Research Article
Viral Hepatitis

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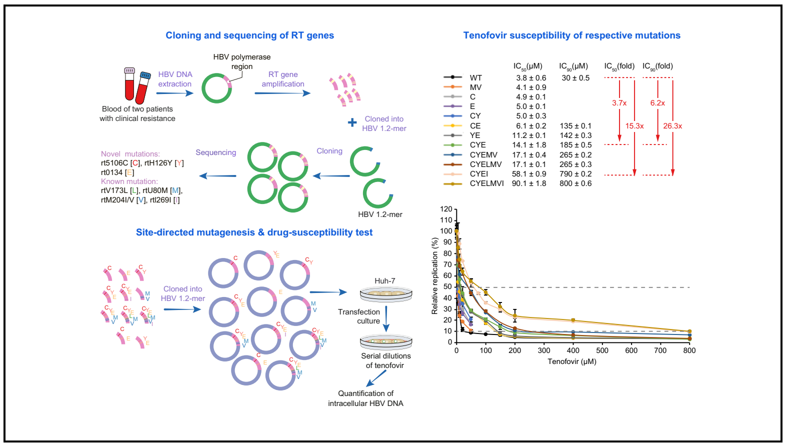
Identification of a quadruple mutation that confers tenofovir resistance in chronic hepatitis B patients

Graphical abstract



Highlights

- Among oral antivirals for HBV infection, only tenofovir has revealed no genotypically resistant HBV.
- However, there are patients with incomplete viral response during tenofovir-containing treatment.
- We consistently identified 7 common mutations including rtS106C (C), rtH126Y (Y), rtD134E (E), and rtL269I (I).
- The mutations C, Y, and E were novel mutations associated with drug resistance.
- The quadruple CYEI mutation increases the amount of tenofovir required to inhibit HBV by 15.3-fold in IC_{50} and 26.3-fold in IC_{90}.
- All tenofovir-resistant mutants with/without entecavir resistance were susceptible to a novel capsid assembly modulator.

Authors

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Lay summary

Tenofovir is the most potent nucleotide analogue for the treatment of chronic hepatitis B virus infection and there has been no hepatitis B virus mutation that confers >10-fold resistance to tenofovir up to 8 years. Herein, we identified, for the first time, a quadruple mutation that conferred 15.3-fold (IC_{50}) and 26.3-fold (IC_{90}) resistance to tenofovir in 2 patients who experienced viral breakthrough during tenofovir treatment.

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Identification of a quadruple mutation that confers tenofovir resistance in chronic hepatitis B patients

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Background & Aims: Tenofovir disoproxil fumarate (TDF) is one the most potent nucleot(s)ide analogues for treating chronic hepatitis B virus (HBV) infection. Phenotypic resistance caused by genotypic resistance to TDF has not been reported. This study aimed to characterize HBV mutations that confer tenofovir resistance.

Methods: Two patients with viral breakthrough during treatment with TDF-containing regimens were prospectively enrolled. The gene encoding HBV reverse transcriptase was sequenced. Eleven HBV clones harboring a series of mutations in the reverse transcriptase gene were constructed by site-directed mutagenesis. Drug susceptibility of each clone was determined by Southern blot analysis and real-time PCR. The relative frequency of mutants was evaluated by ultra-deep sequencing and clonal analysis.

Results: Five mutations (rtS106C [C], rtH126Y [Y], rtD134E [E], rtM204I/V, and rtL269I [I]) were commonly found in viral isolates from 2 patients. The novel mutations C, Y, and E were associated with drug resistance. In assays for drug susceptibility, the IC_{50} value for wild-type HBV was 3.8 ± 0.6 μM, whereas the IC_{50} values for CYE and CYEI mutants were 14.1 ± 1.8 and 58.1 ± 0.9 μM, respectively. The IC_{90} value for wild-type HBV was 30 ± 0.5 μM, whereas the IC_{90} values for CYE and CYEI mutants were 185 ± 0.5 and 790 ± 0.2 μM, respectively. Both tenofovir-resistant mutants and wild-type HBV had similar susceptibility to the capsid assembly modulator NVR 3–778 (IC_{50} <0.4 μM *vs.* IC_{50} = 0.4 μM, respectively).

Conclusions: Our study reveals that the quadruple (CYEI) mutation increases the amount of tenofovir required to inhibit HBV by 15.3-fold in IC_{50} and 26.3-fold in IC_{90}. These results demonstrate that tenofovir-resistant HBV mutants can emerge, although the genetic barrier is high.

Lay summary: Tenofovir is the most potent nucleotide analogue for the treatment of chronic hepatitis B virus infection and there has been no hepatitis B virus mutation that confers >10-fold resistance to tenofovir up to 8 years. Herein, we identified, for the first time, a quadruple mutation that conferred 15.3-fold (IC_{50}) and 26.3-fold (IC_{90}) resistance to tenofovir in 2 patients who experienced viral breakthrough during tenofovir treatment.

Introduction

Worldwide, an estimated 2 billion people are infected with hepatitis B virus (HBV), and 686,000 people die from complications due to HBV each year.[1] Despite the development of nucleos(t)ide analogue (NA) drugs, antiviral therapy of HBV infection remains a major clinical issue. Due to both the viral persistence and heterogeneity of the HBV genome, the emergence of drug-resistant mutants is inevitable. The development

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Keywords: Capsid assembly modulator; Entecavir; Nucleotide analogue; CYEI; HBV; Antivirals.

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of drug resistance is associated with poor prognosis. Problems arising from drug resistance include hepatitis flares, reversion of histologic improvement, and sometimes severe exacerbation of illness, hepatic decompensation, or death.[2]

Both entecavir and tenofovir (which is an active moiety of tenofovir disoproxil fumarate [TDF] or tenofovir alafenamide [TAF]) are approved as first-line therapeutic options for chronic hepatitis B (CHB) in current international guidelines due to their high potency and low resistance of the virus.[3,4] Although entecavir has a high genetic barrier to resistance, resistance to entecavir has been reported with a significant incidence rate, especially in patients with genotypic resistance to lamivudine and a prior history of lamivudine treatment.[5,6]

Meanwhile, HBV that displays clinical resistance to tenofovir has not been reported. Although TDF showed inferior efficacy in adefovir-experienced patients,[7] there was no detectable genotypic resistance to TDF after 8 years of therapy in patients with CHB.[8] Although the rtA194T mutation was reported to decrease tenofovir sensitivity by increasing the IC_{50} value in *in vitro* analysis, it does not confer tenofovir resistance *in vivo* nor is it associated with partial tenofovir drug resistance.[9–11] A recent article reported that rtS78T/sC69* was related to tenofovir resistance, but the IC_{50} values were increased by only 1.6-fold compared to wild-type.[12] Moreover, a recent randomized control study reported that viral response to TDF monotherapy was comparable to that to TDF and entecavir combination therapy in patients with multidrug resistance (MDR).[13,14] However, daily clinical practice shows that there are patients with a partial response to TDF varying from 0.8% to 24% and some patients developed viral breakthrough despite good adherence to TDF.[15,16] Therefore, we suspected the existence of genotypic resistance to tenofovir.

In this study, we aimed to identify the presence of tenofovir-resistant HBV by collecting blood samples from patients who showed viral breakthrough during TDF-based treatment and to characterize the responsible mutations *in vitro*.

Patients and methods

Patients

This study included 2 patients who developed viral breakthrough during TDF-containing treatment from 2 university-affiliated hospitals in Korea. Detailed flow of patient enrollment is provided in Supplementary methods and Fig. S1. Viral breakthrough is defined as an increase in the HBV DNA level of more than 1 $\log_{10}$ IU/ml compared to the nadir during therapy.[17] All participants provided written informed consent before enrollment and blood of each patient was sampled at the time of viral breakthrough. The study protocol conformed to the ethical guidelines of the World Medical Association Declaration of Helsinki and was approved by the Institutional Review Boards of Seoul National University Hospital (Seoul, Korea) and Chungbuk National University Hospital (Cheongju, Korea).

The clinical courses of both patients are provided in Fig. 1. Both patients developed viral breakthrough with >4 $\log_{10}$ IU/ml increase in serum HBV DNA level compared to the nadir. A 57-year-old woman with CHB (Patient #1) started lamivudine monotherapy (100 mg/day) in 2007. In August 2009, adefovir dipivoxil (10 mg/day) was added to the regimen because of viral breakthrough accompanied by the emergence of a lamivudine-resistant mutation (rtL80I). In December 2012, the antiviral regimen was changed to lamivudine (100 mg/day) plus TDF (300 mg/day) due to another viral breakthrough. At that time, rtM204I was also found in direct sequencing analysis. However, viral breakthrough re-occurred in February 2014. An additional mutation, rtA181T, was found together with the pre-existing mutations (rtL80I + rtM204I). After viral breakthrough, treatment was changed to entecavir (1 mg/day) and TDF (300 mg/day). After 14 months of entecavir/TDF treatment, viral breakthrough developed again and reached 43,500,000 IU/ml. Blood was sampled for the current study in July 2015 when the HBV DNA level was 17,400,000 IU/ml (Fig. 1A).

A 66-year-old man with CHB (Patient #2) took lamivudine treatment for 1 year between January 2006 and December 2006. He was treated again with entecavir (0.5 mg/day) for HBV e antigen (HBeAg)-positive hepatitis from June 2007 and developed entecavir-resistance in May 2009. He underwent adefovir (10 mg/day) plus lamivudine (100 mg/day) combination therapy from June 2009 to July 2012, but he developed viral breakthrough again in July 2012. He then started TDF (300 mg/day) plus telbivudine (600 mg/day) combination treatment in July 2012 and follow-up was lost after September 2013. He started TDF monotherapy in June 2014 and achieved complete viral suppression. However, he developed viral breakthrough again in June 2017, when his serum HBV DNA was 6,936,000 IU/ml. Blood was sampled for the current study at that time (Fig. 1B). He died of hepatocellular carcinoma in September 2017. He had no available prior results from antiviral resistance tests now as he was mainly treated at local private clinics.

Assessment of drug compliance

Drug compliance was assessed for each patient in 3 ways: (i) inquiry by attending physician at each visit, (ii) medication possession ratio (MPR), and (iii) measurement of serum trough concentrations of tenofovir and entecavir by modifying a previously reported method (Supplementary methods).

Direct sequencing analysis

Direct PCR-based DNA sequencing was performed to identify genotypic resistance (Supplementary methods).

Construction of HBV reverse transcriptase mutant replicons by site-directed mutagenesis

The reverse transcriptase (RT) gene was amplified by PCR and cloned into the HBV 1.2-mer replicon and sequenced (Supplementary methods). To identify the mutation(s) critical for drug resistance, the sequences were compared with the RT sequence of a wild-type genotype C HBV genome (NCBI GenBank accession no. GQ872210) isolated from the serum of an HBeAg-positive asymptomatic patient with CHB (Table 1). The following 11 artificial HBV 1.2-mer replicons were generated by site-directed mutagenesis of the wild-type HBV 1.2-mer: rtS106C (C), rtD134E (E), rtS106C + rtH126Y (CY), rtS106C + rtD134E (CE), rtH126Y + rtD134E (YE), rtS106C + rtH126Y + rtD134E (CYE), rtS106C + rtH126Y + rtD134E + rtL269I (CYEI), rtL180M + rtM204V (MV), rtS106C + rtH126Y + rtD134E + rtL180M + rtM204V (CYEMV), rtS106C + rtH126Y + rtD134E + rtV173L + rtL180M + rtM204V (CYELMV), and rtS106C + rtH126Y + rtD134E + rtV173L + rtL180M + rtM204V + rtL269I (CYELMVI). All artificial mutant clones were confirmed by sequencing.

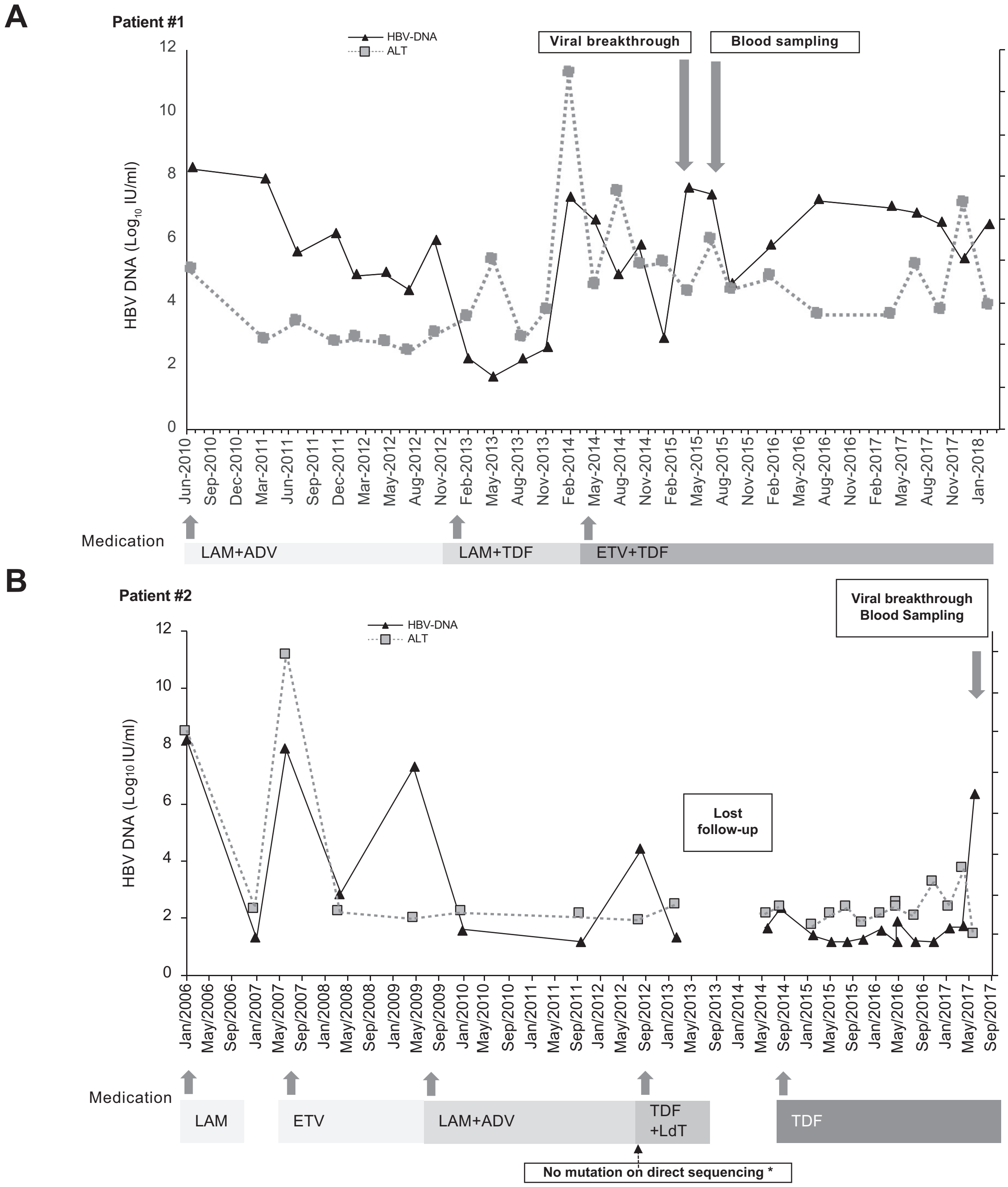


Fig. 1. Clinical courses of 2 chronic hepatitis B patients with clinical resistance to TDF-containing regimens. Serum samples were analyzed for HBV DNA and ALT. The time points of serum sampling are indicated by arrows. ADV, adefovir dipivoxil; ALT, alanine aminotransferase; ETV, entecavir; HBV, hepatitis B virus; LAM, lamivudine; LdT, telbivudine; TDF, tenofovir disoproxil fumarate.

Cell culture, transfection, and drug treatment

Huh7 cells, a human hepatocellular carcinoma cell line, were seeded into 6-well plates and transfected with 2 µg of each replicon (Supplementary methods and Supplementary CTAT Table). After 4 h, the medium was changed to fresh medium containing various concentrations of tenofovir (kindly provided by Gilead Science, Foster City, CA, USA), entecavir (kindly provided by Bristol-Myers Squibb, New York, NY, USA), or NVR 3-778, a capsid assembly modulator. NVR 3-778 was synthesized according to the literature procedure (US20140178337A1 by Novira Therapeutics, Inc., Doylestown, PA, USA). After drug treatment for 4 days, the supernatants and cells were harvested for analysis.

Southern blotting

Southern blot analysis was performed to determine the *in vitro* drug susceptibility and genome replication of each clone (Supplementary methods).

Table 1. HBV RT mutations identified in suspected tenofovir-resistant patients.

Clones		Amino acid position															
		33	78	80	98	**106**	118	**126**	**134**	**173**	**180**	204	207	238	267	**269**	301
Wild-type (GQ872210)		N	S	L	M	**S**	T	**H**	**D**	**V**	**L**	**M**	V	N	Q	**L**	T
Patient #1	1,3,7,8			I		**C**		**Y**	**E**			**I**			L	**I**	
	2		P			**C**		**Y**	**E**	**L**	**M**	**V**			L	**I**	
	4,6			I	T	**C**		**Y**	**E**			**I**			L	**I**	
	13					**C**		**Y**	**E**	**L**	**M**	**V**			L	**I**	A
Patient #2	1,3,6,7,8,9,10,11					**C**		**Y**	**E**			**I**	I	K		**I**	
	2	S				**C**		**Y**	**E**			**I**	I	K		**I**	
	4							**Y**	**E**			**I**	I	K		**I**	
	5					**C**	A	**Y**	**E**			**I**	I	K		**I**	
Conserved mutations						**C**		**Y**	**E**			**I/V**				**I**	

HBV, hepatitis B virus; RT, reverse transcriptase.

Real-time PCR

HBV DNA was extracted from the core particles and quantitated by real-time PCR (Supplementary methods).

Measurement of HBsAg and HBeAg

The levels of secreted HBV surface antigen (HBsAg) and HBeAg were measured as the indicators of transfection yield (Supplementary methods).

Ultra-deep sequencing

We classified the haplotypes of HBV strains based on the phasing of substitution mutations and counted their relative frequency. Two PCR amplicons from HBV were ultra-deep (>1,000,000-fold) sequenced using Illumina MiSeq platform (Supplementary methods).

Statistical analyses

At least 3 independent experiments were done for analysis. Statistical analyses were performed by the Student's *t* test using SPSS 22.0 software (IBM, Chicago, IL, USA). The *p* values of <0.05 were considered statistically significant.

Role of the funding source

The funding sources were not involved in this study. The corresponding authors had full access to all study data and had final responsibility for the decision to submit for publication.

Results

Mutation profiles of the HBV polymerase RT isolated from TDF-resistant patients

HBV DNA was isolated from the serum of the first patient (Patient #1) after she developed viral breakthrough during treatment with TDF-containing regimens. We constructed the HBV 1.2-mers where the original RT gene was replaced with the patient-derived ones and obtained 8 clones (Clones 1-1, 1-2, 1-3, 1-4, 1-6, 1-7, 1-8, and 1-13) from Patient #1. Each clone was sequenced to analyze the quasispecies of the entire RT gene.

Among the 8 clones isolated from Patient #1 after TDF treatment, 4 clones had the same sequence in their RT domains, indicating that a dominant HBV clone had evolved (Table 1). Notably, all 8 clones derived from Patient #1 shared common mutations, namely rtS106C, rtH126Y, rtD134E, rtM204I/V, rtQ267L, and rtL269I. In addition, the rtV173L and rtL180M mutations were commonly found in one-fourth of clones from Patient #1. Considering that the rtQ267L mutation is frequently detected in genotype C even before antiviral therapy,[18] the 7 common mutations (rtS106C [C], rtH126Y [Y], rtD134E [E], rtV173L [L], rtL180M [M], rtM204I/V [V], and rtL269I [I]) identified in Patient #1 could be associated with tenofovir resistance. Of these, C, Y, and E were novel mutations associated with drug resistance.

RT mutations in patient-derived HBV confer resistance to tenofovir

To evaluate whether the isolated RT mutants were resistant to tenofovir, we performed an *in vitro* drug-susceptibility assay using a representative RT mutant. Clones 1-1 and 1-13 were selected as representative RT mutants for the *in vitro* drug-susceptibility assay as they harbored the common mutations (Table 1). After transfection of Huh7 cells with the HBV

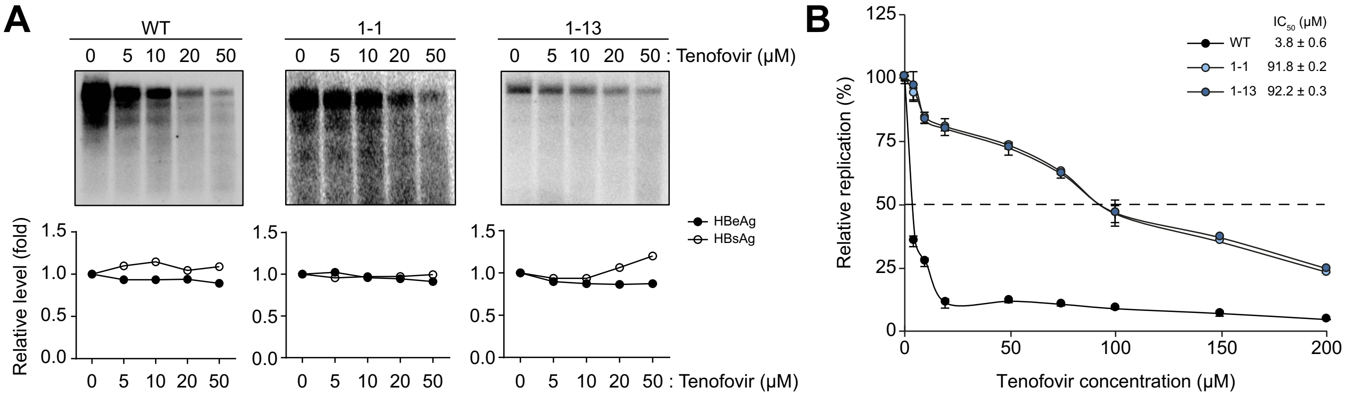


Fig. 2. *In vitro* tenofovir susceptibility of patient-derived RT mutants using Southern blot and quantitative real-time PCR. (A) The tenofovir susceptibility assay. Each panel shows representative autoradiograms from at least 3 independent experiments. The levels of secreted HBsAg and HBeAg were determined by ELISA to assess transfection yield. (B) Determination of IC$_{50}$ values by quantitative real-time PCR. The level of HBV replication without drug treatment was set at 100%. The IC$_{50}$ values were obtained by interpolation of the data from at least 3 independent experiments. HBeAg, HBV e antigen; HBsAg, HBV surface antigen; HBV, hepatitis B virus; RT, reverse transcriptase; WT, wild-type.

1.2-mers (wild-type, Clone 1-1, or 1-13), the replicative capacity and drug susceptibility were examined (Fig. 2A). In the absence of antiviral drugs, Clone 1–13 had lower replicative capacity compared to wild-type and Clone 1–1 showed similar replication capacity to wild-type (the leftmost lanes in each blot of Fig. 2A). The replication of HBV wild-type was greatly reduced by tenofovir treatment in a concentration-dependent manner, indicating that wild-type HBV is susceptible to tenofovir. However, Clones 1–1 and 1–13 showed no considerable decrease in replication following tenofovir treatment. There were no considerable differences in the levels of HBsAg and HBeAg between the samples (Fig. 2A). Quantitative real-time PCR showed that IC$_{50}$ values for wild-type, Clone 1–1, and Clone 1–13 were 3.8 ± 0.6, 91.8 ± 0.2, and 92.2 ± 0.3 μM, respectively (Fig. 2B). These results indicate strong tenofovir resistance of Clones 1–1 and 1–13.

A quadruple mutation is associated with tenofovir resistance

To identify and characterize the responsible mutations in Clone 1–1 and 1–13, we constructed 11 clones that harbored the 7 common mutations in various combinations (*i.e.*, C, E, CY, CE, YE, CYE, CYEI, MV, CYEMV, CYELMV, and CYELMVI) by site-directed mutagenesis. All mutant clones used in this study had the same sequence in the coding region of the HBeAg; however, the coding region of the HBsAg was mutated because it overlaps with the RT gene. The conserved HBV RT and corresponding surface mutations of each constructed clone are depicted in Fig. 3A. The absence of notable differences in the levels of both HBsAg and HBeAg regardless of tenofovir treatment (Fig. 2A and 3B) indicated that i) the transfection yield of each clone was consistent; therefore, the replicative capacity of each clone could be attributed solely to the RT mutations; and ii) the mutations in the corresponding surface protein have no considerable effect on surface antigenicity.

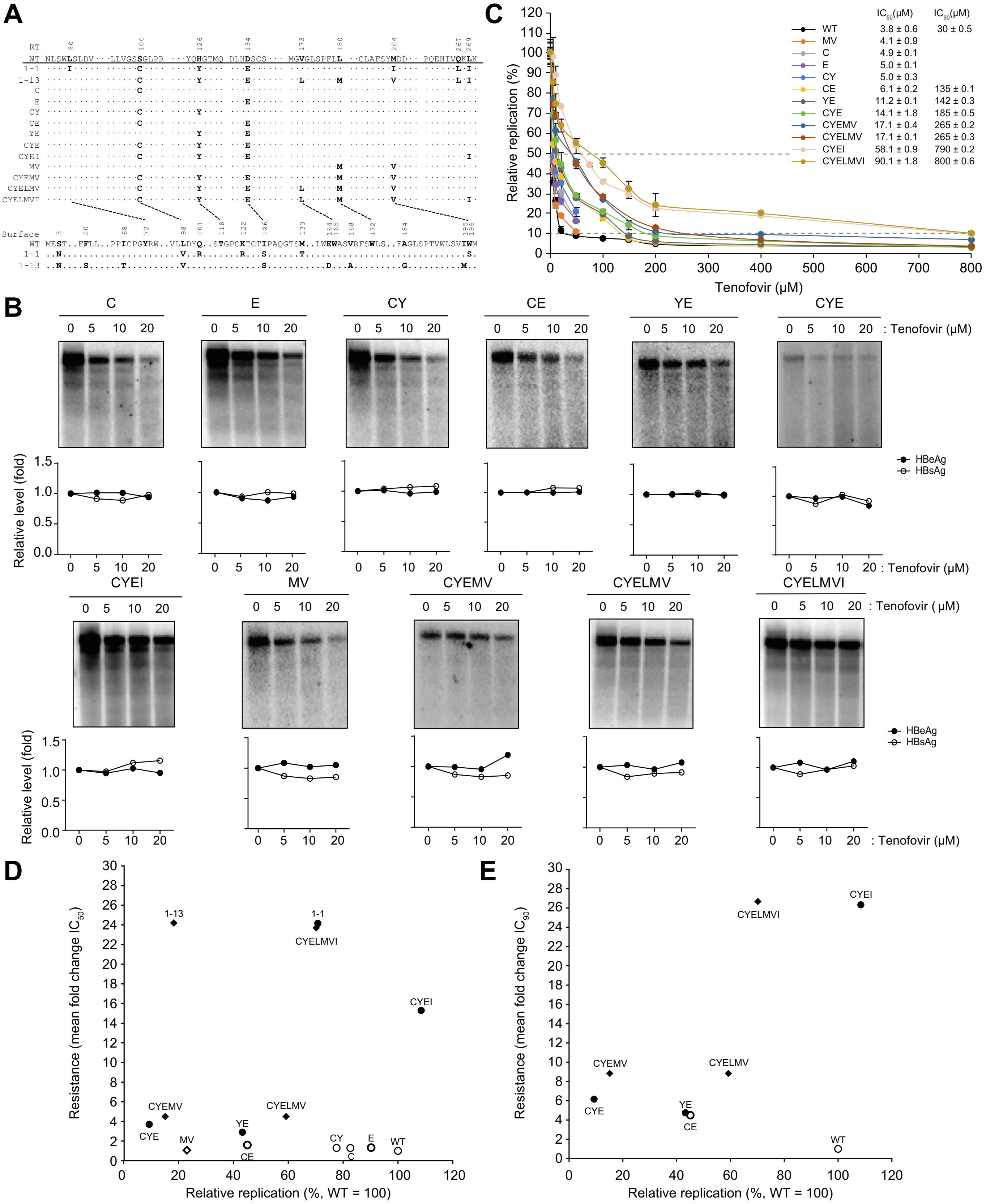
Clone 1–13 harbored 7 suspicious mutations in the RT gene. To characterize the role of each mutation in HBV replication and tenofovir resistance, we tested the replicative ability and tenofovir susceptibility using constructed clones. The mutants harboring C, E, CY, CE, YE, and MV were susceptible to tenofovir treatment in both Southern blot analysis (Fig. 3B) and real-time PCR (Fig. 3C). However, the mutant harboring a novel triple CYE mutation significantly reduced susceptibility to tenofovir. When the rtL269I mutation was combined with the CYE mutation (*i.e.*, CYEI), the replication capacity was strongly enhanced regardless of tenofovir treatment and showed phenotypic resistance (Fig. 3B). Quantitative real-time PCR showed that IC$_{50}$ values for wild-type, CYE, and CYEI mutants were 3.8 ± 0.6, 14.1 ± 1.8, and 58.1 ± 0.9 μM, respectively (Fig. 3C). These results indicate that the CYE mutation reduced tenofovir susceptibility (by 3.7-fold) and the CYEI mutation conferred complete resistance (by 15.3-fold) to tenofovir. The 90% inhibitory concentration (IC$_{90}$) values for the wild-type HBV, CYE, and CYEI mutants were 30 ± 0.5, 185 ± 0.5, and 790 ± 0.2 μM, respectively. The IC$_{90}$ value for the CYEI mutant was 26.3-fold higher than that of wild-type HBV (Fig. 3C). An additional entecavir-resistant LMV mutation enhanced tenofovir resistance when it was added to the CYEI mutation (*i.e.*, CYELMVI) (Fig. 3B and 3C).

To compare the effects of different mutations on replicative capacity and drug resistance, fold resistance (IC$_{50}$ and IC$_{90}$) *vs.* relative replication of each clone was plotted using real-time PCR data (Fig. 3D and 3E, respectively). It was evident that CYE conferred resistance to tenofovir and the addition of I to CYE backbone greatly enhanced tenofovir resistance and replicative capacity. Addition of E mutation to CY backbone strongly inhibited replication; however, the addition of the L or I mutation greatly increased replication, suggesting their roles as complementary mutations in the CYE backbone.

To validate whether the CYEI mutation is clinically relevant in tenofovir resistance, clonal analysis of HBV isolated from 1 additional patient (Patient #2) who developed viral breakthrough during TDF treatment was conducted. Likewise, the CYEI mutation was also detected in this patient. The HBV mutants with all the 4 CYEI mutation were predominant in both Patients #1 and #2. According to ultra-deep sequencing analysis (Table 2), CY mutation is co-localized in HBV clones obtained from Patient #1, suggesting mutations C and Y were acquired almost simultaneously, but earlier than mutation E.

Susceptibility tests using other antiviral agents

The wild-type and CYE or CYEI viral mutants proved susceptible to entecavir (all IC$_{50}$ ≤0.02 μM), whereas the CYELMVI mutant (as expected) was strongly resistant to entecavir alone (IC$_{50}$ = 4.37 μM; Fig. 4A) and entecavir/tenofovir in combination (Fig. 4B). Clone 1–1, harboring the CYEI and other mutations


A
RT 80 106 126 134 173 180 204 267 269
WT NLSWLSLDV··LLVGSSGLPR ··YQHGTMQ DLHDSCS··· MGVGLSPFLL ··CLAFSYMDD·· PQEHIVQKLK
1-1 ····I······ ·····C···· ····Y··· ··E····· ·········· ·······I··· ·····L·I·
1-13 ····· ·····C···· ····Y··· ··E····· ·L·····M ·······V··· ·····L·I·
C ·····C····
E ··E·····
CY ·····C···· ····Y···
CE ·····C···· ··E·····
YE ····Y··· ··E·····
CYE ·····C···· ····Y··· ··E·····
CYEI ·····C···· ····Y··· ··E····· ·······I·
MV ·····M ·······V···
CYEMV ·····C···· ····Y··· ··E····· ·····M ·······V···
CYELMV ·····C···· ····Y··· ··E····· ·L·····M ·······V···
CYELMVI ·····C···· ····Y··· ··E····· ·L·····M ·······V··· ·······I·
Surface 3 20 68 72 98 101 118 122 126 133 164 165 168 172 184 195 196
WT MEST..FFLL..PPICPGYRW..VLLDYQ..STGPCKTCTIPAQGTSM..LWEWASVRFSWLS..FAGLSPTVWLSVIWM
1-1 ..N.....................V..R.......R...S.....T...........................S.
1-13 ..N....S......T..........V...............S...........D...A.........G..........M..
B
C E CY CE YE CYE
0 5 10 20 : Tenofovir (μM)
Relative level (fold)
1.5 1.0 0.5 0.0
HBeAg
HBsAg
CYEI MV CYEMV CYELMV CYELMVI
C
Relative replication (%)
Tenofovir (μM)
0 100 200 300 400 500 600 700 800
IC50(μM) IC90(μM)
WT 3.8 ± 0.6 30 ± 0.5
MV 4.1 ± 0.9
C 4.9 ± 0.1
E 5.0 ± 0.1
CY 5.0 ± 0.3
CE 6.1 ± 0.2 135 ± 0.1
YE 11.2 ± 0.1 142 ± 0.3
CYE 14.1 ± 1.8 185 ± 0.5
CYEMV 17.1 ± 0.4 265 ± 0.2
CYELMV 17.1 ± 0.1 265 ± 0.3
CYEI 58.1 ± 0.9 790 ± 0.2
CYELMVI 90.1 ± 1.8 800 ± 0.6
D
Resistance (mean fold change IC50)
Relative replication (%, WT = 100)
1-13 1-1 CYELMVI CYEI CYEMV CYE MV YE CE CYELMV CY C E WT
E
Resistance (mean fold change IC90)
Relative replication (%, WT = 100)
CYELMVI CYEI CYEMV CYE CYELMV YE CE WT

(rtL80I + rtM204I + rtQ267L), also showed resistance to both single-agent entecavir (IC_{50} = 1.22 μM, Fig. 4A) and combination entecavir/tenofovir, demonstrating higher replicative capacity compared to Clone 1–13 (Fig. 4B). These results demonstrate why Clone 1–1 was a dominant clone in Patient #1 at the time of viral breakthrough during entecavir/tenofovir combination treatment. All tenofovir-resistant mutants with/without entecavir-resistant mutations were susceptible to the capsid assembly modulator NVR 3–778, with similar susceptibility as wild-type HBV (IC_{50} < 0.40 μM *vs.* IC_{50} = 0.44 μM, respectively) (Fig. 4C).

Discussion

In this study, we identified a quadruple tenofovir-resistant mutation in HBV isolated from the sera of 2 patients with clinical resistance to tenofovir treatment and confirmed *in vitro* tenofovir resistance. To the best of our knowledge, this is the first report of HBV mutants with both clinical and *in vitro* resistance to TDF treatment. A novel CYE triple mutation reduced tenofovir susceptibility and a quadruple CYEI mutation conferred complete resistance to tenofovir. Ultra-deep sequencing and/or clonal analysis showed that CYEI mutation was predominant in those 2 patients, which indicates that our finding is clinically relevant. According to our results, HBV should accumulate at least 4 mutations to develop clinical resistance, which may explain why the genetic barrier to the development of tenofovir resistance is high. Fortunately, the tenofovir-resistant mutant was susceptible to a novel capsid assembly modulator, NVR 3–778.

Tenofovir works against both human immunodeficiency virus (HIV) and HBV because of the shared structure of both viruses, especially the similarity of RT.[19] In HIV, resistance to tenofovir is well established and most often reported in patients treated with lamivudine or nevirapine.[20] In HBV, no resistance to tenofovir was observed during 8 years of TDF therapy in an extended follow-up of clinical trials.[8] From now on, however, physicians need to suspect and identify the occurrence of a tenofovir-resistant mutation in patients with CHB who reveal viral breakthrough despite good adherence to tenofovir-containing regimens.

The role of mutations found in this study has not been understood until now. The C mutation was found in treatment-naïve patients with CHB, but whether NA treatment can increase the expression of the mutation is not well documented.[21] Notably, a recent case report found that 11 mutations in the RT region, including the C mutation, were detected by direct sequencing analysis in a patient developing viral breakthrough during TDF treatment who was previously antiviral-naïve,[22] but an *in vitro* genotypic resistance analysis is warranted to confirm whether the mutant is resistant to tenofovir and which mutations among the 11 involve tenofovir-insensitivity. Although the Y mutation has been found in lamivudine-treated patients and E in adefovir-treated ones, their association with treatment efficacy has not been established.[23,24] Compensatory mutations that recover the replicative ability of replication-defective drug-resistant HBV, such as M204V/I mutants, are important in viral breakthrough. In this regard, the patient harboring the rtA181V + rtN236T mutation showed no viral breakthrough during the tenofovir treatment because of its low replicative capacity, although the rtA181V + rtN236T mutation reduces *in vitro* susceptibility to tenofovir by 10-fold.[25] According to our previous data, the I mutation is associated with MDR and serves as a compensatory mutation for replication-defective MDR genotype C HBV. Although the I mutation alone does not confer resistance to NAs, it enhances replication capacity in the presence of other mutations.[26] In this study, however, we found that the I mutation not only enhanced replication of the CYE mutant by approximately 11-fold regardless of tenofovir treatment, but also strongly increased the degree of tenofovir resistance (3.7-fold increase by CYE mutation *vs.* 15.3-fold increase by CYEI). Interestingly, an MDR HBV mutant that harbored the quintuple rtM129L + rtV173L + rtM204I + rtL269I + rtH337N mutation in the RT domain was completely resistant to lamivudine, clevudine, and entecavir; however, it was susceptible to adefovir and tenofovir.[26] These data suggest that the I mutation alone or rtV173L + rtM204I + rtL269I does not confer resistance to tenofovir. The unexpected ability of the I mutation to greatly increase the degree of tenofovir resistance in the CYE mutation backbone suggests that the effect of this mutation on drug resistance is context-dependent.

Clinical guidelines for MDR HBV strains are still limited.[17,27] Although several studies reported that the efficacy and safety of TDF monotherapy are similar to those of combination therapy in lamivudine-resistant, adefovir-experienced, and even in entecavir-resistant patients with CHB,[28–30] the long-term outcome of TDF monotherapy based on large-scale randomized control trials has not yet been reported. A randomized control trial showed similar efficacy of TDF monotherapy and TDF/entecavir combination therapy in entecavir-resistant HBV, but the observation duration was only 48 weeks.[33] A subsequent follow-up study showed that TDF monotherapy was efficacious but 20.6% of patients failed to achieve viral suppression at week 144.[14] Therefore, at the moment there is insufficient evidence for conclusions about the efficacy of TDF monotherapy. However, our current findings suggest that there is a risk of resistance developing during treatment with all NAs, including

Fig. 3. *In vitro* tenofovir susceptibility assay and determination of the IC_{50} and IC_{90} values of tenofovir against wild-type and mutant HBV by quantitative real-time PCR. (A) A schematic representation of the mutant constructs used in this study. Commonly found HBV RT mutations and corresponding surface mutations in Clones 1–1 and 1–13 obtained were presented. (B) A tenofovir susceptibility assay was performed using Huh7 cells transfected with 2 μg of indicated HBV 1.2-mer mutants following Southern blot analysis. Each panel shows representative autoradiograms from at least 3 independent experiments. The levels of secreted HBsAg and HBeAg were determined by ELISA to assess transfection yield. (C) Determination of the IC_{50} and IC_{90} values of each HBV mutant against tenofovir treatment using quantitative real-time PCR. The level of HBV replication without drug treatment was set at 100%. The IC_{50} and IC_{90} values were obtained by interpolation of the data from at least 3 independent experiments. (D-E) Fold resistance *vs.* relative replication plot for each mutant. Relative viral replication (x-axis) was determined by real-time PCR in the absence of tenofovir at 4 days after Huh7 cell transfection with each mutant HBV 1.2-mer. Relative resistance (y-axis) is presented as the mean fold-change in IC_{50} values (D) or IC_{90} values (E) compared to that of wild-type. The CYEI mutation had higher replication capacity with 15.3-fold increase in IC_{50} and 26.3-fold increase in IC_{90} values compared to wild-type. HBeAg, HBV e antigen; HBsAg, HBV surface antigen; HBV, hepatitis B virus; RT, reverse transcriptase.

Table 2. Distribution of HBV mutations (according to C, Y, E, or I mutations) obtained by clonal analysis and ultra-deep sequencing.

SNVs	Patient #1		Patient #2
	Clonal analysis	UDS	Clonal analysis
CYEI	100%	95.8%[a]	90.9%
CYE	0	0	0
CYI	0	4.2%[b]	0
YE	0	0	9.1%
CE	0	0	0
C	0	0	0
Y	0	0	0
E	0	0	0
Wild-type	0	0	0
Others	0	0	0
Total	100%	100%	100%

HBV, hepatitis B virus; SNV, single-nucleotide variant; UDS, ultra-deep sequencing.

[a] Composition: CYEI + rtM204V (4.2%), CYEI + rtM204I (81.0%), CYEI + rtM204I + rtL269I (3.0%), and CYELMVI (7.6%).

[b] Composition: CYI + rtM204I (4.2%).

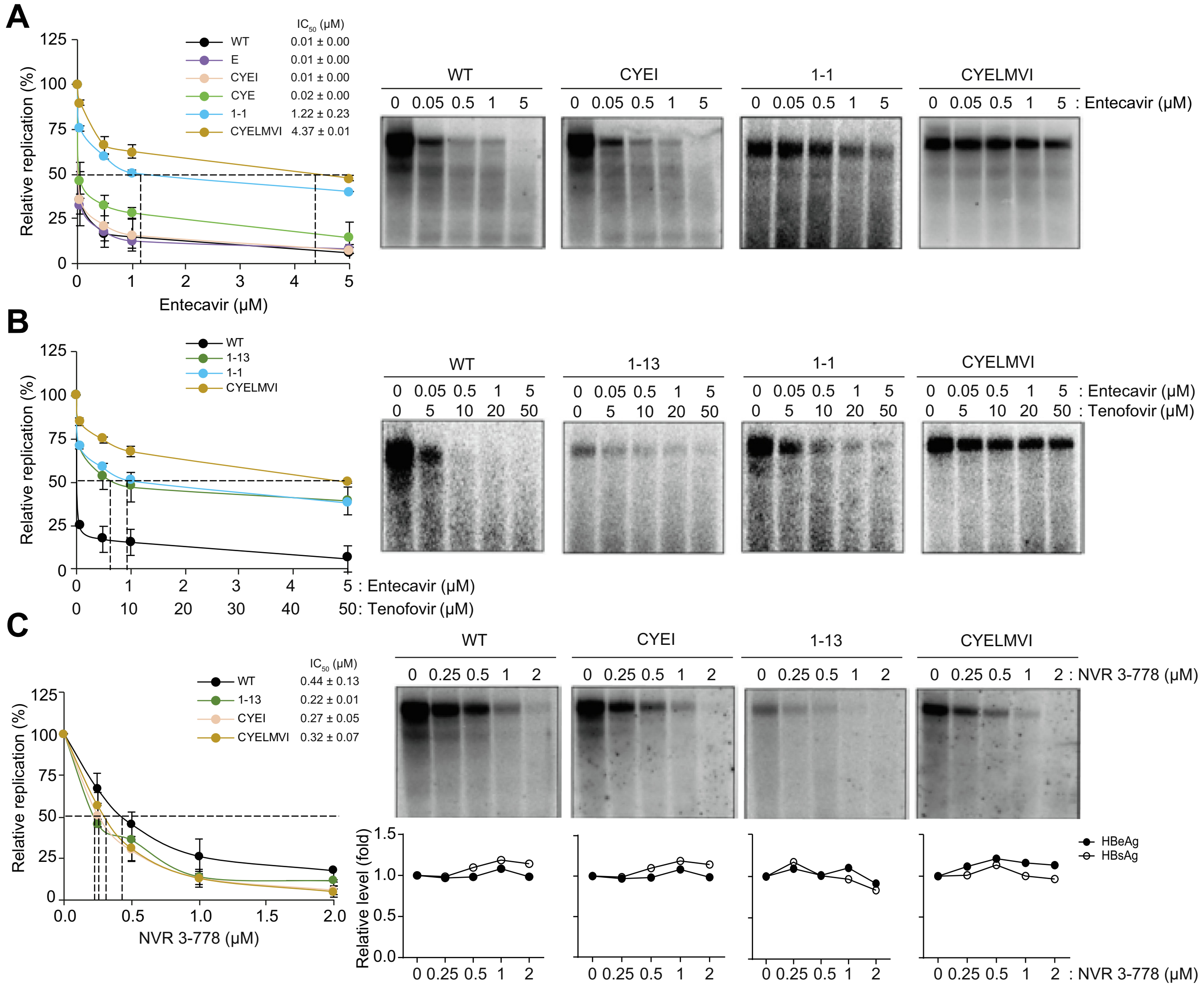


Fig. 4. Susceptibility of tenofovir-resistant mutants to alternative antiviral regimens. Susceptibility to (A) entecavir, (B) entecavir plus tenofovir, and (C) a capsid assembly modulator, NVR 3-778. Determination of the IC$_{50}$ values of each HBV mutant against treatment with indicated drugs. Huh7 cells were transfected with 2 µg of indicated HBV 1.2-mer mutants. At 4 days after transfection, cells were prepared and subjected to Southern blot analysis. The level of HBV replication without drug treatment was set at 100%. The IC$_{50}$ values were obtained by interpolation of the data from at least 3 independent experiments. *In vitro* susceptibility assay of indicated HBV mutants to treatment with (A) entecavir, (B) entecavir plus tenofovir combination, or (C) a capsid assembly modulator, NVR-3778. HBeAg, HBV e antigen; HBsAg, HBV surface antigen; HBV, hepatitis B virus; WT, wild-type.

TDF, particularly in MDR HBV strains. Considering that, among tenofovir-resistant quadruple mutations, the Y mutation was found in lamivudine-treated patients and the E mutation in those treated with adefovir, only the NAs with a high genetic barrier (such as TDF, TAF, or entecavir) should be recommended as a first-line treatment to prevent the development of any NA-resistance mutations.[31] Notably, there was no NA treatment option available for Patient #1 in our study. She revealed no response to the TDF (300 mg/day) and entecavir (1 mg/day) regimen, which is currently the most potent combination therapy. Fortunately, however, all mutants with tenofovir resistance, with/without entecavir-resistance, were susceptible to a novel capsid assembly modulator (Fig. 4C), suggesting that the results are conducive to designing novel antiviral strategies for patients with CHB. Whether the predominance of CYEI mutation persists during tenofovir treatment and whether treatment with new antiviral agents other than NAs might be effective for these patients also needs to be further evaluated.[32]

TAF reaches a therapeutic concentration in hepatocytes at a lower oral dose (25 mg/day), with a > 90% lower serum tenofovir level compared to TDF (300 mg/day). In a dose-finding study, the short-term safety of TAF was tested up to a dose of 120 mg/day,[33] which is 4.8-fold higher than the usual dose. However, high-dose TAF might not be an optimal rescue therapy for patients who develop TDF-resistance, considering that the IC_{50} and IC_{90} values of the CYEI mutant were 15.3- and 26.3-fold higher than those of wild-type HBV, respectively. Notably, the fold-change in IC_{90} of the CYEI mutant (resistant factor $[RF]_{90} = 26.3$) was higher than that in IC_{50} ($RF_{50} = 15.3$) compared to wild-type HBV, suggesting strong residual replication capacity of the CYEI mutant under the TDF treatment; a similar observation was reported for the rtM204 mutation against entecavir-susceptibility.[34]

In conclusion, although the use of TDF as CHB therapy was very successful for more than 10 years because of a high genetic barrier to resistance, we herein report that HBV isolated from 2 patients who developed phenotypic resistance during TDF therapy showed genotypic resistance in an *in vitro* study. To develop clinical resistance to TDF, the accumulation of at least 4 mutations is required, meaning that tenofovir has a higher genetic barrier to resistance than other NAs. However, since there is no proven rescue therapy option with NAs for patients with the tenofovir-resistance mutation, TDF monotherapy needs to be used with caution for patients with MDR HBV. Novel treatment options including a capsid assembly modulator should be tested as potential rescue therapies for patients with TDF-resistant HBV. Our findings suggest that the development of a new generation of anti-HBV drugs is urgently needed.

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Conflict of interest

These authors disclose the following: Dr. Lee JH reports receiving lecture fee from GreenCross Cell, Daewoong Pharmaceuticals, and Gilead Korea; Dr. Yu SJ reports lecture fee from Bayer HealthCare Pharmaceuticals; Dr. Kim YJ, research grants from Bristol-Myers Squibb, Roche, JW Creagene, Bukwang Pharmaceuticals, Handok Pharmaceuticals, Hanmi Pharmaceuticals, Yuhan Pharmaceuticals, Samjin Pharmaceuticals, and Pharmaking, and lecture fees from Bayer HealthCare Pharmaceuticals, Gilead Science, MSD Korea, Yuhan Pharmaceuticals, Samil Pharmaceuticals, CJ Pharmaceuticals, Bukwang Pharmaceuticals, and Handok Pharmaceuticals; Dr. Yoon JH, research grants from Bayer HealthCare Pharmaceuticals, Daewoong Pharmaceuticals, and Bukwang Pharmaceuticals. No other potential conflict of interest relevant to this article was reported.

Authors' contributions

Study concept and design: Lee JH and Kim KH. Provision of study patients and acquisition of clinical data: Lee JH, Lee YB, Chae HB, Yu SJ. Provision of study materials: Jeong N, Choi BS, and Park YK. *In vitro* experiments: Park ES, Lee AR, Kim DH, Ahn SH, Sim H, Park S, Kang HS, Won J, Ha YN, Shin GC, and Kim KH. Biostatistical analysis and interpretation: Lee JH, Kim KH, Park ES, Lee AR, Kim DH, Yu KS, An Y, and Ju YS. Drafting of the manuscript: Park ES, Yoo JJ, Lee JH, and Kim KH. Critical revision of the manuscript for important intellectual content: Kwon SY, Kim YJ, Yoon JH, and Zoulim F. Final approval of manuscript: all authors.

Supplementary data

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Author names in bold designate shared co-first authorship

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