	85	–	63	92	–	73	–
P (mmol/l)	0.9	–	1.32	1.19	–	0.99	–
HBsAg (COI)	4,714	–	8,431	6,078	–	5,614	–
HBeAg status	Negative	–	Positive	Positive	–	Positive	–
anti-HBe (COI)	1.35	–	1.86	1.5	–	1.06	–
In 6 months							
HBV DNA (log_{10} IU/mL)	1.60	5.71	–	2.20	–	1.60	1.90
ALT (U/l)	44	57	–	18	–	39	15
CRE (μmol/l)	–	94	–	98	–	84	77
P (mmol/l)	–	1.05	–	–	–	0.99	1.02
HBsAg (COI)	–	4,177	–	6,089	–	5,913	2,542
HBeAg status	–	Positive	–	Positive	–	Positive	Negative
anti-HBe (COI)	–	5.3	–	1.55	–	1.22	1.37
In 9 months							
HBV DNA (log_{10} IU/mL)	1.60	3.58	1.60	2.17	–	1.60	–
ALT (U/l)	27	27	33	20	–	39	–
CRE (μmol/l)	78	97	68	84	–	78	–
P (mmol/l)	0.89	0.68	1.31	1.18	–	1.27	–
HBsAg (COI)	4,818	4,118	7,807	7,371	–	6,870	–
HBeAg status	Negative	Positive	Positive	Positive	–	Positive	–
anti-HBe (COI)	1.32	3.84	1.81	1.56	–	1.12	–
In 12 months							
HBV DNA (log_{10} IU/mL)	1.60	–	1.60	2.21	–	1.60	1.90
ALT (U/l)	47	–	67	15	–	42	19
CRE (μmol/l)	–	–	68	87	–	82	74
P (mmol/l)	–	–	1.36	0.94	–	1.16	1.03
HBsAg (COI)	4,707	–	6,427	7,194	–	5,973	3,175
HBeAg status	Negative	–	Positive	Positive	–	Positive	Negative
anti-HBe (COI)	1.08	–	1.64	1.69	–	1.12	1.44
At the end of follow-up							
HBV DNA (log_{10} IU/mL)	1.60	1.60	1.60	1.60	1.60	1.60	1.60
ALT (U/l)	22	23	33	21	44	57	31
CRE (μmol/l)	74	91	66	79	75	80	79
P (mmol/l)	0.91	1.04	1.29	1.19	1.32	1.1	1.01
HBsAg (COI)	4,336	3,877	5,805	7,007	6,603	7,856	1,986
HBeAg status	Negative	Positive	Positive	Positive	Positive	Positive	Negative
anti-HBe (COI)	1.23	1.54	2	1.24	1.06	1.15	1.36

TDF: tenofovir disoproxil fumarate; ALT: alanine aminotransferase; CRE: creatinine; P: phosphorus; –: without detection record.

Supplementary Figure 1. Comparison of the inhibitory effect of different drugs on the wild-type and two MDR strains.



MDR: multidrug-resistant; ETV: entecavir; ADV: adefovir dipivoxil; TDF: tenofovir disoproxil fumarate. Data are presented as mean ± standard deviation. Experiments were performed at least three times, independently. *$P < 0.05$.