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Prevalence, virology and antiviral drugs susceptibility of hepatitis B virus rtN238H polymerase mutation from 1865 Chinese patients with chronic hepatitis B

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ABSTRACT

Amino acid substitutions at positions rtN238T/D of the hepatitis B virus (HBV) polymerase have been reported as potential mutations associated with adefovir (ADV) resistance. In this study, we characterized the prevalence of the rtN238H mutation and determined the susceptibility to LAM and ADV using phenotypic analyzes *in vitro*. One thousand eight hundred and sixty-five HBsAg-positive patients with chronic HBV (CHB) infection were included in this study. HBV genotypes and reverse transcriptase (RT) mutations were determined by direct sequencing. Replication-competent HBV constructs containing the naturally occurring rtN238H mutation were generated and replication capacity and susceptibility to LAM and ADV in transiently transfected hepatoma cell lines were determined. Among 1865 enrolled HBV infected patients, 8.8% (165/1865) showed mutations in the rtN238 locus (143 males/22 females, 91 treatment-naive, 42 ADV-treated, 16 LAM-treated and 16 ADV + LAM-treated), namely 86% rtN238H (142/165), 5.5% rtN238S (9/165), 5.5% rtN238T (9/165) and 3% rtN238D (5/165). Among the rtN238H mutant strains, there were no significant differences between ADV- or/and LAM- treated patients and treated-naive patients (42% vs. 58%). Compared with wild-type HBV, this mutant displayed an equivalent susceptibility to LAM or ADV in phenotypic assays. Importantly, we found that the incidence rate of rtN238H was higher in HBV genotype B infected patients than HBV genotype C subsets (80.3% vs. 19.7%), even without exogenous selection pressures. As rtN238H did neither impair the viral replication efficiency nor susceptibility to LAM or ADV *in vitro*, rtN238H likely represents background polymorphisms rather than resistance mutations with clinical implications. The incidence of rtN238H may be associated with HBV genotype.

1. Introduction

Chronic hepatitis B virus (HBV) infection remains a major health problem affecting approximately 400 million people worldwide (Akman et al., 2010; McMahon, 2009). As a highly endemic area for HBV infection, there is an estimated 93 million of population infected with HBV in China (Li et al., 2010). The HBV is a small, partially double stranded DNA virus that uses the HBV polymerase for its replication via RNA intermediate. Due to lack of proofreading function, mutations and polymorphisms are frequently observed in the HBV genome of chronically infected patients (Chotiyaputta and Lok, 2009). The emergence of mutations within the polymerase gene is of exceptional clinical relevance, because these mutations can be associated with progression of liver disease due to effects on the viral replication efficiency with resistance to antiviral drugs (Yuen et al., 1999; Kao et al., 2003; Sumi et al., 2003; Yim et al., 2006). During treatment with nucleoside or nucleotide analogs, resistance mutations are commonly selected at typical positions. For instance, lamivudine (LAM) and other L-nucleosides select mutations in the catalytic domain (YMDD) of the reverse transcriptase, usually resulting in an amino acid exchange at position rt204 (rtM204I/V) ± rtL180M. Resistance to adefovir dipivoxil (ADV), on the other hand, is characteristically mediated by mutations at rtN236T or rtA181V/T (Lok et al., 2007). The current literature suggests that rtN238T/D mutations which may appear alone or in conjunction with rtN236T/rtA181V/T may be associated with ADV resistance *in vitro* (Shaw et al., 2006; Gallego et al., 2008; Sheldon et al., 2006; Ghany and Liang, 2007). However, the prevalence and relationship between the rtN238H mutation with LAM or ADV resistance has remained unclear.

In this study, we analyzed the polymerase region of 1865 HBV infected patients from China and report a relatively high prevalence of rtN238H in both treatment-naive and ADV treated patients. We constructed replication-competent HBV vector harboring the observed naturally occurring rtN238H and compared its replication efficacy as well as its susceptibility to LAM and ADV in comparison to wild-type, LAM- and ADV-resistant viruses *in vitro*.

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2. Materials and methods

2.1. Patient cohort

One thousand eight hundred and sixty-five HBsAg-positive patients with chronic HBV (CHB) infection who visited Beijing 302 Hospital from August 2007 to September 2010 were enrolled in the study. Patients were mainly from different regions of China, including 59.3% males and 40.7% females with a median age of 39 years (range 4–75 years). Nine hundred and eighty patients were treatment-naive, while 520 of the patients were currently treated with ADV (10 mg/day) and 247 of the patients were currently treated with LAM (100 mg/day) when the samples were obtained. One hundred and eighteen patients were on treatment with LAM and ADV due to the emergence of LAM-resistance. No other nucleoside or nucleotide analogs were administered. The diagnostic criteria were based on Management Scheme of Diagnostic and Therapy Criteria of Viral Hepatitis, which were issued by the Chinese Society of Infectious Diseases and Parasitology, and the Chinese Society of Hepatology, which have been described in detail in our previous studies (Li et al., 2010; Liu et al., 2011). All patients were serum HBsAg positive for at least 6 months, there was no evidence for HCC, concomitant with HCV, HDV, and HIV infection, or autoimmune liver disease. Excluded were patients with acute hepatitis A, B, HCV, HDV, or HIV co-infection, and drug-induced acute hepatitis, existence of renal failure, hepatic decompensation or psychiatric disorders, and central nervous system disease such as epilepsy, or patients that had received bone marrow or organ transplants, or had received immunosuppressive, nephrotoxic, or hepatotoxic medications within 2 months of enrollment.

The study protocol was approved by the Beijing 302 Hospital Research Ethnics Committee, written informed consent for therapy and study was obtained from each patient.

2.2. Detection of serological markers and analysis of HBV genotype

Serological markers and quantitation of HBV DNA, serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), and other biochemical parameters were measured by standard procedures. HBeAg/anti-HBe, HBsAg/anti-HBs and anti-HBc were detected by enzyme-linked immunosorbent assay (Kewei Diagnostic Ltd., Beijing, China) or chemiluminescent assays (Abbott Laboratories, North Chicago, IL). HBV DNA level was determined by a real-time quantitative polymerase chain reaction (PCR) kit (Fosun Pharmaceutical Co. Ltd., Shanghai, China) with a lower limit of detection of 500 copies/mL (about 100 IU/mL). HBV genotype assignment was based on analysis of the 1225 bp-long S/Pol-gene sequence (nt 54–1278) as described previously (Zhong et al., 2011). The sense and antisense primers for the first-round PCR were 5′-AGTCAGGAAGACAGCCTACTCC-3′ (nt 3146–3167) and 5′-AGGTGAAGCGAAGTGCACAC-3′ (nt 1577–1596), respectively (Li et al., 2010). Direct sequencing was performed using an ABI 3730xl DNA Analyzers (Applied Biosystems, Foster City, CA). Phylogenetic and molecular evolutionary analyses were performed in MEGA version 4.0 (Koichiro et al., 2007). The gene bank number of reference sequences used for the definition of mutations was DQ448619 for genotype B and Y1855 for genotype C. Drug-resistance-associated mutations in RT region of HBV genome were analyzed as previously described (Zhong et al., 2011).

2.3. Histopathological analysis

The stage of fibrosis and the degree of inflammatory activity were evaluated by the Metavir score system (Tonetto et al., 2009), which classifies fibrosis into five stages: F0 – no fibrosis, F1 – portal fibrosis without septa, F2 – portal fibrosis with few septa, F3 – portal fibrosis with numerous septa, without cirrhosis, F4 – cirrhosis. The degree of inflammatory activity was divided into: A0 – lack of histological activity, A1 – mild histological activity, A2 – moderate histological activity, A3 – severe histological activity.

2.4. HBV constructs for phenotypic analysis

HBV DNA was extracted from the patient's serum sample using QIAamp Ultrasens virus kit (Qiagen). PCR fragments containing N238H were obtained and cloned into a pTriEX-HBV vector including 1.1 HBV genome unit length (Durantel et al., 2004). Site-directed mutagenesis using the Quick Change II XL Site-Directed Mutagenesis kit (Stratagene) was employed to generate replication-competent HBV vectors with the rtN236T (ADV-resistance) polymerase mutations. LAM-resistant mutant strains carrying the rtM204I mutation or the rtL180M + rtM204V mutation were constructed.

2.5. Cell culture and transfection

The HepG2 hepatoblastoma cells were cultured in 24-well culture dishes (BD Biosciences, Franklin lake, NJ) with Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U of penicillin/ml, and 10 μg of streptomycin/ml. HepG2 cells were maintained in an incubator at 37 °C with 5% CO_2. The cells were transfected with 0.25 μg per well of plasmid by using the Fugene 6 transfection reagent (Roche, Indianapolis, IN). Transfected cells were harvested after 5 days. Cell lysates from 24-well culture dishes were pooled for further analysis and treated. All experiments were performed at least three times.

2.6. HBV viral load

HBV particles were harvested from the cell culture supernatant. HBV DNA was digested by DNase I, followed by extraction according to the instructions (Fosun Pharmaceutical Co. Ltd.). To measure HBV viral load in the cell culture supernatant, a real-time PCR was carried out by Fosun Pharmaceutical Co. Ltd. (Shanghai, China).

2.7. HBV proteins in supernatant

Hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) were measured in the supernatant by enzyme-linked immunosorbent assay (Kewei Diagnostic Ltd., Beijing, China).

2.8. Antiviral compounds and drug susceptibility analysis

LAM and ADV were obtained from Gilead Sciences. 50 mM stock solutions for LAM and ADV were prepared in sterile distilled water, aliquoted and stored frozen at −20 °C.

The analyzes of drug susceptibility were performed after transient transfection of HepG2 cells with recombinant wild-type (WT) and mutant HBV clones. Sixty hours after transfection, the medium was changed and cells were cultured with drug-free medium or medium containing 0, 0.033, 0.1, 0.33, 1.0, and 3.3 μM of ADV and 0, 0.01, 0.1,1.0, 10, and 100 μM of LAM. Treatments were renewed every day for 5 days.

Intracellular HBV replicative intermediates were isolated by lysis of cells. Cell lysates from two parallel wells were transferred to microcentrifuge tubes and spun for 5 min at 10,000*g*, respectively. Supernatants were transferred to clean tubes, adjusted to 10 mM $MgCl_2$, and incubated with 30 U of DNase I with 1.5 μg of RNase (Roche) at 37 °C for 1.5 h. After digestion of capsids with proteinase

Table 1
Characteristics of the patients with rtN238 polymerase substitutions.

	N238H	N238S	N238T	N238D
n (%)	142 (86)	9 (5.5)	9 (5.5)	5 (3)
Male/female, *n*	124/18	8/1	8/1	3/2
Age, median (range) [years]	39 (4–75)	46 (14–60)	38 (6–71)	48.6 (41–66)
Clinical status, n (%)				
Chronic hepatitis	80 (56.3)	3 (33.3)	5 (55.6)	1 (20)
Cirrhosis	62 (43.7)	6 (66.7)	4 (44.4)	4 (80)
Current therapy, n (%)				
Lamivudine	14 (9.9)	0 (0)	0 (0)	2 (40)
Adefovir	30 (21.1)	8 (88.9)	4 (44.4)	0 (0)
Lamivudine + adefovir	16 (11.3)	0 (0)	0 (0)	0 (0)
Treatment-naive	82 (57.7)	1 (11.1)	5 (55.6)	3 (60)
ALT, median (range) [U/L]	153.9 (10–808)	57.4 (15–177)	153.9 (21–713)	31.2 (21–54)
AST, median (range) [U/L]	108.7 (12–678)	54.6 (22–104)	103.2 (38–253)	33.8 (31–35)
Total bilirubin, median (range) [μmol/L]	34.5 (1.5–176.6)	18.0 (6.6–44.3)	48.9 (4.2–62.1)	20.8 (8.2–38.5)
Inflammation degree, median (range)	1.7 (0.5–3)	0.1 (0–1)	0.1 (0–1)	0.1 (0–1)
Fibrosis stage, median (range)	1.8 (0.5–4)	0.1 (0–0.5)	0.2 (0–1.5)	0.1 (0–0.5)
Genotype, n (%)				
Genotype B	114 (80.3)	0 (0)	0 (0)	1 (20)
Genotype C	28 (19.7)	9 (100)	9 (100)	4 (80)

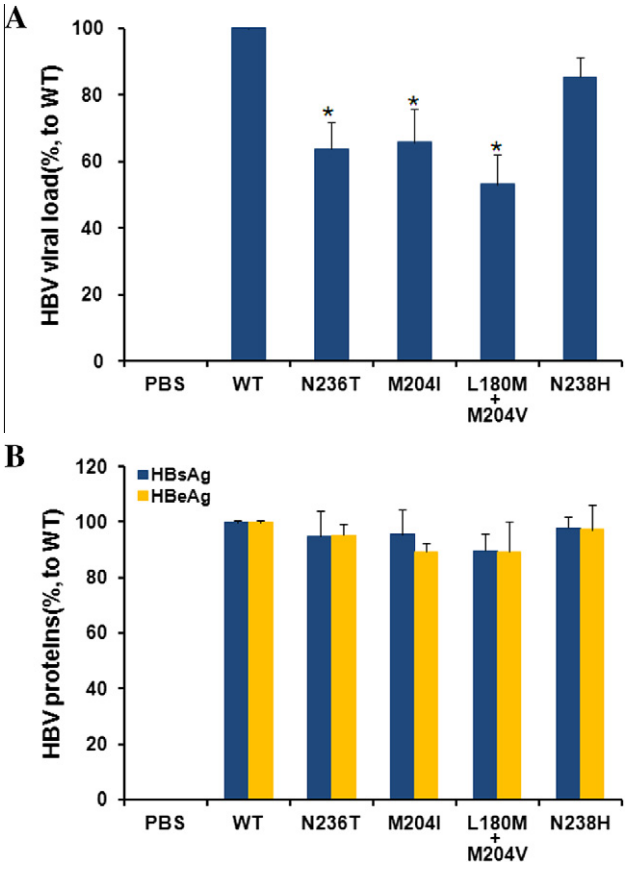

Fig. 1. Release of HBV virions and proteins. (A) HBV viral load was quantified from cell culture supernatant by real-time PCR. In comparison to WT, ADV-resistant (rtN236T) and LAM-resistant (rtM204I or rtL180M + rtM204V) mutants showed significantly lower HBV copy numbers in supernatant, while N238H substitution did not affect HBV viral load. (B) HBsAg and HBeAg were detected by enzyme-linked immunosorbent assay or chemiluminescent assays. $^{*}p < 0.05$ (compared to WT).

K-SDS (Roche), HBV DNA was extracted by phenol–chloroform, followed by precipitation with alcohol. Viral DNA was resuspended in 10 mM Tris–1 mM EDTA buffer. HBV DNA was quantified by a real-time PCR. All experiments were performed at least three times. For each drug, the concentration inhibiting by 50% the amount of intracellular viral DNA (IC_{50}) detected in treated cells at the end of the treatment, compared with untreated cells, was determined by Phosphor Imager analysis.

2.9. Statistical analyses

Measurement data were expressed as means ± standard error of the mean (SEM). Differences in measurement data were examined by Student's *t* test and analysis of variance; the numeration data were analyzed by chi-square test. All statistical analysis was carried out with SPSS 16.0 software. A *P* value < 0.05 was considered statistically significant.

3. Results

3.1. Relatively high prevalence of rtN238 polymerase mutations in CHB patients

We analyzed HBV isolates from a cohort of 1865 CHB patients from China by direct sequencing of the polymerase region. Amino acid mapping of HBV polymerase gene revealed a relatively high frequency of substitutions at position rtN238. 8.8% (165/1865) showed mutations in the rtN238 locus (143 males/22 females, 91 treatment-naive, 42 ADV-treated, 16 LAM-treated and 16 ADV + LAM-treated), namely 86% rtN238H (142/165), 5.5% rtN238S (9/165), 5.5% rtN238T (9/165) and 3% rtN238D (5/165).

Patients with rtN238 polymerase mutations did not display significant differences as compared to patients with the wild-type sequence with respect to epidemiological factors (age, sex), the clinical status (chronic hepatitis, cirrhosis) or biochemical markers of liver inflammation. Among rtN238H mutation strains, there were no significant differences between ADV- or/and LAM- treated patients and treated-naive patients (42% vs. 58%). Importantly, we found that the incidence rate of rtN238H was higher in HBV genotype B infected patients than HBV genotype C subsets (80.3% vs. 19.7%) (Table 1).

3.2. Different with adefovir- or lamivudine-resistant clones, rtN238H polymerase mutations displayed regular viral replication levels

Mutations of the HBV polymerase impact the virus' replicative capacity and susceptibility to nucleoside or nucleotide analogs (Tacke et al., 2004a). In order to understand the impact of rtN238H

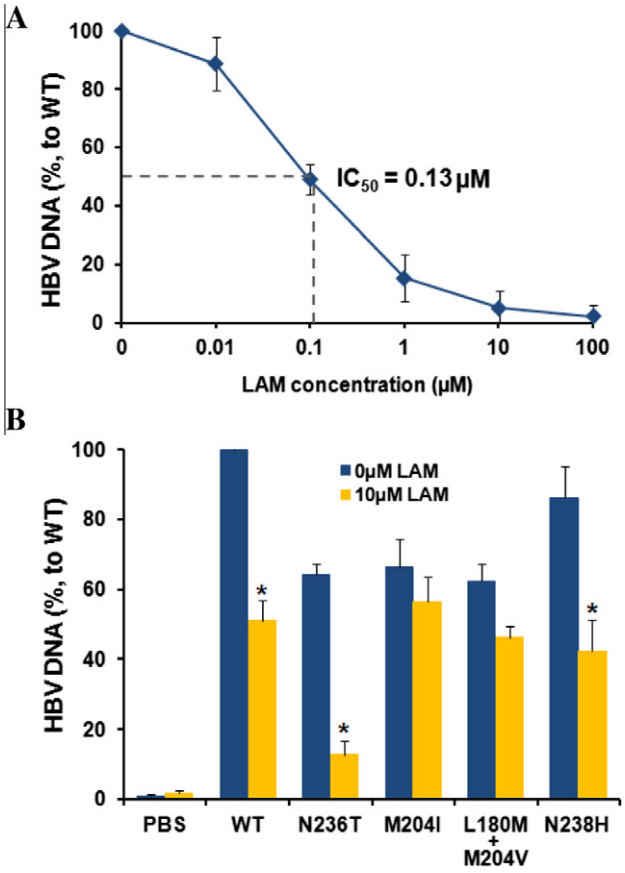

Fig. 2. Susceptibility to lamivudine. (A) The 50% inhibitory concentration (IC_{50}) for lamivudine (LAM) was extrapolated by incubating series of transfected WT constructs with increasing concentrations of LAM and quantifying HBV DNA by real-time PCR. (B) The replication competent HBV constructs were then cultured in the absence or in the presence of 10 μM LAM for 5 days. In contrast to the LAM-resistant rtM204I or rtL180M + rtM204V constructs, WT and N238H mutants were susceptible to LAM in vitro. $^{*}p < 0.05$ (compared to 0 μM).

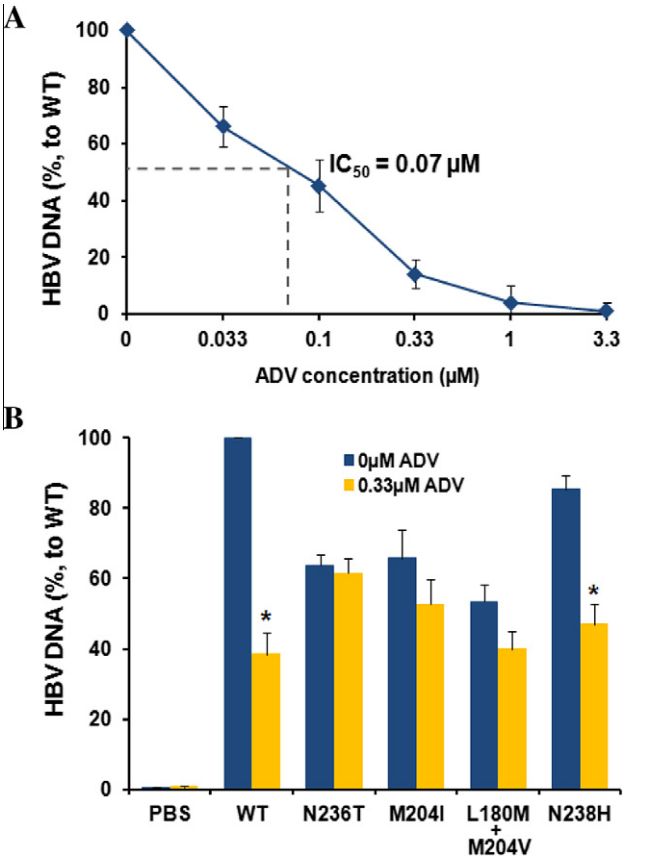

Fig. 3. Susceptibility to adefovir. (A) The 50% inhibitory concentration (IC_{50}) for adefovir dipivoxil (ADV) was extrapolated by incubating series of transfected WT constructs with increasing concentrations of ADV and quantifying HBV DNA by real-time PCR. (B) The replication-competent HBV constructs were then cultured in the absence or in the presence of 0.33 μM ADV for 5 days. In contrast to the ADV-resistant rtN236T constructs, WT and N238H mutants were susceptible to ADV in vitro. $^{*}p < 0.05$ (compared to 0 μM).

polymerase mutation on viral replication, we constructed replication-competent HBV vectors harboring the rtN238H mutation. These were compared to WT as well as ADV- (rtN236T) and LAM resistant (rtM204I or rtL180M + rtM204V) constructs.

The constructs were transiently transfected to the HepG2 cells. Intracellular replication was assessed by real time PCR. The replication level of rtN238H mutants was equal to WT (Fig. 1A). In accordance, the copy number of HBV virions that were released into the supernatant did not differ between WT and rtN238H mutants.

Furthermore, amino acid substitution at rtN238H did not affect the concentrations of HBsAg and HBeAg in the supernatant (Fig. 1B). Of note, the molecular assays quantifying the replicative capacities of the different constructs did not reveal a significant difference between rtN238H and WT. These data showed that rtN238H substitution of the HBV polymerase significantly differed on a functional level from 'classical' LAM- or ADV-resistance mutations, as they did not impair the viral replication efficiency.

3.3. rtN238H polymerase mutations did not confer resistance against lamivudine and adefovir

To test if rtN238H substitution affect the susceptibility of the mutants towards LAM or/and ADV, we exposed transfected WT constructs with increasing concentrations of LAM (Fig. 2A) or ADV (Fig. 3A) and combination of LAM and ADV (Fig. 4A) in order to calculate the 50% inhibitory concentration (IC_{50}) of both antiviral drugs *in vitro-system*. Replication of all constructs was tested in the presence of efficient concentrations of LAM or/and ADV. As shown in Figs. 2 and 4B, LAM and combination of LAM and ADV significantly reduced the viral replication of WT and rtN238H mutants, whereas the rtM204I and the rtL180M + rtM204V constructs were LAM-resistant. On the other hand, rtN236T constructs were resistant to ADV and combination of LAM and ADV (Figs. 3 and 4B), while increasing concentrations of ADV significantly impaired replication of WT as well as rtN238H mutants (Fig. 3B). Although these experiments did not exclude minor differences of the IC_{50} between WT and rtN238H mutants, constructs with rtN238H mutation remained largely susceptible to LAM as well as ADV, indicating that rtN238H substitution did not confer drug resistance.

4. Discussion

In this study, we reported the prevalence and clinical character of HBV variants harboring the rtN238H substitution in 1865 CHB patients. Our results indicated that variations at rtN238H of the HBV polymerase more frequently occurred compared with rtN238T/S or rtN238D (86% vs. 5.5% or 3%).

The rtN238 mutation could be detected in the patients receiving ADV in independent studies (Borroto-Esoda et al., 2007). The rtN238T/D mutations had been described as potential mutations for ADV resistance (Bartholomeusz and Locarnini, 2006; Ghany and Liang, 2007; Yim et al., 2006). However, the prevalence and relationship between rtN238H and ADV-resistance has remained

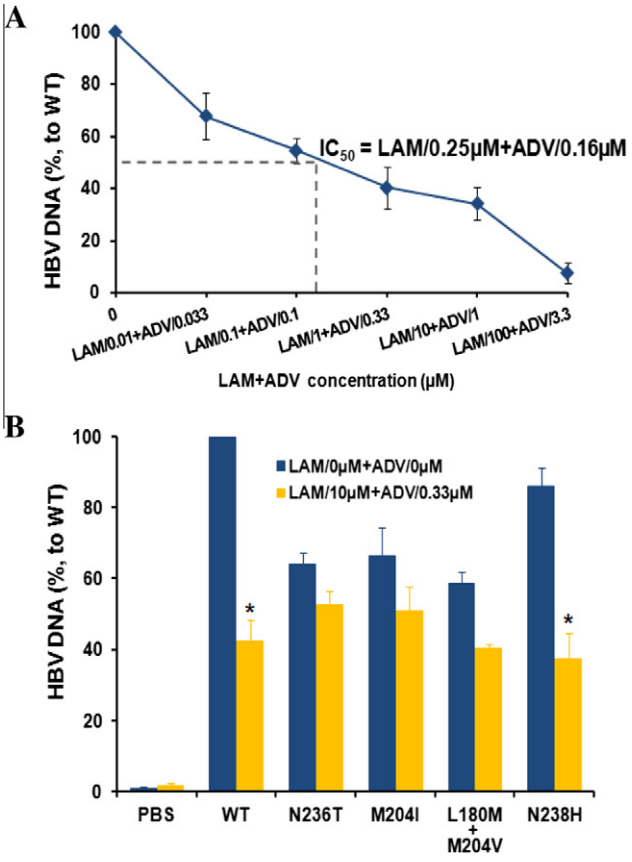

Fig. 4. Susceptibility to combination of lamivudine and adefovir. (A) HBV DNA was quantified by real-time PCR. The 50% inhibitory concentration (IC_{50}) for lamivudine (LAM) and adefovir dipivoxil (ADV) was extrapolated by incubating series of transfected WT constructs with increasing concentrations of LAM and ADV, respectively. (B) The replication-competent HBV constructs were then cultured in the absence or in the presence of 10 μM LAM and 0.33 μM ADV for 5 days. In contrast to the LAM-resistant rtM204I or rtL180M + rtM204V constructs and the ADV-resistant rtN236T constructs, WT and N238H mutants were susceptible to combination of LAM and ADV in vitro. $^*p < 0.05$ (compared to LAM/0 μM + ADV/0 μM).

unclear. In our cohort, there was no significant association between rtN238H mutation and clinical complications as well as ADV resistance.

In this study, we found that the incidence rate of rtN238H was higher in HBV genotype B infected patients than HBV genotype C subsets (80.3% vs. 19.7%). In order to understand the molecular and functional consequences of rtN238H substitution, rtN238H viral replication was tested *in vitro*. Classical mutations conferring resistance to LAM (rtM204I, rtL180M + rtM204V) and ADV (rtN236T) were compared with N238H. The results showed that rtM204I, rtL180M + rtM204V and rtN236T displayed significantly impaired viral replication efficiencies, confirming previous results of other investigators (Tacke et al., 2004b; Angus et al., 2003; Brunelle et al., 2005). In contrast, there was no significant difference between rtN238H and wild-type in HBV replication. These data demonstrated that rtN238H substitution did not impair viral replication, possibly explaining why they can naturally occur at a significant rate in treatment-naive HBV-infected individuals.

Our analysis showed that rtN236T constructs were at least partially cross-resistant to LAM as well. This had been described before by other investigators (Locarnini and Mason, 2006; Qi et al., 2007). Our results demonstrated that rtN238H mutants does neither impair the viral replication efficiency nor susceptibility to LAM or ADV *in vitro*. These findings were supported by our clinical study demonstrating that rtN238H mutations did not influence initial or overall treatment responses to LAM and ADV or the kinetics of viremia. However, further investigations need to evaluate whether the combination of rtN238H substitution together with 'classical' LAM- or ADV-resistance impacts the replication capacity or drug susceptibility of these viral strains. Taken together, our study reveals that rtN238H substitution in the HBV polymerase frequently occurs in genotype B virus-infected patients, even without exogenous selection pressures. As this substitution did neither impair the viral replication efficiency nor susceptibility to LAM or ADV *in vitro*, it is more likely that rtN238H substitution represent background polymorphisms rather than resistance mutations with clinical implications.

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