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Prevalence and Significance of Hepatitis B Reverse Transcriptase Mutants in Different Disease Stages of Untreated Patients

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Abstract

Aims—Hepatitis B virus (HBV) reverse transcriptase (RT) mutants, which have not been well characterized according to different disease stages. This study aimed to characterize the profiles of naturally occurring mutations in the HBV RT region and their associated clinical outcomes.

Methods—HBV RT region mutations and genotypes were determined by PCR-direct sequencing and compared with p-distance model.

Results—Among 467 consecutive eligible patients (262 chronic hepatitis B patients, 105 cirrhotic patients and 100 hepatocellular carcinoma patients), the nucleos(t)ide analogues related mutations (rtI169T, rtV173L, rtL180M, rtA181T, rtS202C, rtM204I/V, rtN236T) were found. The p-distance value reached a peak in the age of 20 to 30 years in the CHB patients and in the age of 40 to 45 years in the cirrhotic patients and hepatocellular patients. The naturally occurring mutation, rtS106C mutation was higher in chronic B patients (14/100, 14.0%) and cirrhotic patients (14/100, 14.0%) patients than that in hepatocellular carcinoma patients (4/100, 4.0%, $P=0.013$). And the rtD134E/G/N/S mutations were also higher in chronic hepatitis B patients

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Competing interests

All authors declare that they have no competing interests.

Authors' contributions

JXZ did most of the experimental work and drafted the manuscript. FW set up database. DYZ helped to collect the plasma and record of patients, and other experimental support. YYY analyzed the data. ZZ designed and supervised the research. ZZ analyzed the data and revised the manuscript for important content and gave final approval of the version to be published. CQP revised the manuscript. All authors read and approved the final manuscript. Authors thank the HBV study consortium for collection of samples [Department of Infectious Diseases, Peking University First Hospital, Beijing, P.R. China; Institute of Liver Diseases Research, Beijing Military General Hospital, Beijing, P.R. China; Beijing Ditan Hospital; Department of Surgery, Beijing Institute of Tumor Prevention and Therapy Beijing, P.R. China; Department of Infectious Diseases, Shanxi Medical University, Taiyuan, P.R. China; Department of Infectious Diseases, Qinhuaangdao No. 3 Hospital, Qinhuaangdao, P.R. China; Department of Surgery, Inner Mongolia Medical College, Hohhot, P.R. China; Department of Infectious Diseases, Xuzhou No. 3 Hospital, Xuzhou, P.R. China; Department of Infectious Diseases, Xinjian Medical University, Urumoqi, P.R. China; Department of Infectious Diseases, the Second Affiliate Hospital of China Medical University, Shenyang, P.R. China; Institute of Liver Diseases Research, Peking University Second Hospital, Beijing, P.R. China; Department of Surgery, Peking Union Medical College, Beijing, P.R. China].

(22/100, 22.0%) and cirrhotic patients 21.0%) than that in hepatocellular carcinoma patients (10/100, 10.0%, $P=0.021$ and $P=0.032$, respectively). The mutation frequencies in A–B interdomain were higher in cirrhotic (101/1900, 5.3%) than that in hepatocellular carcinoma patients (68/1900, 3.6%) ($P=0.009$).

Conclusions—The nucleos(t)ide analogues related mutations do exist in treatment naïve patients with different disease stages. rtS106C, rtD134E/G/N/S and A–B interdomain mutations may be associated with necro-inflammation, immune response and cirrhosis development at ages older than 40.

Keywords

hepatitis B virus; reverse transcriptase; mutations; naïve; clinical outcomes

INTRODUCTION

Hepatitis B virus (HBV) infection remains a global health problem, and more than 350 million people worldwide are chronic carriers of the virus (1). HBV infection could cause variety chronic liver diseases, including acute self-limiting hepatitis, chronic hepatitis (CHB) or inactive carrier state, with progression to cirrhosis or hepatocellular carcinoma (HCC) (2).

Nucleos(t)ide analogue (NA) antiviral therapy can provide the long-term suppression of HBV replication with improvement on liver histology(3). Since 1999, lamivudine (LMV), adefovir dipivoxil (ADV), entecavir (ETV) and telbivudine (LdT) have all been approved for the treatment of chronic hepatitis B in China. However, viral resistance has become a major barrier for the antiviral treatments. Published data showed that approximately 65% of patients on lamivudine monotherapy developed YMDD motif mutations (rtM204I/V) after 5 years of treatment (4). Further study on virological mechanisms of HBV drug resistance reported that potential antiviral resistance mutations were localized into the reverse transcriptase (RT) region of HBV (5). Based on in-vitro phenotypic analyses confirmation, drug-resistance mutations are classified into two categories, primary resistance mutations and secondary/compensatory mutations (6). In addition, studies have observed that amino acid (AA) substitutions in other positions of RT, such as rtV191I and rtT128I/N, were selected during prolonged NA therapy and affected the replication capacity of HBV (7, 8).

Despite adherence to antiviral medications, nucleos(t)ide analogue resistant (NAr) mutants were commonly found in patients who developed viral breakthrough or suboptimal viral reduction during the therapy. Recent studies demonstrated that these mutants also existed in treatment naïve patients (9–16). Controversy remains on whether treatment naïve patients with pre-existing NAr mutations are more likely to develop antiviral drug resistance during the therapy(17–19). In addition, the relationship between pre-existing NAr mutants and disease progression has not been fully explored. The study populations in published studies are limited to patients with compensated CHB or inactive CHB carriers; data on patients with cirrhosis and HCC are lacking. This study aims to determine the virologic features of pre-existing mutants and explore their relationship among treatment naïve patients with different disease stages of CHB.

MATERIAL AND METHODS

Patients

Study subjects were recruited from 12 hospitals in China during the period of 2002 to 2010 after meeting eligibility criteria and providing informed consent for participation (see Authors' contributions). The relevant institutional ethics review committee at each site

approved the study prior to enrollment. Major eligible criteria included age of 18–70 years treatment naïve and hepatitis B surface antigen (HBsAg)-positive for at least 12 months: serum HBV DNA levels were > 200 IU (10^3 copies)/ml. Exclusion criteria were the followings: co-infection with hepatitis A virus (HAV), hepatitis C virus (HCV), hepatitis D virus (HDV), hepatitis E virus (HEV), and human immunodeficiency virus (HIV); pregnancy; diagnosis of other liver diseases including autoimmune hepatitis and Wilson disease, or evidence of hepatic tumor; history of renal, cardiovascular, pulmonary or neurological diseases; concurrent treatment with immune-modulators, cytotoxic drugs, or steroids; history of nucleos(t)ide antiviral drug or interferon related treatment; drug abuse including alcohol abuse; or hepatic encephalopathy (degree IV). Clinical data and serum samples were obtained from consecutive patients who had CHB, cirrhosis or HCC and met the above criteria.

The clinical definition of disease stages in this study were the followings: CHB is diagnosed by two positive tests for HBsAg and antibodies to HBcAg at least 6 months apart with ALT and/or AST levels greater than 2 times upper limits of normal (based on local reference lab range) prior to enrollment. Patients with histologic (stage IV fibrosis) or clinical evidence of cirrhosis were not considered in this group. Cirrhosis is diagnosed by either histologic (stage IV fibrosis) or clinical evidence of cirrhosis (in the event which liver biopsy was not performed). Clinical evidence included cirrhosis suggested by sonogram, CT, or MRI; varices in the upper gastrointestinal tract; as cites or edema; hepatic encephalopathy; serum albumin < 35 g/L, total bilirubin > 35 μ mol/L. Hepatocellular carcinoma (HCC) is diagnosed by at least one of the following: 1) liver biopsy; or 2) elevated AFP levels and evidence of HCC suggested by sonogram, CT, or MRI (20).

Viral markers and HBV DNA assays

HBV serum markers, such as hepatitis B surface antigen (HBsAg), hepatitis e antigen antibody to hepatitis e antigen (anti-HBe), antibody to hepatitis c virus (anti-HCV), were detected with commercially kits (ABBOTT, Max-Planck-Ring, Wiesbaden, Germany). Anti-HIV was detected with commercially kits (E-Y Laboratories (HK) Ltd, Tsuen wan, New territories, HK). Anti-HAV, anti-HDV, and anti-HEV were detected with commercially ELISA kits (Livzon diagnostics, Zhuhai, P.R. China; Beijing Kewei Clinical Diagnostic Reagents Co., Ltd, Beijing, P.R. China; and MP Diagnostics, The Cavendish, Singapore; respectively). HBV DNA levels were determined by commercial real-time PCR kit (DaAn Gene Co., Ltd, Guangzhou, Guangdong, P.R. China). The linear range is from 10^3 copies/ml to 10^{10} copies/ml, and the limit is greater than 10^3 copies/ml

HBV DNA extraction, amplification and sequencing

HBV DNAs were extracted by QIAamp DNA blood mini kit (QIAGEN China Co., Ltd, Zhangjiang Hi-Tech Park Pudong, Shanghai, P.R. China). RT region sequences of HBV were amplified by semi-nested polymerase chain reaction (PCR) with the following primers: P-F1: 5' ACTCGTGGTGGACTTCTC3' (254–271), P-R1: 5' AGGCAGGATADCCACATT3' (D=T, A, G) (1053-1036), and P-R2: 5' TAAAAGGGGCRGCAAASC3' (R=A, G) (S=C, G) (1032-1015). All of the above primers designed according to the Gene Bank sequence AY206384. The sense primer P-F1 and antisense primer P-R1 were used for the first round amplification of P gene. The PCR solution includes in 1.5 mM MgCl₂, 0.2 mM dNTP (dATP, dTTP, dCTP, dGTP) (Promega, Madison, WI, USA), 0.2 mM sense primer and antisense primer, and 2.5 units Go-Taq DNA Polymerase (Promega, Madison, WI, USA). The following cycling profile was carried out for the first round PCR: 95°C for 2 min, then 35 cycles for 40 seconds at 95°C, 60 seconds at 53°C, 90 seconds at 72°C and final extension for 72°C for 7 min. The second round PCR was carried out by sense primer P-F1 and antisense primer P-R2. And the amplification

cycling conditions for the second round PCR was 95°C for 2 min, followed by 35 cycles of 95°C for 40s, 55°C for 60s, and 72°C for 60s and final extension for 72°C for 7 min. The first round or second round PCR product was purified and sequenced directly by forward primer P-F1 using DNA sequencer (ABI Prism 3730, Perkin Elmer, Foster City, CA).

HBV genotyping

HBV genotypes were determined by using NCBI viral genotyping tool (<http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi>) and phylogenetic analysis with MEGA 5.0 software.

Mutation analysis and definitions

The mutations analysis and definitions of HBV reverse transcriptase were referenced to previous literatures and by using CLC sequence viewer and MEGA 5.0 software (21, 22). Briefly, the HBV reverse transcriptase (RT) amino acid (AA) mutations were identified by comparing HBV RT sequences with the genotype-matched consensus sequence generated based on the HBV sequences obtained in this study (HBV sequences from all the chronic hepatitis B patients) and the reference sequences in previous investigation (genotype C, X01587) (23, 24). Conserved positions were defined as those with a single AA identity observed across all isolates, while polymorphic positions were those with multiple AA identities (21, 22). A mutation type was referred to the replacement of the consensus AA of the corresponding genotype with a novel one; for instance, rtM204I and rtM204V were described as two mutation types (24). The primary drug resistant mutations were defined as nucleotide mutation(s) caused an amino acid substitution that resulted in reduced susceptibility to an antiviral drug, such as rtL180M, rt204I/V for lamivudine, rtL180M+rtM204I/V+rtS202C or rtM250I/V for entecavir; rtA181T/V, rtN236T for adefovir dipivoxil; rtM204I for telbivudine. The secondary drug mutations were defined as nucleotide mutation(s) caused an amino acid substitution that were not above primary mutations in this study.

HBV reverse transcriptase sequence analysis by bioinformatics

HBV reverse transcriptase sequences were handled with software MEGA 5.0 and the average evolutionary divergence over sequence pairs within groups was analyzed by p-distance model (25). The estimation of variance over sequence pairs within groups was analyzed by bootstrap method in the p-distance model, and the number of bootstrap replications was 500.

Statistical analysis

Continuous data are expressed as mean $\pm$ SD and analyzed by Student's *t* test, one-way factorial analysis of variance (ANOVA) or nonparametric Mann–Whitney “U”-test. Categorical data are expressed as number (percentage) and compared by the chi-square test or Fisher's exact test. P values of <0.05 were regarded as statistically significant. All data were analyzed by the statistical software SPSS (version 14.0, Chicago, IL, USA).

RESULTS

Clinical characteristics of the patients

527 patients were enrolled in the study and all subjects' clinical data with serum samples were available for analyses. The analysis of HBV genotypes from the 527 patients showed genotype B, C and D in all groups, which included 311 patients with CHB (genotype B: 46; C: 262; D: 3); 109 patients with cirrhosis (genotype B: 3; C: 105; D: 1) and 107 patients with HCC (genotype B: 5; C: 100; D: 2). The small number of patients with genotype B and

D precluded further analyses, as the results would lack statistic power. Thus, 467 patients with genotype C were analyzed in the present study. The demographical, biochemical and virological data of these subjects are shown in Table 1. The mean ages of cirrhotic and HCC patients were significantly higher than CHB patients ($P < 0.001$). The mean level of serum ALT was lower in HCC patients compared with CHB patients ($P = 0.001$). The mean HBV DNA levels in CHB and cirrhotic patients were higher than patients with HCC ($P = 0.009$ and $P < 0.001$, respectively). In addition, the HBeAg⁻ to HBeAg⁺ ratios in cirrhotic and HCC patients were higher than those in CHB patients ($P < 0.001$).

Nucleotide analogues related resistance mutations

The prevalence of nucleos(t)ide analogues related resistance mutations are shown in Table 2. All mutations in the RT sequences of HBV were grouped into 4 categories, (1) primary drug resistance mutation, (2) compensatory mutation, (3) putative NAr mutation and, (4) other mutation (22). The mutations were observed in different disease stage of HBV. Among patients with lamivudine related resistance mutations, eleven had rtM204I/V/S (four with rtM204I, six with rtM204V and one with rtM204S), nine had rtL180M and three had rtV173L mutation. Eight patients had double mutations of rtM204V/I+rtL180M and one patient with triple mutations of rtM204V+rtL180M+rtV173L. Among patients with adefovir related resistance mutations, five had rtA181T/V, one had rtN236T and eighteen had rtV214A/E/I. Other related resistance mutations were found in two patients with rtI169L/T/N and one patient with rtS202I.

The average evolutionary divergence in CHB, cirrhotic and HCC patients

The average evolutionary divergence in groups is shown in Table 3. The p-distance value averages among the groups with CHB, cirrhosis and HCC are similar. The p-distance value averages between male/female and different HBV DNA levels in the CHB, cirrhotic and HCC patients were also similar. However, the p-distance value average was higher in HBeAg negative patients (0.024 ± 0.002) than that in HBeAg positive patients (0.019 ± 0.002) in the cirrhosis group ($P < 0.05$). The average of p-distance value was higher in patients with ALT ≥ 160 IU/L (0.029 ± 0.003) than those with ALT < 80 IU/L (0.019 ± 0.002) in the cirrhosis group ($P < 0.05$).

We analyzed the average evolutionary divergence adjusted by age in the CHB, cirrhosis group and HCC groups with p-distance model. As showed in Fig. 1, the average p-distance value reached a peak in the range of age from 20 to 30 years in CHB patients. However, the average p-distance value in cirrhotic and HCC patients reached a peak in the range of age from 40 to 45 years. Thus, the average of p-distance value in cirrhotic and HCC patients were higher than those in CHB patients.

Mutation distribution and frequency in RT regions and different clinical outcomes of HBV infection

To further delineate the relationship between HBV reverse transcriptase mutations and its clinical significance, the age and sex of patients in the CHB, cirrhosis and HCC group were paired. As the result, 100 patients from each group were selected (by age and sex paired) and analyzed for their HBV reverse transcriptase mutations. A significant portion of patients with rtS106C mutations were detected in the CHB group (14/100, 14.0%) or cirrhosis group (14/100, 14.0%) when compared to those in the HCC group (4/100, 4.0%, $P = 0.013$). Additionally, A significant portion of patients with rtD134E/G/N/S mutations were detected in the CHB group (22/100, 22.0%) or cirrhosis group (21/100, 21.0%) when compared to those in the HCC group (10/100, 10.0%, $P = 0.021$ and $P = 0.032$, respectively).

HBV RT region is composed of 6 functional domains (F, A, B, C, D and E) and 5 interdomains (F–A, A–B, B–C, C–D and D–E) connecting domains (23). The analyses of mutation distributions and frequencies in these RT regions were based on the protocol by Liu et al (22). The result of analyzing the domains in our subjects is shown in table 4. The mutation frequencies of non-A–B interdomains (2.9%, 4.2%, 4.2%) were higher than those of RT domains (A, B, C, D, E) (1.4%, 1.4%, 1.3%) in the CHB, cirrhotic and HCC patients ($P=0.005$, $P<0.001$ and $P<0.001$, respectively). The mutation frequencies of A–B interdomain (4.3%, 5.3%, 3.6%) were also higher than those of RT domains (A, B, C, D, E) (1.4%, 1.4%, 1.3%) in the CHB, cirrhotic and HCC patients ($P<0.001$, $P<0.001$ and $P<0.001$, respectively). No significant differences in the distributions among the RT domains (A, B, C, D, E), non-A–B interdomains and A–B interdomain mutations were found during stratification of data by sex, age, HBV DNA level, or HBeAg positivity. However, the mutation frequencies of A–B interdomain were significantly higher in cirrhotic patients (101/1900, 5.3%) when compared to those in the HCC group (68/1900, 3.6%) ($P=0.009$).

DISCUSSION

Nucleos(t)ide analogues have been proven to be effective in suppressing of HBV replication with the benefits of delaying the disease progression and possibly decreasing the incidence of hepatocellular carcinoma. However, antiviral resistance may occur with long-term therapy either from the second line agents or non-adherence to the first line agents. Mutations induced by lamivudine therapy have been shown to reduce the efficacy of nucleos(t)ide therapy with entecavir with over 30% resistance rate in three years. Pre-existing mutants in treatment naïve patients have been reported in asymptomatic HBV carriers or chronic hepatitis B patients (9–16). However, the impact of pre-existing mutations in treatment naïve patients remains controversial. In addition, pre-existing mutations in treatment naïve patients with cirrhosis or HCC have not been fully studied. To our knowledge, this was the first report of pre-existing mutations detected in patients with cirrhosis and HCC. This study confirmed that pre-existing mutations could be found in all stages of CHB.

The impact of mutations in RT region is not clear yet. It was interesting that we observed rtS106C and rtD134E/G/N/S mutations were more frequently detected in the CHB and cirrhotic patients when compared to those in the HCC patients. And rtD134E/G/N/S mutation could also generate the sI126N/V mutation in the S gene of HBV. Up to now, there were no research reports about the relationship between these mutations and the progression of chronic liver diseases. The CHB and cirrhotic patients may have a stronger immune response towards active necro-inflammation than HCC patients. And the rtS106C and rtD134E/G/N/S mutations may be associated with necro-inflammation, immune response and cirrhosis development in these treatment naïve patients. The immune response towards active necro-inflammation seen in patients with CHB or cirrhosis may also induce the rtS106C and rtD134E/G/N/S mutations. HCC patients tend to have advance stages of fibrosis and less amounts of necro-inflammation.

In order to investigate the evolutionary divergence of HBV sequences, we analyzed the variation of HBV reverse transcriptase nucleos(t)ides with the p-distance model. Our result showed that the HBV reverse transcriptase variation was more frequently detected in patients with HBeAg negative when compared to those with HBeAg positive in the cirrhosis group. In addition, a significant portion of HBV RT variation was observed between patients with ALT 160IU/L and ALT<80IU/L in the cirrhosis group. Therefore, HBV RT variation may be the produce of immune pressure, especially the hepatic necro-inflammation was under the partial immune control at the stage of prolong immune clearance (also leading to

cirrhosis). Such scenario is commonly presented in patients with HBeAg negative with active necro-inflammation (elevated ALT). The relationship between age and HBV RT variation was also explored in this study. Interestingly, HBV RT variation reached a peak in the different ages in CHB, cirrhotic and HCC patients. In CHB patients, the HBV reverse transcriptase variation was mostly detected in the age range of 20 to 30 years. In contrast, the HBV reverse transcriptase variation in patients with cirrhosis and HCC was detected more often in the age range of 40 to 45 years (Fig. 1). These findings indicated that immune response pattern might occur with two phases of surge with an early one in the age of 20–30 years and the later on in the age of 40–50 years. It appears that the progression of CHB to cirrhosis or development of HCC at the age of 40 to 45 years may be associated with HBV reverse transcriptase variation. Further studies are needed to verify such correlation and provide the evidence to support the treatment candidacy selection in CHB patients with age over 40.

Another interesting finding in present study was that amino acid (AA) substitutions in A–B interdomain and non-A–B interdomain were higher than those of RT domains (A, B, C, D, E) in the CHB, cirrhotic and HCC patients (Table 4). This was accord with a recent study by Liu et al (22). However, the differences of mutation frequency in cirrhotic and HCC patients were not investigated in Liu's study. And our data also emphasized the RT domains (A, B, C, D, E) displaying the lowest mutation frequency in RT regions of HBV in the CHB, cirrhotic and HCC patients. As table 4 showed, the mutation frequencies of A–B interdomain were higher in cirrhotic patients than those in HCC patients, which led to the concept of the A–B interdomain mutations being associated with the cirrhosis development.

A–B interdomain mutations were structurally distant from other significant functional domains (26). Due to the overlapping structure of HBV genomes, the most of RT-HBsAg concomitant mutations were found centered in the A–B interdomain, corresponding to the overlapping 'a' determinant with a cluster of affirmed immune-escape mutations, such as s126A/N/S, sT131N and sG145R (27). A–B interdomain may not be crucial for RT function and antiviral resistance, but the mutations were driven mainly by host immune pressures (22, 28). Thus, RT domains (A, B, C, D, E) seemed critical for RT function and antiviral resistance. Our data also showed that RT domains (A, B, C, D, E) had the lowest frequency of naturally occurring mutations in RT regions of HBV and indicated that A–B interdomain mutations may associate with the cirrhosis development (Table 4) as the result of inefficient but over reacting host immune response and severe hepatic necro-inflammation.

In conclusion, the nucleos(t)ide analogues related mutations not only exist in treatment naive patients with CHB, but also in patient with cirrhosis and HCC. In cirrhotic stage, a significant portion of patients with HBeAg negativity and high levels of ALT have HBV reverse transcriptase variations when compared to those with HBeAg positivity and lower levels of ALT. The HBV reverse transcriptase variations were more frequently detected in patients with CHB at the age group of 20 to 30, which were 15 to 20 years younger than the age group in patients with cirrhosis and HCC. The host immune response to HBV may occur with two phases of surge including an early one at the age of 20–30 and a later one at the age of 40–50. The time points when patient reach the age of 40 to 45 seem to be a critical period for the chronic liver diseases progression from CHB to cirrhosis and/or HCC. The rtS106C and rtD134E/G/N/S mutations may be an induced consequence from the overacting immune response towards severe necro-inflammation seen during different disease stages of CHB. A–B interdomain mutations may be linked to hepatic necro-inflammation, immune response and cirrhosis development.

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Lists of abbreviation

anti-HBe	antibody to hepatitis e antigen
ADV	adefovir dipivoxil
CHB	chronic hepatitis B
ETV	entecavir
HBeAg	hepatitis e antigen
HBV	hepatitis B virus
HBsAg	hepatitis B surface antigen
HCC	hepatocellular carcinoma
LdT	telbivudine
LMV	lamivudine
NA	nucleos(t)ide analogue
NAr	nucleos(t)ide analogue resistances
RT	reverse transcriptase

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Fig 1. Estimates of average evolutionary divergence over different age groups within CHB, Cirrhosis and HCC patients were analyzed by p-distance model

In age of 20–30 in CHB, 40–50 in Cirrhosis and HCC groups, the HBV reverse transcriptase variations were higher.

Table 1

Epidemiologic, biochemical and virological characteristics of the 467 untreated patients

	CHB	<u>Cirrhosis</u>	HCC	<i>P</i>
N	262	105	100	
Age (mean ± SD)	40±9.9	51±8.2	49.8±8.0	<0.001 ^a , <0.001 ^b
Male/female	192/70	78/27	85/15	NS
ALT(IU/L)	144±10	115±14	89±14	0.001 ^b
HBV DNA	6.3±1.4	6.0±1.1	5.5±1.2	0.009 ^b , <0.001 ^c
HBeAg+/HBeAg–	184/78	47/58	33/67	<0.001 ^a , <0.001 ^b

CHB: chronic hepatitis B; HCC: hepatocellular carcinoma; ALT: alanine aminotransferase (mean ± SD); HBV DNA: log10 copies/ml (mean ± SD); NS: no significant.

^aCHB vs Cirrhosis;

^bCHB vs HCC;

^cCirrhosis vs HCC;

Table 2

Nucleos(t)ide resistance related mutations in HBV reverse transcriptase region

	CHB (N=262)	Cirrhosis (N=105)	HCC (N=100)	Total (N=467)
<i>Primary drug resistance mutations</i>				
rtI169L	2 (0.8)	1 (0.9)		3 (0.6)
rtA181T			1 (1.0)	1 (0.2)
rtT184A				0
rtA194T				0
rtS202I	1 (0.4)	1 (0.9)		2 (0.4)
rtM204I/V/S	5 (1.9)	5 (4.8)	1 (1.0)	11 (2.4)
rtN236T	1 (0.4)			1 (0.2)
rtM250I/L/V				0
<i>Compensatory mutations</i>				
rtL80I/V	2 (0.8)		1 (1.0)	3 (0.6)
rtV173L	2 (0.8)		1 (1.0)	3 (0.6)
rtL180M	5 (1.9)	4 (3.8)		9 (1.9)
<i>Putative mutations</i>				
rtH126D/Y	5 (1.9)	3 (2.8)	1 (1.0)	9 (1.9)
rtT128A/N/P	21 (8.0)	11 (10.5)	8 (8.0)	40 (8.6)
rtN139A/D/H/K/T/Y	19 (7.3)	10 (9.5)	7 (7.0)	36 (7.7)
rtR/W153G/Q	8 (3.1)	1 (0.9)		9 (1.9)
rtV191I	13 (4.9)	11 (10.5)	6 (6.0)	30 (6.4)
rtS213N/T	8 (3.1)	3 (2.8)	2 (2.0)	13 (2.8)
rtV214A/E/I	7 (2.7)	4 (3.8)	7 (7.0)	18 (3.8)
rtQ215H	2 (0.8)		2 (2.0)	4 (0.8)
rtL229M/V/W	4 (1.5)	5 (4.8)	5 (5.0)	14 (3.0)
rtN238A/D/H/Q/S/T	11 (4.2)	4 (3.8)	9 (9.0)	24 (5.1)
<i>Other mutations</i>				
rtS78T	3 (1.1)	8 (7.6)	6 (6.0)	17 (3.6)
rtV84I/M	1 (0.4)	1 (0.9)		2 (0.4)
rtI91L	12 (4.6)	3 (2.8)	1 (1.0)	16 (3.4)
rtS106C	23 (8.8)	15 (14.3)	4 (4.0)	42 (9.0)
rtR110G/Q	1 (0.4)	2 (1.9)	2 (2.0)	5 (1.1)
rtT118A/N/S	4 (1.5)	1 (0.9)	1 (1.0)	6 (1.3)
rtN121I	3 (1.1)	4 (3.8)	1 (1.0)	8 (1.7)
rtI122F/K/L/N/S	13 (4.9)	5 (4.8)	3 (3.0)	21 (4.5)
rtN123D/F/H/Y	4 (1.5)	5 (4.8)	3 (3.0)	12 (2.6)
rtY124D/H/N	19 (7.3)	10 (9.5)	9 (9.0)	38 (8.1)
rtQ125H/K/N/R	4 (1.5)	1 (0.9)	1 (1.0)	6 (1.3)
rtG127R/W	5 (1.9)	2 (1.9)	2 (2.0)	9 (1.9)
rtM129L/V	7 (2.7)	1 (0.9)	5 (5.0)	13 (2.8)
rtD134E/G/N/S	37 (14.1)	21 (20.0)	10 (10.0)	68 (14.6)

	CHB (N=262)	Cirrhosis (N=105)	HCC (N=100)	Total (N=467)
rtS135F/P/T/Y	8 (3.1)	2 (1.9)	4 (4.0)	14 (3.0)
rtS137Q/T	2 (0.8)	1 (0.9)	4 (4.0)	7 (1.5)
rtR138K/Q	3 (1.1)	2 (1.9)	2 (2.0)	7 (1.5)
rtL145M/V	2 (0.8)	5 (4.8)	2 (2.0)	9 (1.9)
rtI163V	7 (2.7)	1 (0.9)	2 (2.0)	10 (2.1)
rtL199F/M/V	1 (0.4)	3 (2.8)	1 (1.0)	5 (1.1)
rtV207L/M	4 (1.5)			4 (0.8)
rtS219A/T	3 (1.1)	2 (1.9)	2 (2.0)	7 (1.5)
rtF221Y	14 (5.3)	11 (10.5)	13 (13.0)	38 (8.1)
rtI233V	1 (0.4)	1 (0.9)		2 (0.4)
rtP237H	1 (0.4)			1 (0.2)
rtN248H	7 (2.7)	2 (1.9)	3 (3.0)	12 (2.6)
rtV253A/E/R	2 (0.8)	1 (0.9)	2 (2.0)	5 (1.1)

CHB: chronic hepatitis B; HCC: hepatocellular carcinoma; Data represented as: n (%);

Table 3

Estimates of average evolutionary divergence by p-distance model

Groups	d ± SE(nt)	Groups	d ± SE(nt)
<u>Diseases</u>		<u>HBV DNA Levels</u>	
CHB(N=262)	0.020±0.002	CHB(DNA<10 ⁵ copies/ml, N=64)	0.021±0.002
<u>Cirrhosis</u> (N=105)	0.022±0.002	CHB(DNA 10 ⁵ copies/ml, N=198)	0.020±0.002
HCC(N=100)	0.022±0.002	<u>Cirrhosis</u> (DNA<10 ⁵ copies/ml, N=20)	0.021±0.003
		<u>Cirrhosis</u> (DNA 10 ⁵ copies/ml, N=85)	0.022±0.002
<u>M/F</u>		HCC(DNA<10 ⁵ copies/ml, N=36)	0.024±0.003
CHB-M(N=192)	0.020±0.002	HCC(DNA 10 ⁵ copies/ml, N=64)	0.020±0.002
CHB-F(N=70)	0.022±0.003		
<u>Cirrhosis</u> -M(N=78)	0.023±0.002	<u>ALT Levels</u>	
<u>Cirrhosis</u> -F(N=27)	0.019±0.003	CHB-ALT<80(N=113)	0.021±0.002
HCC-M(N=85)	0.022±0.002	CHB-80 ALT<160(N=77)	0.021±0.003
HCC-F(N=15)	0.020±0.003	CHB-ALT 160(N=72)	0.019±0.002
		<u>Cirrhosis</u> -ALT<80(N=68)	0.019±0.002
<u>HBeAg Status</u>		<u>Cirrhosis</u> -80 ALT<160(N=16)	0.023±0.003
CHB-HBeAg+(N=184)	0.020±0.002	<u>Cirrhosis</u> -ALT 160(N=21)	0.029±0.003 ^b
CHB-HBeAg-(N=78)	0.022±0.003	HCC-ALT<80(N=71)	0.021±0.002
<u>Cirrhosis</u> -HBeAg+(N=47)	0.019±0.002	HCC-80 ALT<160(N=18)	0.023±0.003
<u>Cirrhosis</u> -HBeAg-(N=58)	0.024±0.002 ^a	HCC-ALT 160(N=11)	0.025±0.003
HCC-HBeAg+(n=23)	0.022±0.003		
HCC-HBeAg-(n=67)	0.021±0.002		

CHB: chronic hepatitis B; HCC: hepatocellular carcinoma; M/F: M: Male; F: female; HBeAg+: HBeAg positive; HBeAg-: HBeAg negative; ALT: alanine aminotransferase (IU/L), ALT ULN (upper limit of normal)=40IU/L; d ± S.E: p-distance (mean ± SD); nt: HBV reverse transcriptase nucleotide;

^a Cirrhosis-HBeAg-(0.024±0.002) vs Cirrhosis-HBeAg+(0.019±0.002), P<0.05 (analyzed by 95% confidence interval);

^b Cirrhosis-ALT 160(0.029±0.003) vs Cirrhosis-ALT<80(0.019±0.002), P<0.05(analyzed by 95% confidence interval);

Table 4

Mutation site distributions and frequencies in different sections of HBV RT region.

RT Sections	CHB(N=100)	Cirrhosis (N=100)	HCC(N=100)
Domains (A,B,C,D,E) ^a			
Sites with mutations	12/21	11/21	10/21
Mutation frequency %	1.4 (29/2100)	1.4 (30/2100)	1.3 (27/2100)
Non-A-B interdomains ^b			
Sites with mutations	8/9	7/9	8/9
Mutation frequency %	2.9 (26/900)	4.2 (38/900)	4.2 (38/900)
<i>P</i> value	0.005 [*]	<0.001 [*]	<0.001 [*]
A-B interdomain ^c			
Sites with mutations	16/19	19/19	18/19
Mutation frequency %	4.3 (82/1900)	5.3 (101/1900) ^d	3.6 (68/1900)
<i>P</i> value	<0.001 [#]	<0.001 [#]	<0.001 [#]

CHB: chronic hepatitis B; HCC: hepatocellular carcinoma; The number of mutations detected within a specific RT section divided by the total sites number studied in this section in 300 patients (3 groups) was defined as the mutation frequency. For instance, the calculation of the mutation frequency of “domains (A, B, C, D, E)” section in CHB group was as follows: 29 mutations detected/(21 studied sites × 100 isolates) × 100% = 29/2100 × 100% = 1.4%.

^a Mutation sites in this section include A: rt78, rt80, rt84, rt85, rt91; B: rt163, rt169, rt173, rt180, rt181, rt184; C: rt202, rt204, rt207; D: rt233, rt236, rt237, rt238; E: rt248, rt250, rt253;

^b Mutation sites in this section include rt191, rt194, rt199, rt213, rt214, rt215, rt219, rt221, rt229.

^c Mutation sites in this section include rt106, rt110, rt118, rt121, rt122, rt123, rt124, rt125, rt126, rt127, rt128, rt129, rt134, rt135, rt137, rt138, rt139, rt145 and rt153.

^d A-B interdomain mutation frequency: Cirrhosis (101/1900, 5.3%) vs HCC (68/1900, 3.6%), P=0.009;

^{*} P values: Non-A-B interdomains mutation frequency vs Domains (A, B, C, D, E) mutation frequency.

[#] P values: A-B interdomain mutation frequency vs Domains (A, B, C, D, E) mutation frequency.