

RESEARCH ARTICLE

Pre-Existing Mutations in Reverse Transcriptase of Hepatitis B Virus in Treatment-Naive Chinese Patients with Chronic Hepatitis B

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Abstract

High rate of viral replication and lacking of proofreading activity in hepatitis B virus (HBV) polymerase lead to the generation of mutations in HBV virus. Mutations in the reverse transcriptase (RT) region of HBV polymerase are demonstrated to be strongly associated with drug resistance during antiviral treatment. However, the presence of mutations as well as its clinical significance in treatment-naïve hepatitis patients (defined as pre-existing mutations) need to be further investigated. In the present study, a total of 168 serum samples from treatment-naive chronic hepatitis B (CHB) patients were collected, and the RT region of HBV polymerase was sequenced. The results showed that pre-existing mutations in the RT region of HBV polymerase were detected in 43 of 168 (25.6%) treatment-naive CHB patients within which there were no well-characterized primary nucleotide analogs (NAs) resistance sites. Three dominant sites at rt191, rt207 and rt226 were found mutant in 7(16.28%), 8(18.60%), and 14(32.56%) samples respectively among these 43 patients. No significant correlation was found between pre-existing mutations and gender, age, HBV genotype, ALT, HBeAg or HBV DNA loads. However, patients with pre-existing RT mutations under HBeAg sero-negative status exhibited decreased HBV DNA loads, which contributed to the decreased HBV DNA loads in the total HBeAg sero-negative patients. The above investigation indicated that there was a prevalence of pre-existing mutations in RT region of HBV polymerase which might affect the serum HBV DNA level in treatment-naive CHB patients. Its effects on the occurrence of NAs resistance and the prognosis after treatment need to be further investigated.

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Introduction

Chronic hepatitis B virus (HBV) infection, a major cause of cirrhosis and hepatocellular carcinoma, afflicts approximately 350 million people worldwide[1] and 93 millions of Chinese population[2]. HBV is a partially double-stranded DNA virus containing 3200 nucleotides with four open reading frames (ORFs), encoding pre-S/S, pre-C/C, HBX and polymerase. It replicates DNA genome through RNA intermediate during viral life cycle using reverse transcriptase encoded by DNA-P region[3]. Lacking of proof reading ability of reverse transcriptase, the error rate after the replication of viral genome has thus been mounted as 10^{-7} per nucleotide which is 10-fold higher than other DNA virus[4]. Mutations accumulated in the individual genome reflect the selection pressure either from virus or host immune responses. In addition, antiviral treatment represents exogenous selection to determine the survival advantage of HBV mutants[5]. High rate of mutations in HBV genome compromises the antiviral therapy of nucleotide analogs (NA), leading to the generation of drug resistant viral strains and disease progression.

HBV polymerase contains four domains as terminal protein, spacer, reverse transcriptase and ribonuclease H. According to the latest consensus of nomenclature system[6], reverse transcriptase of HBV polymerase consists of 344 amino acids starting with the highly conserved EDWGPCDEHG motif and partially overlaps with HBV surface antigens. Mutations within reverse transcriptase probably affect the replication capacity of HBV, which in turn might alter the antigenicity, encapsidation and virulence of virus as well as the generation of drug resistance[7–9]. Indeed, the drug resistant mutations in reverse transcriptase were extensively explored during antiviral therapy of NAs, including lamivudine(LMV)[10], adefovir (ADV) [11], entecavir (ETV) [12] and telbivudine (LdT) [13]. It is reported that LMV-resistant strains were detected in about 70% of patients after 5 years of treatment with mutant sites at rtM204I/V [14]. Mutant strains with ADV resistance anchor at rtA181T/V and rtN236T [15]. The virological significance of some mutants is also investigated [16, 17].

However, the genetic variability in treatment-naïve chronic hepatitis B patients was unthoroughly investigated. Defining the mutations in treatment naïve patients as pre-existing mutations, the clinical characteristics and significance of these pre-existing mutations were investigated in the present study.

Results

Clinical features of treatment-naïve CHB patients

Samples from 168 treatment-naïve CHB patients were enrolled in this retrospective study. Their clinical features were summarized in Table 1. The patient cohort consisted of 77.38% males and 22.62% females with a median age of 40 years (range 19–81 years). High serum ALT level ($\geq 10N$) was found in 33.73% patients. 61.31% of the subjects displayed high HBV DNA level in the serum ($\geq 10^6$ copies/mL) while 64.88% of them were HBeAg sero-positive.

Correlation between pre-existing mutations and clinical features

Through gene sequencing and sequence alignment with 10 standard genotype (A–J) strains in Genbank, HBV genotypes of the subjects were determined with either type B or C in which type C was the majority (59.62%) (Table 1). Among 168 samples tested, 43 samples (25.6%) had pre-existing mutations in the HBV RT region. The correlation between pre-existing mutations and clinical features, including HBV genotype, age, gender, HBeAg, ALT level and baseline serum HBV DNA in 168 treatment-naïve CHB patients were further analyzed. As

Table 1. Clinical features of the 168 treatment-naïve CHB patients.

Characteristic	Number (Percentage)
Gender (male/female)	130/38 (77.38%/22.62%)
years of Age, median (range)	40 (19–81)
Genotype (type B/C)	68/100 (40.48%/59.62%)
ALT* ($\geq 10N$ / $< 10N$)	56/110 (33.73%/66.27%)
AST* ($\geq 10N$ / $< 10N$)	33/133(24.81%/75.19%)
TBIL* ($\mu\text{mol/L}$), median (range)	33(6.2–612.6)
HBV DNA ($\geq 10^6$ / $< 10^6$ copies/mL)	103/65 (61.31%/38.69%)
Serum HBeAg (positive/negative)	109/59 (64.88%/35.12%)

ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen;

*N = 166.

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indicated in Table 2, no significant correlations were found between the incidence of pre-existing mutations and those clinical features (all $P > 0.05$, Table 2).

Characterization of pre-existing mutations in RT regions from treatment-naïve CHB patients

All of the amino acid substitutions detectable in 43 samples with pre-existing mutations in RT region are listed in Table 3. No well-characterized primary nucleotide analogs (NAs) resistant mutations (i.e. I169T, A181T/V, T184A/C/F/G/I/L/M/S, A194T, S202C/G/I, M204I/V/S, N236T, M250I/L/V) or secondary/compensatory mutations (i.e. L80I/V, V173L, L180M) [19, 20] were found in these mutations. Within 43 samples, 35 of them (81.40%) had single amino acid substitution, 7 patients (16.28%) had two amino acids substitution, and 1 patient (2.33%) had three amino acid substitutions. There were a total of 17 pre-existing mutant sites detectable among 43 samples, in which three dominant mutation sites rt191, rt207 and rt226 were present in 7(16.28%), 8(18.60%), and 14(32.56%) samples respectively (Fig 1).

Table 2. Epidemiological analysis of pre-existing mutations in HBV RT region.

	Group with mutations (n = 43)	Group without mutations (n = 125)	P-value
Genotype Type B	17	51	0.884
Type C	26	74	
Gender Male	36	94	0.249
Female	7	31	
HBeAg Positive	27	82	0.739
Negative	16	43	
Age ≥ 40 years old	25	63	0.381
< 40 years old	18	62	
HBV DNA $\geq 10^6$ copies/mL	22	81	0.113
$< 10^6$ copies/mL	21	44	
ALT * $\geq 10N$	12	44	0.348
$< 10N$	31	79	

HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; ALT, alanine aminotransferase;

*N = 166.

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Table 3. Characteristics of amino acid mutations in HBV RT region.

Characteristics	Genotype	No.
rtA181S	C	2
rtI187V	B	1
rtI187V + rtN226H	B	1
rtV191I	C	7
rtL207V	B	1
rtL207V + rtN226H	B	1
rtV207M	C	1
rtV207M + rtV214A	C	1
rtV207M + rtN226H	C	3
rtV207I + rtN226H	C	1
rtK212R	B	1
rtV214A	C	1
rtE218D	B	1
rtS219A	C	1
rtS219A + rtT225V + rtN226H	B	1
rtL220I	B	1
rtN226H	B	6
rtN226T	C	1
rtL228F	B	2
rtL228I	C	1
rtL229V	C	2
rtH234R	B	1
rtH234Q	C	1
rtL235I	C	2
rtN238G	C	1
rtS246C	B	1

RT, reverse transcriptase.

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Patients with pre-existing Mutations in RT region showed decreased HBV DNA levels within HBeAg sero-negative group

In this study, pre-existing RT mutations were associated with decreased serum HBV DNA level within the total treatment-naïve patients, but the difference didn't reach significance (mutant vs. non-mutant: $10^{6.08}$ vs. $10^{6.47}$, $P = 0.113$, Fig 2). However, when we sub-grouped the patients into HBeAg sero-positive and HBeAg sero-negative groups, it turned out that the average HBV DNA loads were significantly lower in patients with pre-existing RT mutations when comparing with non-mutant ones within HBeAg sero-negative group (mutant vs. non-mutant: $10^{5.45}$ vs. $10^{6.23}$, $P = 0.0318$, Fig 3B). The serum HBV DNA loads were comparable between mutant and non-mutant patients within HBeAg sero-positive group (mutant vs. non-mutant: $10^{6.46}$ vs. $10^{6.60}$, $P > 0.05$, Fig 3A).

Pre-existing RT Mutations contribute to the decreased viral loads in treatment-naïve HBeAg sero-negative patients

It is known that there is a direct relationship between HBeAg positivity status and high levels of HBV DNA loads. But in HBeAg negative CHB patients, there are always precore/core mutations which could restore or increase the viral loads. Within the total 168 samples in this study,

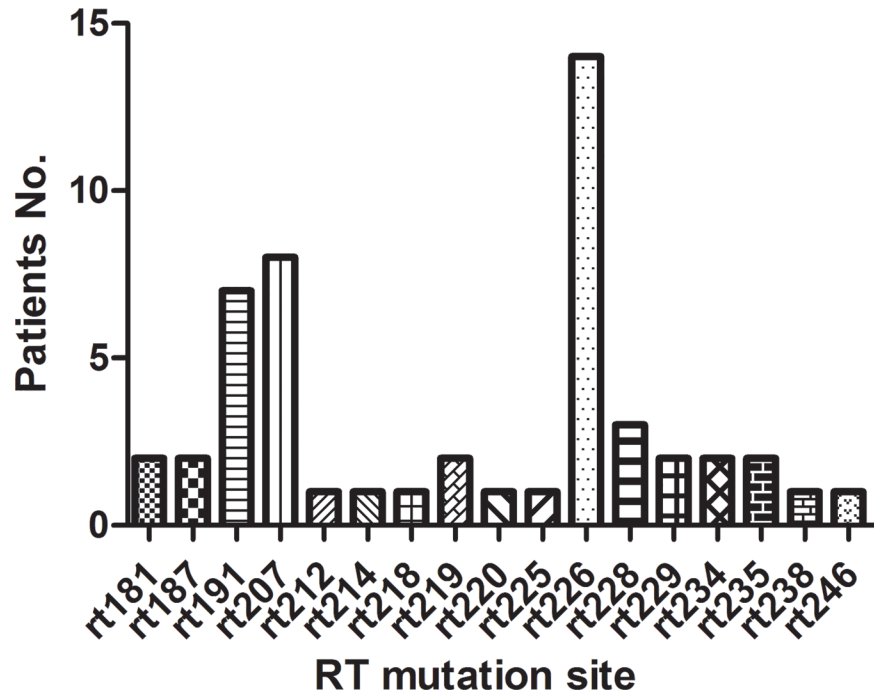


Fig 1. Distribution of pre-existing mutations in HBV RT region of 43 samples.

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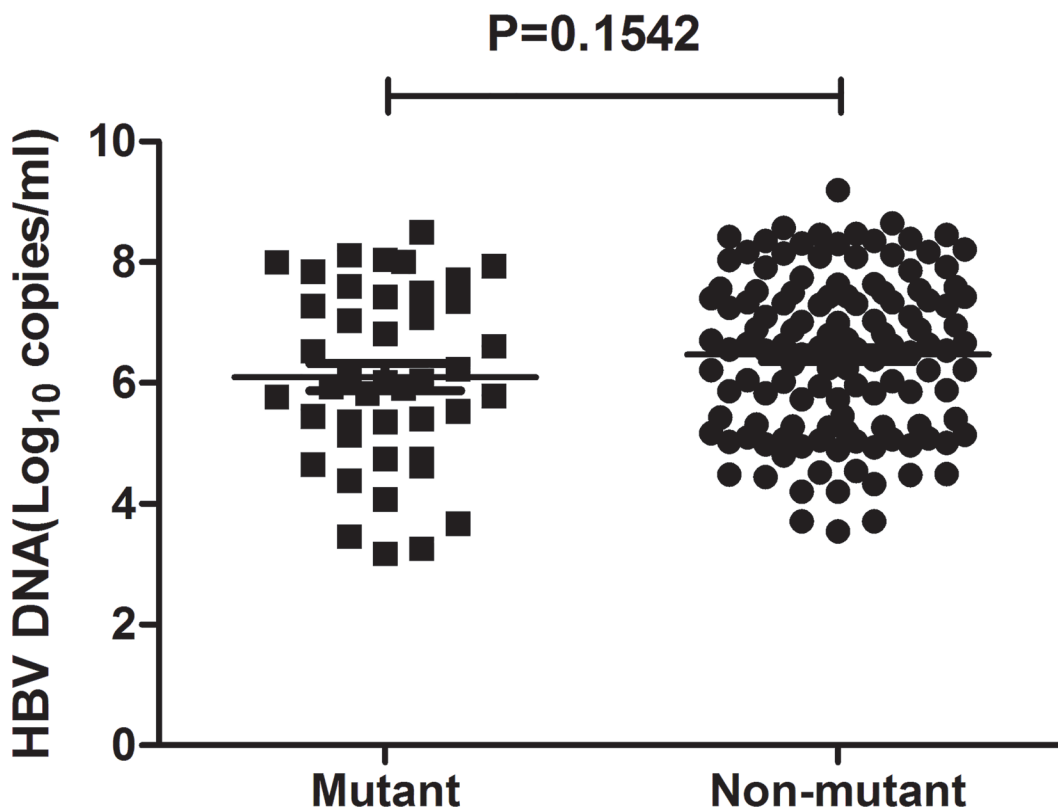


Fig 2. HBV DNA levels in 168 treatment-naïve CHB patients with or without pre-existing RT mutations.

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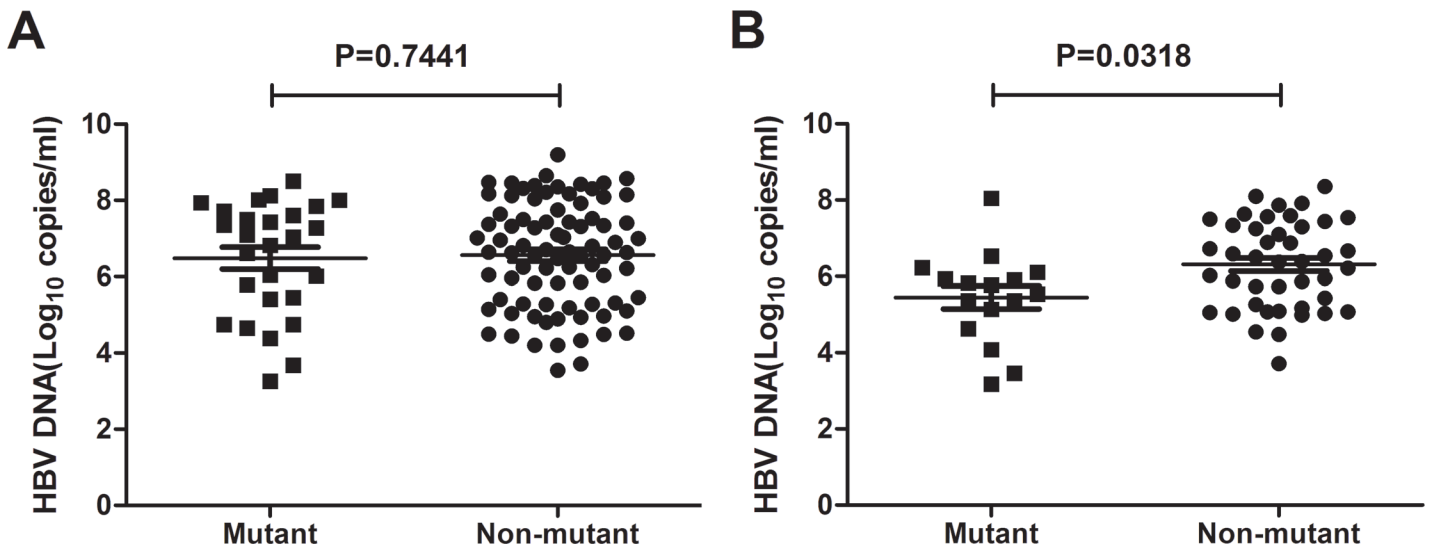


Fig 3. HBV DNA levels in CHB patients with or without pre-existing RT mutations among HBeAg sero-positive group (A) and HBeAg sero-negative group(B).

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HBeAg sero-negative patients showed decreased viral loads (HBeAg⁺ vs HBeAg⁻: $10^{6.565}$ vs $10^{6.020}$, $P = 0.0112$, Fig 4). On the other hand, when excluding the 43 patients with pre-existing RT mutations and comparing HBV DNA loads in the 125 patients without mutations, no significant difference were found between HBeAg sero-positive group and HBeAg sero-negative group (HBeAg⁺ vs HBeAg⁻: $10^{6.646}$ vs $10^{6.252}$, $P = 0.1932$, Fig 5A). Within the 43 patients with pre-existing RT mutations, HBeAg sero-negative group showed decreased viral loads (HBeAg⁺ vs HBeAg⁻: $10^{6.463}$ vs $10^{5.445}$, $P = 0.023$, Fig 5B). These results demonstrate that pre-existing RT mutations contribute to decreased HBV DNA loads in treatment-naïve HBeAg sero-negative patients.

Discussion

Determination of mutant sites in HBV RT region is extensively applied to monitor drug resistance to NAs in the long-term treatment for CHB patients. There are certain well-known sites in HBV RT region as indicators for the resistance to lamivudine (rtV173L, rtL180M, rtM204V/I and rtA181V/T), adefovir (rtA181V/T and rtN236T) or entecavir (rtI169T, rtI184G, rtS202G/I, and rtM250V) etc, which provide important guidance to drug selection for clinicians[21, 22]. The existence of pre-existing mutations in RT region of HBV polymerase was also reported in previous literatures, for instance, secondary/compensatory but not primary mutations were found in treatment-naïve Italian population[23], while there were 8.8% Indian treatment-naïve CHB patients carrying primary drug resistant mutations[24]. Based on our retrospective study, we have demonstrated that pre-existing mutations in HBV RT gene exist in 25.6%(43/168) of treatment-naïve hepatitis B patients. Primary NAs resistance mutations or secondary/compensatory mutations were not found in this study, which is consistent with similar studies on Chinese population[19]. This might be due to the limitation of the sequencing approach which can not detect minor variants with a frequency below 20% or due to the property of the patient cohort enrolled in this study, because YMDD mutations were found in 26.9% Chinese CHB patients if the inclusion criteria were non-taking antiviral drugs within the last one year[25]. However, our result indicated that the naturally occurring NAs resistant

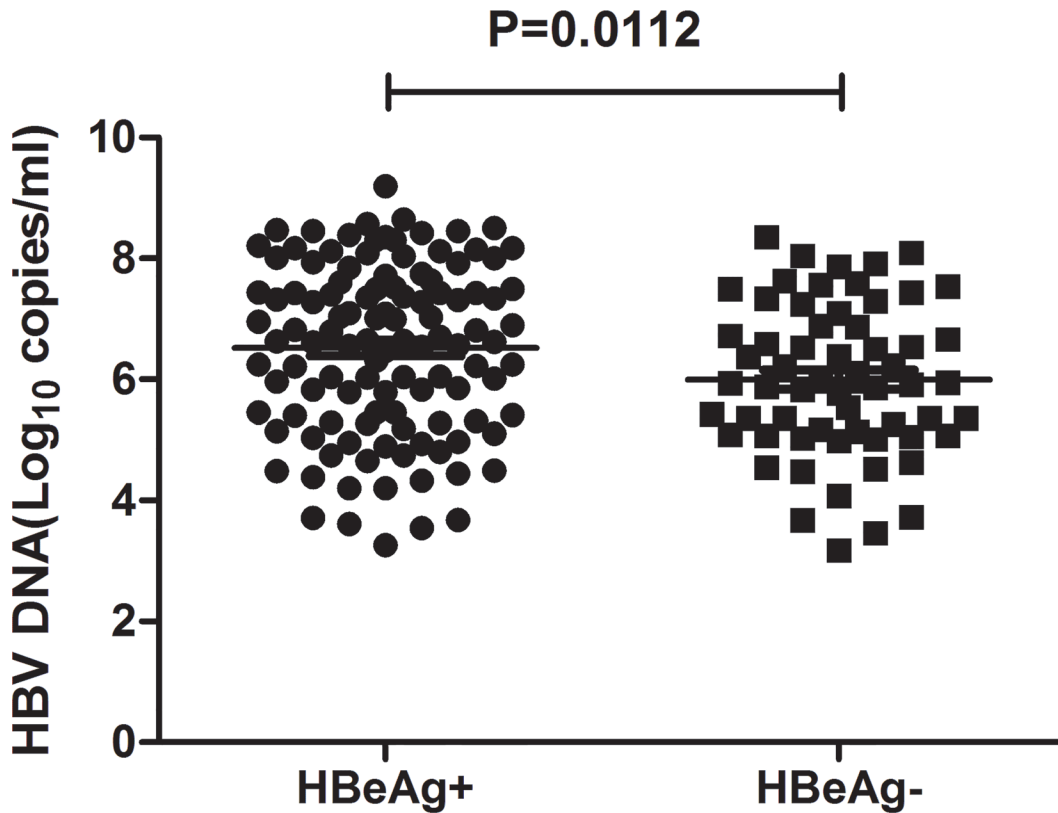


Fig 4. HBeAg sero-negative patients displayed lower HBV DNA level than HBeAg sero-positive patients.

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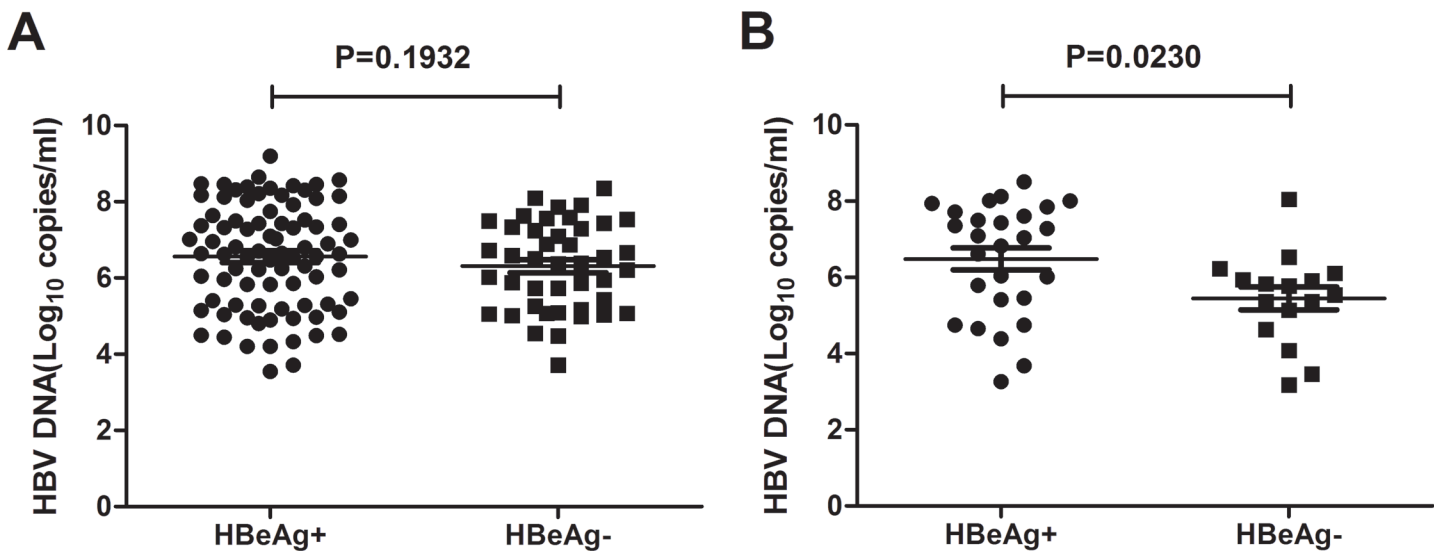


Fig 5. HBV DNA levels in HBeAg sero-positive and HBeAg sero-negative groups among patients without pre-existing RT mutations (A), and patients with pre-existing RT mutations (B).

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strains were not prevalent or predominant in treatment-naïve Chinese CHB patients infected by B- or C-genotype virus.

Although primary or secondary/compensatory NAs resistance mutations were not found in this studied population, there were a lot of putative NAs resistant mutations which have been reported to be selected during prolonged NAs treatment or potentially associated with NAs resistance or compensatory replication capacity. For instance, rtV191I, rtV207I, rtL229V were associated with lamivudine resistance[26–28], and rtA181S, rtV214A, rtE218D were associated with adefovir resistance[29–31]. Besides NAs resistance, many mutations such as rtV191I, rtV207I, rtK212R, rtS219A, rtT225V, rtN226H caused accompanied mutations on the overlapped s gene and produced amino acids mutant or truncated HBsAg[32, 33]. This may support survival advantages by reducing antigenicity of the HBsAg protein under the natural selection pressure of hosts' immune system in these treatment-naïve patients. What's more, rtA181S, rtI187V, rtV191I and rtL229V were proved *in vitro* with decreased replication efficacy[7,28,34,35], while rtL220I, rtL228F/I, rtH234R and rtL235I were novel mutations of which biological or clinical significance has not been reported so far as we know.

Pre-existing mutations in HBV RT region are not apparently related to most of the clinical features, including HBV genotypes, age, gender, serum HBeAg, ALT level as well as baseline serum HBV DNA level. However, when we sub-grouped the patients into HBeAg sero-positive and sero-negative groups, the presence of pre-existing mutations in HBV RT region is dramatically related to the decrease of HBV DNA level within HBeAg sero-negative patients but not HBeAg sero-positive patients. This is quite unexpected. How HBeAg sero-negativity affects HBV DNA level in patients with pre-existing mutations remains unclear.

HBeAg is a non-structural protein encoded by gene HBV preC/C gene. It is derived from HBV core antigen (HBcAg) with the loss of some amino acid residues[36]. After synthesized in the cytoplasm, it is secreted out of liver cells through endoplasmic reticulum and distribute in the periphery blood and the whole body fluid[37, 38]. HBeAg is dispensable for the assembly and the replication of HBV, but it is demonstrated to be an indicator for rapid replication of HBV *in vivo* and high contagion. Serological conversion of HBeAg is a critical indicator for effectiveness of anti-viral drugs. After treatment, the decrease of virus DNA level accompanied by sero-conversion of HBeAg always indicates a good prognosis. Biologically, it is suggested that HBeAg induces immune tolerance when it is secreted into the serum[39]. HBeAg was reported to be able to induce the secretion of Th2 cytokines such as IL- 4, IL-10 in peripheral blood lymphocytes[40]. Peripheral circulation of HBeAg in transgenic mice could eliminate HBcAg- and HBeAg-specific Th1 cells through Fas-FasL mediated mechanism[41]. Whether the decline of HBV DNA level in HBeAg negative patients is owing to the recovery of Th1 cellular immune responses against HBeAg or related protein (such as HBcAg) still needs to be further investigated.

It has been reported that HBV RT mutations can affect HBV DNA replication. For instance, when the proline residue at position306 (rtP306) in HBV RT region is replaced by serine or other amino acid residues, virus replication efficacy declines[42]. It is also reported that the substitution of YMDD motif of ayw subtypes into YIDD, YVDD reduced the capacity of virus replication *in vitro*[43]. In addition, rtA194T mutation in HBV polymerase region results in a decreased HBV DNA replication capacity. However, when pre-C/BCP mutations co-exist with rtA194T mutation in HBeAg sero-negative status, the virus replication level will recover to the comparable levels of wild type strain[44]. But recent studies showed that pre-C/BCP mutations could only counteract the effects of rtA194T mutation. If other mutant sites exist in RT region meanwhile, the capacity of HBV DNA replication reduced as well[45]. In our study, we found that pre-existing mutations in HBV RT region was closely related to lower HBV DNA levels in

HBeAg sero-negative subjects, which is consistent with impaired replication capacity of mutant stains such as rtA181S, rtI187V, rtV191I and rtL229V mutant stains[7,28,34,35].

In conclusion, we demonstrate that the presence of pre-existing mutations in RT region in treatment naïve CHB patients. These mutations might contribute to the decreased HBV DNA loads in HBeAg sero-negative patients. Its effects on the occurrence of NAs resistance and the prognosis after treatment need to be further investigated.

Materials and Methods

Clinical samples

This study was retrospective. A total of 168 treatment-naïve chronic HBV patients who visited Shanghai 3rd People's Hospital Affiliated to Shanghai Jiao Tong University School of Medicine from July 2008 to May 2010 were enrolled in the study. All of the patients were first diagnosed as chronic hepatitis B and confirmed without taking any Nucleos(t)ide analogues. The diagnostic criteria were based on 2000 Xi'an Viral Hepatitis Management Scheme issued by the Chinese Society of Infectious Diseases and Parasitology, and the Chinese Society of Hepatology, Chinese Medical Association. The inclusion criteria of chronic hepatitis B patients included hepatitis B surface antigen (HBsAg) positive for more than 6 months and abnormal ALT level; while the exclusion criteria included hepatitis C virus or human immunodeficiency virus co-infection, autoimmune liver disease, and alcohol or drug abuse. Patients were informed and agreed to authorize the hospital to deal with their blood, tissue fluid and tissue sample for diagnosis and research with written consent. The study was approved by Ethics Committee of Shanghai 3rd People's Hospital. Patients' serum was collected and frozen at -80°C.

HBV serology and Liver biochemistry assays

The serology and liver biochemistry assays were routinely detected in the clinical laboratory of Shanghai 3rd People's Hospital affiliated to Shanghai Jiao Tong University School of Medicine. Serum hepatitis B s antigen (HBsAg), hepatitis B e antigen (HBeAg), anti-HBs, anti-HBe, anti-HBc were detected according to the instruction of the attached original commercial kits on the wholly automatic immune fluorescence analyzer Abbott Type I2000 (Abbott Laboratories, Illinois, USA). Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and other liver biochemistry markers were assayed by Beckman's wholly-automatic biochemical analyzer (Beckman Coulter, CA, USA). Two patients' ALT data were excluded because of inconsistent time point of sampling.

Quantitative detection of HBV DNA

HBV DNA was extracted from the serum of chronic hepatitis B patients. DNA level in the blood was detected by the real-time fluorescent quantitative polymerase chain reaction (PCR) (Da'An GENE, Guangzhou, China) on ABI7500 (Applied Biosystems, Foster City, USA). The detection sensitivity was as low as 500 copies/mL.

Amplification and sequencing of HBV RT region

HBV DNA was extracted from the sera of patients using QIAamp DNA Blood Kit (Qiagen, Hilden, Germany). RT region was amplified by nested PCR as previously described[18]. The primers used in the first round PCR were 5-AGTCAGGAAGACAGCCTACTCC-3 (nt 3146–3167) and 5-AGGTGAAGCGAAGTGCACAC-3(nt 1577–1596); the primers used in the second round PCR were 5-TTCCTGCTGGTGGCTCCAGTTC-3 (nt 54–75) and 5-TTCCGCAG TATGGATCGGCAG-3 (nt 1258–1278). PCR products were purified using the QIAquick Gel

Extraction Kit (Qiagen, Hilden, Germany) and sequenced commercially (Sangon Biotech, Shanghai, China). Nucleotide sequences were analyzed using DNASTar 5.0 and MEGA 4.0 softwares. Mutations in HBV RT region were determined by sequence alignment of PCR products with the reference stains in GenBank[6].

Statistical analysis

Statistical analyses were performed using SPSS17.0 software (SPSS, Chicago, IL). Between-group comparisons were performed using one-way ANOVA *t* test or chi-square test, as appropriate. Serum HBV DNA concentrations were expressed on a logarithmic scale. A *P* value of less than 0.05 was considered statistically significant.

Author Contributions

Conceived and designed the experiments: YW GPZ. Performed the experiments: JHW BW. Analyzed the data: YW JX BW. Contributed reagents/materials/analysis tools: YW JX BW. Wrote the paper: YW GPZ. Collected the clinical samples and manifestations: JX LZ. Clinical sample collection and analysis: LH.

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