


## RESEARCH ARTICLE

# Effect of mutations across reverse transcriptase region on HBV replication and progression of liver diseases in Chinese patients

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## Abstract

It was known that mutations in the RT region were mainly related to nucleot(s)ide analogs resistance. Increasing studies indicated that RT mutations were related to advanced liver diseases (ALD) and had effects on HBV replication, but the distribution characteristics of mutations across RT region in the development of liver diseases and the effect of RT mutations on HBV replication were not fully clarified. HBV RT region was direct-sequenced in 1473 chronic HBV-infected patients. Mutation frequencies were analyzed to identify the specific mutations differing between groups classified by genotypes, loads of HBV DNA, or progression of liver diseases. In the range of rt145-rt290, rt145, rt221, rt222, rt267, and rt271 were the genotype-polymorphic sites, while rt238 was the genotype-specific sites. Mutations at rt163, rt173, rt180, rt181, rt184, rt191, rt199, and rt214 were more frequent among patients with C-genotype HBV, while those at rt220, rt225, rt226, rt269, and rt274 were more frequent among patients with B-genotype HBV. RtM204V/I could reduce the HBV DNA loads while rtQ/L267H/R could increase the HBV DNA loads. RtV214A/E/I (OR 3.94, 95% CI 1.09 to 14.26) was an independent risk factor for advanced liver diseases. In summary, the hotspots of mutations were different between B and C genotypes. Besides the effect on the S region, RT mutations had effects on HBV replication by other unknown ways. RtV214A/E/I was found to be an independent risk factor for ALD, suggesting that mutations at rt214 site could be used as a potential virological marker for the liver disease progression.

## KEYWORDS

HBV, liver disease progression, mutation, replication, RT region

## 1 | INTRODUCTION

Hepatitis B virus (HBV) is a DNA virus, belonging to the family of *Hepadnaviridae* DNA viruses, which is the pathogen that causes hepatitis B.<sup>1</sup> At present, there are about 257 million people infected chronically with HBV in the world, accounting for about 3.5% of the global population.<sup>1</sup> Uncontrolled chronic HBV infection can develop

into life-threatening advanced liver diseases, including liver cirrhosis (LC) and hepatocellular carcinoma (HCC), posing great challenges to global public health.

The genome of HBV consists of 3.2 kb incomplete double-stranded relaxed circular DNA (rcDNA) and contains 4 open reading frames (ORF), namely P, PreC/C, PreS/S, and X.<sup>2</sup> The above four genes are responsible for encoding polymerase protein, HBeAg,

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LHBsAg, MHBsAg, SHBsAg, and X protein. Among them, P protein is involved in the process of reverse transcription of HBV pre-genomic RNA into negative-strand DNA.<sup>2</sup>

Since HBV replication lacks a mismatch correction mechanism, the new genome generated by replication is prone to mutations.<sup>3</sup> Among them, mutations in the PreC/C region and X region have been reported to be related to the occurrence of hepatocellular carcinoma.<sup>4,5</sup> Mutations in the PreS/S region are related to immune escape.<sup>6</sup> Besides, mutations in the P region (RT region) have been reported to be mainly related to nucleot(s)ide analogs (NAs) resistance.<sup>7</sup>

Although mutations in the RT region are responsible for NAs resistance, studies have reported that the mutation frequency of rtM204V/I, which was the classic NAs resistance mutation, was significantly higher in patients with advanced liver diseases, such as LC and HCC.<sup>8</sup> Therefore, RT mutations may also be related to the occurrence of HCC, but the distribution characteristics of RT mutations in the occurrence and development of liver disease are still unclear. In addition, classic resistance mutations, such as rtM204V/I, have been reported to reduce the replication of HBV, while the compensation mutation rtL180M could restore the impaired replication capacity caused by rtM204V mutation.<sup>9</sup> Nevertheless, the effect of other RT mutations on HBV replication is still unknown.

In this study, Sanger sequencing was used to detect the HBV RT region sequences from patients with chronic HBV infection. Through analyzing the RT mutations at different HBV replication levels and comparing the characteristics of RT mutations in different liver disease progression, this study aimed to understand the effect of HBV RT mutations on HBV replication and liver disease progression.

## 2 | MATERIALS AND METHODS

### 2.1 | Patients and blood samples

A total of 1473 chronic HBV-infected patients were enrolled at the First Affiliated Hospital of Fujian Medical University from January 2011 to December 2016 and informed consent on entry into the trials was obtained from all patients. The inclusion criteria were the presence of hepatitis B surface antigen (HBsAg) and HBV DNA at least 6 months prior to treatment. Patients co-infected with the hepatitis A/C/D virus, human immunodeficiency virus, autoimmune liver disease, primary biliary cirrhosis, and alcohol or drug abuse were excluded. All the serum samples were collected and stored at  $-80^{\circ}\text{C}$ . The study was approved by the Ethics Committee of the Second Affiliated Hospital of Fujian Medical University.

### 2.2 | Laboratory tests

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined by automated biochemical technique (Siemens Healthcare Diagnostics). Serum HBsAg, anti-HBs, hepatitis B e antigen (HBeAg), anti-HBe, and anti-HBc were measured on the wholly automatic immune fluorescence analyzer Abbott Type

i4000 (Abbott Laboratories) using the original, corollary commercial kits. Serum HBV DNA was quantified using the TaqMan polymerase chain reaction (PCR) assay (Sansure Biotech) on Roche LightCycler 480 Real-Time PCR System (Roche) with a low limit of quantification of 500IU/ml.

### 2.3 | HBV DNA extraction and Sanger sequencing

HBV DNA was extracted from 500 $\mu\text{l}$  patient serum according to the manufacturer's instructions of the TIANamp Genomic DNA Kit (Tiangen Biotech). The HBV RT gene was amplified by PCR using forward primer 5-CTCATGTTGCTGTACAAAACC-3 (nt559-nt579) and reverse primer 5-CAATTCKTTGACATACTTTCCA-3 (nt1000-nt979). PCR was performed in a 50 $\mu\text{l}$  mixture containing 2.5mM MgCl<sub>2</sub>, 400 $\mu\text{M}$  dNTP and 2.5 U of Taq polymerase (TAKARA, Japan). PCR conditions were as follows: 94 $^{\circ}\text{C}$  for 5 min; 35 cycles of 94 $^{\circ}\text{C}$  for 1 min, 60 $^{\circ}\text{C}$  for 1 min, and 72 $^{\circ}\text{C}$  for 1 min; then 72 $^{\circ}\text{C}$  for 10 min. The PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen) and directly sequenced (The Beijing Genomics Institute).

### 2.4 | Sequence alignment and HBV genotyping

Mutations were analyzed by aligning HBV RT sequences with the consensus sequence generated based on the HBV RT sequences in this study and the reference sequences in previous studies according to published literature.<sup>10</sup> In brief, 100 B-genotype DNA sequences (Supplementary material 1) and 100 C-genotype DNA sequences (Supplementary material 2) were aligned to obtain the consensus sequence as the reference sequence, and then each of the sequences was aligned with the reference sequence described above to analyze the mutation. We used the phylogenetic analysis for HBV genotyping (Figure S1). Meanwhile, an online tool Genotyping (<https://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi>) was also applied to verify the accuracy of HBV genotyping.

### 2.5 | Statistical analysis

Differences in measurement data were evaluated by t test, Mann-Whitney *U* test, Kruskal-Wallis test, chi-square test (Fisher's exact test was used when needed), and two-logistic regression with IBM SPSS Statistics software (Version 22.0.0; IBM). All *p*-values were two-tailed. *p* < 0.05 was considered statistically significant.

## 3 | RESULTS

### 3.1 | Clinical characteristics of patients

A total of 1445 subjects with chronic HBV infection who underwent Sanger sequencing were included in this study. The sequencing range of the RT region covers rt145-rt290. This study first

compared the clinical characteristics of patients with chronic HBV infection between B and C genotype and found that the distribution of patients with B- or C-genotype HBV at different age groups, HBeAg status, and disease progression showed significant differences (Table 1). Next, this study grouped chronic HBV carriers (CC) and chronic hepatitis B (CHB) patients into one group, and grouped liver cirrhosis (LC) and hepatocellular carcinoma (HCC) patients into another group. The clinical characteristics of the above two groups were compared. Gender, genotype, age, ALT, HBV DNA, HBsAg, HBeAg, and AFP, except HBcAb, were significantly different between (CC+CHB) and (LC+HCC) groups (Table 2).

### 3.2 | Effect of genotype on RT mutations

Before the analysis of RT mutations, this study first determined the reference sequences of B- and C-genotype HBV. In this study, 100 cases of B-genotype HBV DNA sequences and 100 cases of C-genotype HBV DNA sequences were first aligned respectively to obtain the consensus sequences of the B- and C-genotype HBV. These two consensus sequences were taken as reference sequences for this study.

By using VESPA (<https://www.hiv.lanl.gov/content/sequence/VESPA/vespa.html>), we found that in the range of rt145-rt290,

TABLE 1 Clinical data of the subjects in this study were compared according to the different genotypes

	Genotype-B (%)	Genotype-C (%)	$\chi^2$	<i>p</i>
Gender				
Female	194 (25.1)	200 (28.6)	2.4	0.13
Male	580 (74.9)	499 (71.4)		
Age (years) <sup>a</sup>				
<30	305 (39.4)	173 (24.8)	48.8	<0.001
30–39	232 (30.0)	200 (28.7)		
≥40	237 (30.6)	325 (46.5)		
Serum ALT level (U/L) <sup>a</sup>				
<40 (female) or 50 (male)	116 (20.8)	108 (22.7)	2.0	0.38
40–79 (female) or 50–99 (male)	96 (17.2)	93 (19.5)		
≥80 (female) or ≥100 (male)	346 (62.0)	275 (57.8)		
Serum HBV DNA level (IU/ml) <sup>a</sup>				
<200,000	190 (33.0)	174 (34.3)	0.20	0.66
≥200,000	385 (67.0)	333 (65.7)		
Serum HBsAg level (IU/ml) <sup>a</sup>				
<100	14 (2.5)	16 (3.2)	1.06	0.59
100–999	58 (10.5)	60 (12.0)		
≥1000	482 (87.0)	426 (84.8)		
Serum HBeAg status <sup>a</sup>				
Positive	375 (66.1)	377 (78.4)	19.24	<0.001
Negative	192 (33.9)	104 (21.6)		
Serum AFP status (ng/ml) <sup>a</sup>				
<25	330 (76.9)	285 (78.7)	2.24	0.33
25–399	85 (19.8)	60 (16.6)		
≥400	14 (3.3)	17 (4.7)		
Disease process <sup>a</sup>				
Chronic carrier	240 (31.0)	223 (32.0)	40.18	<0.001
Chronic hepatitis	412 (53.3)	298 (42.8)		
Liver failure	19 (2.5)	5 (0.7)		
Liver cirrhosis	82 (10.6)	136 (19.5)		
Hepatocellular carcinoma	20 (2.6)	34 (5.0)		

Abbreviations: AFP, alpha-fetoprotein; ALT, alanine aminotransferase.

<sup>a</sup>Partial data missing.

rt145, rt151, rt221, rt222, rt223, rt224, rt238, rt267, and rt271 are genotype-dependent sites (Figure 1A and Figure S3). After clarifying the genotype-dependent sites, we analyzed the amino acid changes between B and C genotypes at these above sites. The results showed that rt221, rt222, rt267, and rt271 had significantly more amino acid changes between B and C genotypes (Figure 1B-G), suggesting that these sites are genotype-polymorphic sites. Moreover, in our study, there were no amino acid changes at rt238 and rt263 between B and C genotypes, suggesting that these sites are relatively conservative, that is, genotype-specific sites (Figure 1B).

After analyzing the amino acid changes between B and C genotypes at genotype-dependent sites, this study also investigated the differences of mutations from rt145 to rt290 between B and C genotypes. We first observed the distribution of mutations across RT region of B- and C-genotype HBV, respectively (Figure 1H). The results showed that in the range of rt145-rt217, it seemed that there were more mutations across RT region of C-genotype HBV (Figure 1H). While in the range of rt218-rt290, there seemed to be more mutations across RT region of B-genotype HBV (Figure 1H). After further

statistical analysis, it was found that mutations at rt145, rt163, rt173, rt180, rt181, rt184, rt191, rt199, and rt214 were significantly more in C-genotype HBV (Figure 1I). The mutations at rt220, rt225, rt226, rt269, and rt274 are significantly more in B-genotype HBV (Figure 1I).

### 3.3 | Effect of RT mutations on HBV replication

Some studies have shown that the classic drug-resistant mutation rtM204V/I could reduce the replication capacity of HBV, but the effect of other RT mutations on HBV replication is still not completely clear. Therefore, this study analyzed the effect of mutations at rt145-rt290 on HBV replication.

The average HBV DNA level of subjects in this study was 6.08 ( $\log_{10}$ , IU/ml). Based on this average level, the study divided the subjects into two groups, that is,  $\geq 6.08$  ( $\log_{10}$ , IU/ml) group and  $< 6.08$  ( $\log_{10}$ , IU/ml) group. This study first investigated the distribution of RT mutations in the above two groups. Intuitively from

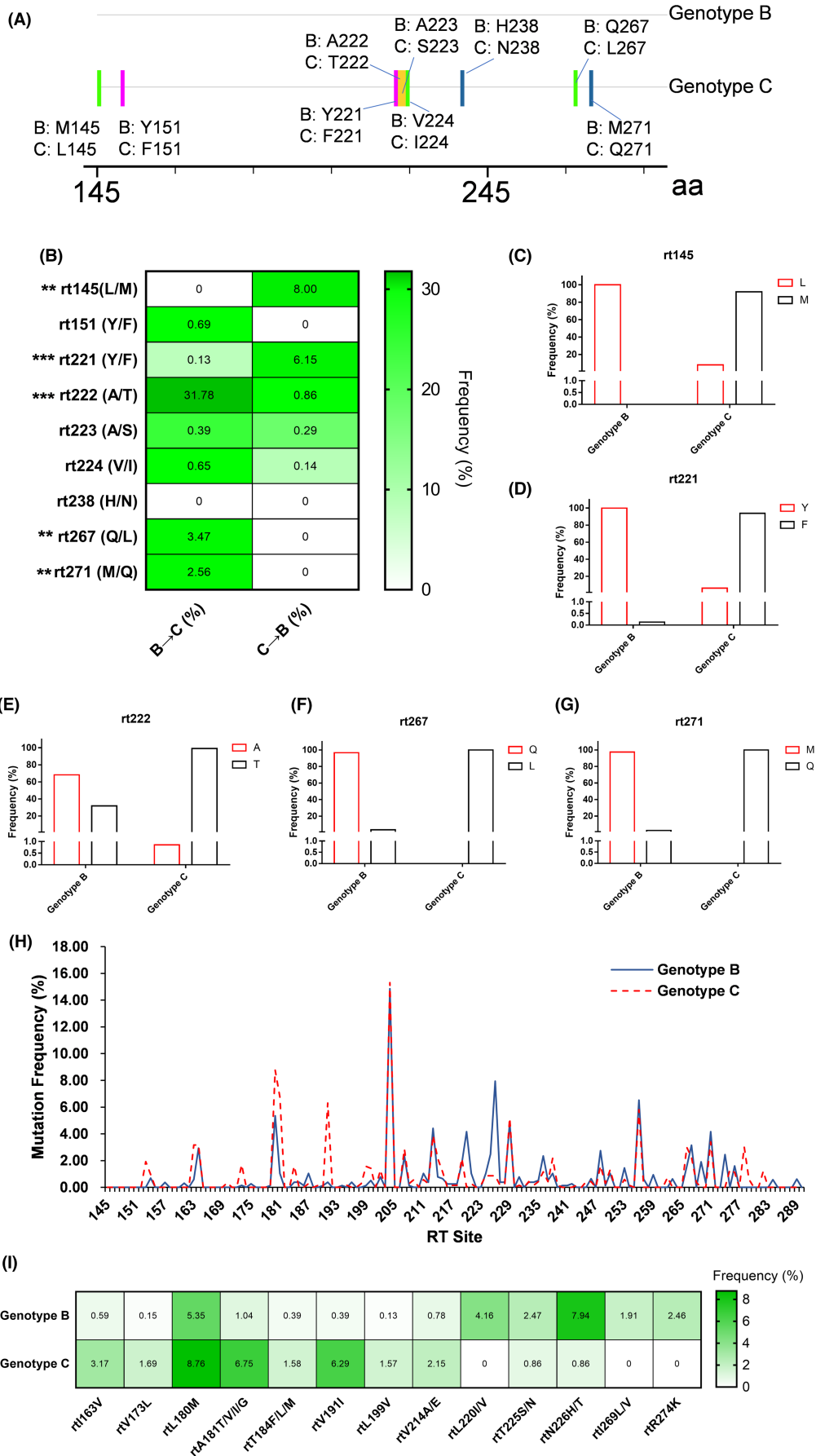
	CC+CHB (n = 1173)	LC+HCC (n = 272)	$\chi^2/t/Z$	p
Gender				
Female (%)	330 (28.1)	56 (20.6)	6.420	0.011
Male (%)	843 (71.9)	216 (79.4)		
Genotype				
B (%)	652 (55.6)	102 (37.5)	28.938	<0.001
C (%)	521 (44.4)	170 (62.5)		
Age (years), (mean $\pm$ 2SD) <sup>a</sup>	34.2 $\pm$ 11.1	49.2 $\pm$ 11.5	-19.916	<0.001
Serum ALT level (ULN), (mean $\pm$ 2SD) <sup>a</sup>	5.9 $\pm$ 7.1	2.9 $\pm$ 4.6	7.217	<0.001
Serum HBV DNA level ( $\log_{10}$ , IU/ml), (mean $\pm$ 2SD) <sup>a</sup>	6.2 $\pm$ 1.5	5.5 $\pm$ 1.4	6.315	<0.001
Serum HBsAg level (IU/ mL), (mean $\pm$ 2SD) <sup>a</sup>	19188.1 $\pm$ 27719.6	9970.5 $\pm$ 22424.0	5.033	<0.001
Serum HBeAg status <sup>a</sup>				
Positive (%)	626 (75.1)	116 (58.6)	21.494	<0.001
Negative (%)	208 (24.9)	82 (41.4)		
Serum HBcAb level (S/CO), (mean $\pm$ 2SD) <sup>a</sup>	12.1 $\pm$ 3.2	11.9 $\pm$ 2.6	0.721	0.471
Serum AFP status (ng/ml), (median, range) <sup>a</sup>	4.56 (0.71-44,756)	7.31 (1-60,500)	-3.645	<0.001

Abbreviations: CC, chronic carrier; CHB, chronic hepatitis B; HCC, hepatocellular carcinoma; LC, liver cirrhosis.

<sup>a</sup>Partial data missing.

TABLE 2 Clinical data of the subjects in this study were compared according to the progression of liver diseases

FIGURE 1 Effects of genotypes on RT mutations were investigated in the range of rt145 and rt290. (A) The genotype-dependent amino acid sites were obtained through the analysis for consensus sequences of 100 B-genotype DNA sequences and 100 C-genotype DNA sequences. (B) Substitutions of amino acids at genotype-dependent sites between B- and C-genotype sequences were analyzed to find the genotype-polymorphic sites and genotype-specific sites. (C-G) Rates of substitutions at the genotype-polymorphic sites between B- and C-genotype sequences were presented. (H) The distributions of mutations across RT region of B- and C-genotype sequences were observed, respectively. (I) The mutants with significant differences between B- and C-genotype sequences were presented



the distribution map, RT mutations from subjects between the two groups may differ only in individual sites (Figure 2A). After further statistical analysis, mutations at rt180, rt204, and rt250 were significantly more in the  $<6.08$  ( $\log_{10}$ , IU/ml) group, while mutations at rt267 were significantly more in the  $\geq 6.08$  ( $\log_{10}$ , IU/ml) group (Figure 2B).

Because rtL180M is a compensatory mutation of rtM204V, it exists in linkage with rtM204V. RtM250L/V, as one of the resistance mutations of entecavir (ETV), is often generated on the basis of the occurrence of rtM204V+rtL180M. However, the correlation between rtQ/L267H/R and rtM204V/I is unknown. Therefore, in this study, the associations of rtM204V/I with rtL180M, rtM250L/V, or rtQ/L267H/R were analyzed respectively by McNemar's test. The results showed that rtL180M and rtM250L/V were significantly correlated with rtM204V/I, while rtQ/L267H/R was significantly uncorrelated with rtM204V/I (Table 3).

Whereafter, we compared the HBV DNA loads of subjects with and without rtM204V/I. The effects of rtL180M and rtM250L/V on the replication of HBV with rtM204V/I were also analyzed respectively. The results showed that the HBV DNA load of subjects with rtM204V/I was significantly lower than that of subjects without rtM204V/I (Figure 2C). Although there was no statistically significance found, rtL180M seemed to slightly increase the HBV DNA load of subjects with rtM204V/I (Figure 2D), while rtM250L/V could slightly reduce the HBV DNA load of subjects with rtM204V/I (Figure 2E). Interestingly, the HBV DNA load of subjects with rtQ/L267H/R was significantly higher than that of subjects without rtQ/L267H/R (Figure 2F).

Some studies reported that the effect of RT mutations on HBV replication might be because that RT mutations could lead to the change of amino acid on S region. Therefore, this study also analyzed the effects of rtL180M, rtM204V/I, rtM250L/V, and rtQ/L267H/R on S region. We found that rtM204V caused sI195M and rtM204I caused sW196L within S region. However, rtL180M, rtM250L/V, and rtQ/L267H/R did not lead to the changes of S region (Figure 2G). Therefore, the effect of RT mutations on HBV replication was not only caused by changes in S region.

### 3.4 | Correlations between RT mutations and liver disease progression

Mutations in the RT region are mainly related to NAs resistance. Some studies have shown that rtM204V was related to the occurrence of HCC. However, the correlation between other RT mutations and the progression of liver diseases is still unknown. Therefore, this study explored the correlation between RT

mutations and liver disease progression within the range of rt145-rt290. In this study, chronic HBV carriers (CC) and patients with chronic hepatitis B (CHB) were grouped into one group, and patients with liver cirrhosis (LC) and those with hepatocellular carcinoma (HCC) were grouped into another group, namely (CC+CHB) group and (LC+HCC) group.

The distribution of mutations across RT region from subjects in the above two groups was analyzed firstly. Intuitively from the distribution map, the subjects from (LC+HCC) group seemed to have more RT mutations (Figure 3A). Further statistical analysis found that there were significantly more mutations at multiple sites in (LC+HCC) group, including rtL180M, rtA181V/T/S/G, rtT184A/I, rtL199V, rtV207F/I/L/M, RtS213T, rtV214A/E/I, rtS219A, and rtL220I (Figure 3B).

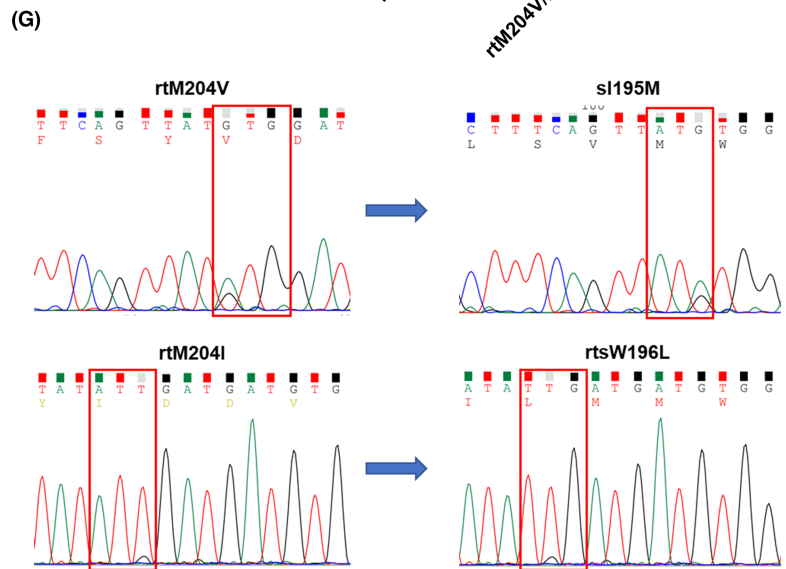
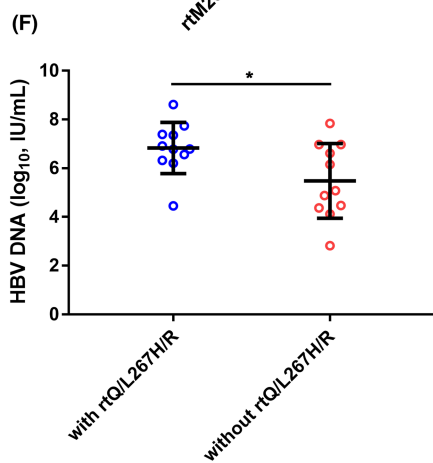
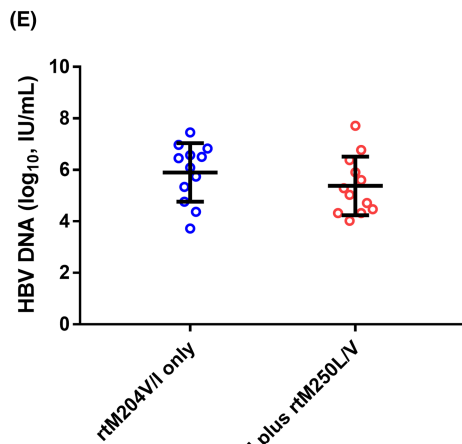
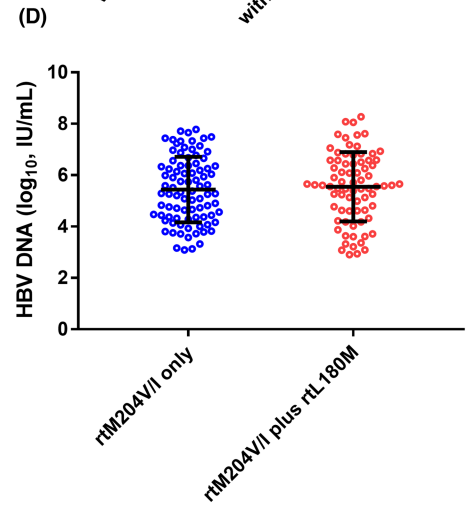
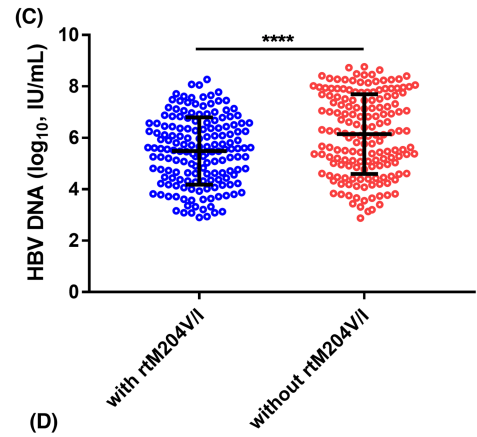
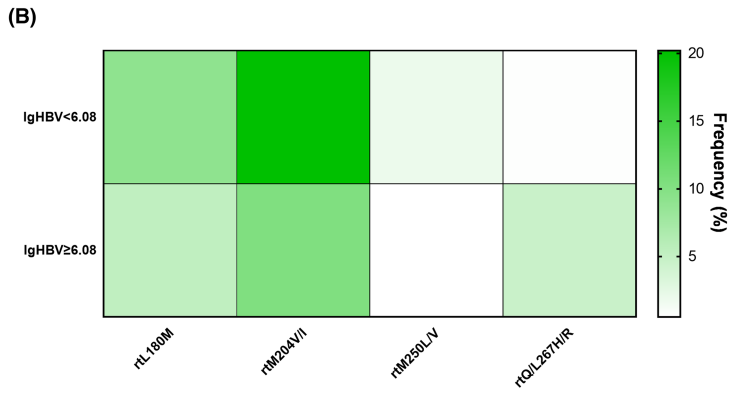
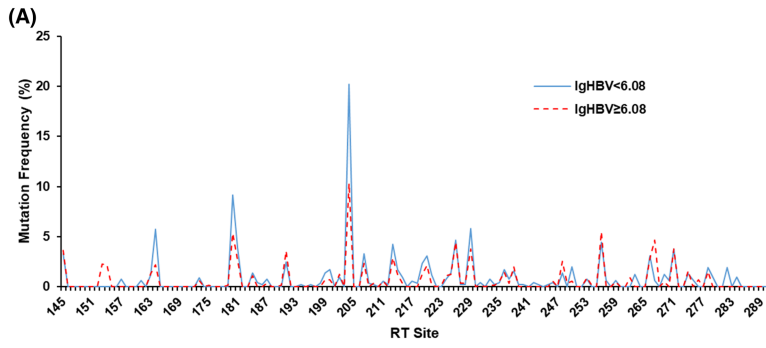
Since some studies have shown that gender, age, HBV genotype, and HBeAg status were related to the advanced liver diseases (such as LC and HCC), the logistic regression analysis was used to investigate significantly different RT mutations between (CC and CHB) group and (LC+HCC) group, and adjusted for gender, age, HBV genotype, and HBeAg status. The results showed that the appearance of rtV214A/E/I (OR 3.94, 95% CI 1.09 to 14.26) was an independent risk factor for advanced liver diseases (Figure 3C).

## 4 | DISCUSSION

Several studies have targeted genotype-dependent sites based on PCR for HBV genotyping, such as the COLD-PCR/FMCA method using specific probes for genotype-specific base sites for genotyping.<sup>11</sup> In this study, we identified 9 genotype-dependent aa sites (Figure 1B). Among these sites, rt145, rt221, rt222, rt267, and rt271 had significant amino acid changes between B and C genotypes (Figure 1B-G), indicating that these sites were genotype-polymorphic sites. In contrast, there were no amino acid changes found at rt238 between genotypes B and C (Figure 1B), indicating that this site was a genotype-specific site. Therefore, the identification for types of amino acids at rt238 site could be used to distinguish genotypes B and C.

Some investigators have reported that NAs-resistant mutations had the bias to different genotypes. Li et al.<sup>12</sup> found that genotype-C HBV had significantly more rtM204V/I, rtA181V, rtM204V/I plus rtT184 substitution or rtS202G/C, while genotype-B HBV harbored significantly more rtN236T and rtM204V/I plus rtM250V/I/L. In this study, our results were generally similar to those of Li et al., but we did not find the difference in the mutation frequency of rtM204V/I and rtN236T between genotypes B and C (Figure 1I). In addition, in this study, it was found that the classic compensatory mutations

**FIGURE 2** RT mutations had effects on the HBV replication. (A) The distributions of mutations across RT region were observed in patients with loads of HBV DNA  $<6.08$  or  $\geq 6.08$  ( $\log_{10}$ , IU/ml), respectively. (B) The mutation frequencies were significantly different at rt180, rt204, rt250, and rt267 between patients with loads of HBV DNA  $<6.08$  and those with loads of HBV DNA  $\geq 6.08$  ( $\log_{10}$ , IU/ml). (C-F) The loads of HBV DNA were compared between patients with and without the corresponding mutations. (G) The effects of rtL180M, rtM204V/I, rtM250L/V, and rtQ/L267H/R on S region were analyzed



Mutations in RT region	Effect on S region
rtM204V	si195M
rtM204I	sw196L
rtL180M	-
rtM250L/V	-
rtQ/L267H/R	-

rtV173L and rtL180M were significantly more in patients infected with genotype-C HBV (Figure 1I). In short, combined with the existing studies and this study, the risk of entecavir resistance in patients infected with genotype-C HBV was higher than that of patients infected with genotype-B HBV. Notably, this study found that in the range of rt145-rt214, patients infected with genotype-C HBV had significantly more RT mutations, while in the range of rt214-rt290, patients infected with genotype-B HBV had significantly more RT mutations (Figure 1H,I), further suggesting that the distribution of mutations across RT region were different between genotypes B and C.

Several studies have reported that mutations within the RT region could affect the replication of HBV.<sup>13-17</sup> rtM204V/I, rtL228W, and rtL229W could reduce the capacity of HBV to replicate, while rtL180M could partially reduce the effect of rtM204V on replication.<sup>17,18</sup> Besides, mutations in the pre-C region (PC) and basal core promoter (BCP) could increase the replication of HBV, but rtA194T could reduce the replication of HBV.<sup>13</sup> In this study, among the subjects with chronic HBV infection, rtM204V was also found to reduce the replication of HBV, but we did not find that rtA194T, rtL228W, and rtL229W had a significant impact on the replication of in vivo (Figure 2A-C). These results suggested that mutations within the RT region did not have the same effect on the replication of HBV in vivo and in vitro, which might be caused by a more complex environment in vivo. Notably, through comparing RT mutations between  $\geq 6.08$  ( $\log_{10}$ , IU/ml) group and  $< 6.08$  ( $\log_{10}$ , IU/ml) group, we have found that rtQ/L267H/R was likely to enhance the capacity of HBV to replicate (Figure 2B). Given the unknown impact of rtQ/L267H/R on the replication of HBV, viral loads of subjects with rtQ/L267H/R were analyzed further to prove in vivo that rtQ/L267H/R could increase the replication of HBV (Figure 2F). In order to determine the role of rtQ/L267H/R, cell and animal experiments needed to be performed to prove the effect of rtQ/L267H/R on the replication of HBV in the future.

TABLE 3 Relations between rt204 and rt180, rt204 and rt250, or rt204 and rt267 were analyzed respectively by McNemar's test

	With rtM204V/I	Without rtM204V/I	<i>p</i>
with rtL180M	76	1	<0.001
without rtL180M	87	919	
with rtM250V/I/L	12	1	<0.001
without rtM250V/I/L	151	919	
with rtQ/L267H/R	1	10	<0.001
without rtQ/ L267H/R	162	910	

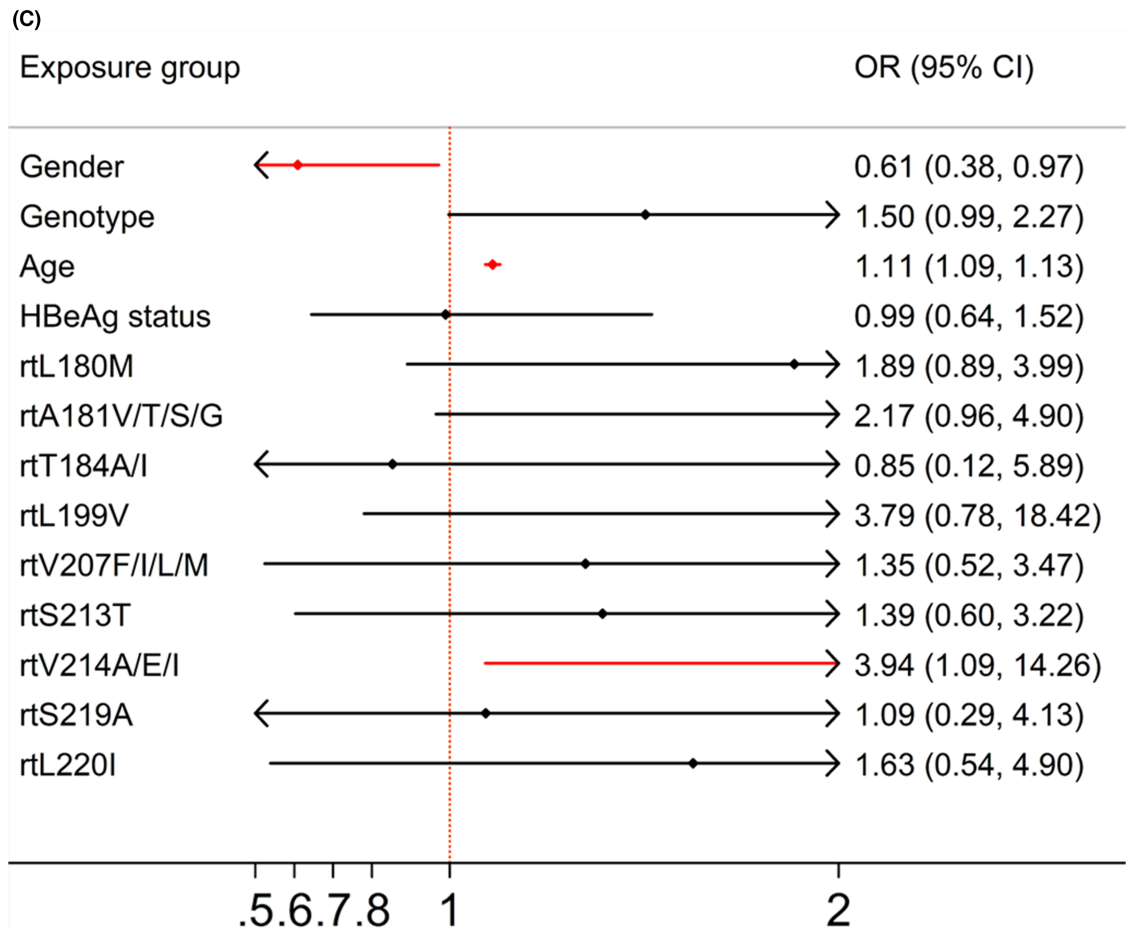
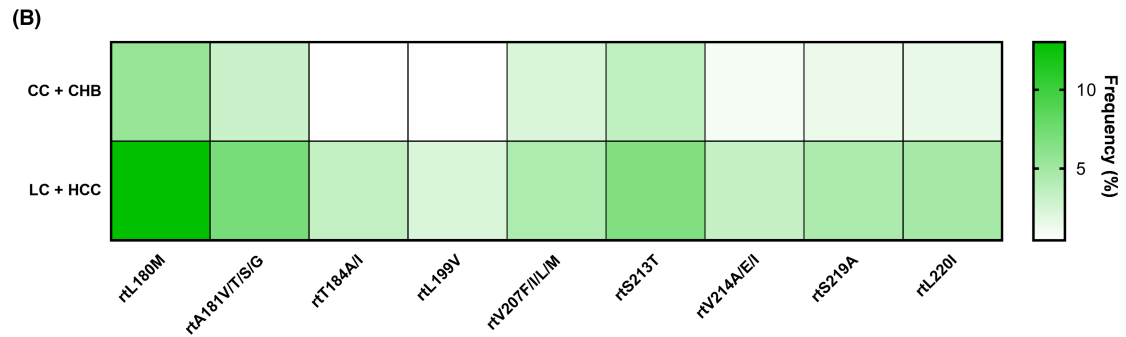
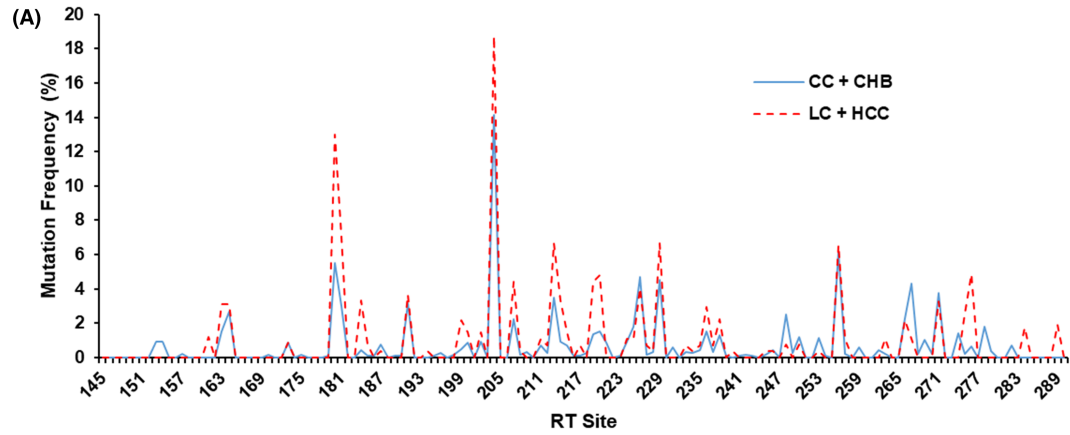
It was reported that the effect of RT mutation on HBV replication might be due to the fact that mutations within the RT region could affect its overlapping S region.<sup>14-16,19,20</sup> For example, rtA181T could cause sW172stop.<sup>15,16</sup> In order to explore the factors affecting replication of HBV by rtM204V/I and rtQ/L267H/R, we analyzed the effects of rtM204V/I and rtQ/L267H/R on the S region. The results showed that rtM204V and rtM204I could cause sI195M and sW196L, respectively (Figure 2G), which was consistent with the previous studies.<sup>20,21</sup> However, rt267 was not located in the overlapping region of RT and S, so rtQ/L267H/R did not affect the protein encoded by preS/S (Figure 2G). Therefore, besides the effect on the S region, mutations located in RT region might also cause changes in the replication of HBV through other unknown ways.

Although HBV X gene was thought to be responsible for the progression of liver diseases,<sup>22,23</sup> increasing studies revealed that RT mutations were also related to the advanced liver diseases.<sup>8,24-27</sup> For example, rtM204V/I was found to be more frequent among HCC patients than that among CHB patients.<sup>8</sup> Although rtF221Y was identified as a polymorphic change between B and C genotypes in this study (Figure 1B), it was thought to be a risk factor for poor prognosis of HCC.<sup>25</sup> In addition, rtL80I, rtD134N, rtY141F, rtI224V, etc. were also reported to be related to HCC development.<sup>27</sup> In this study, we found that rtL180M, rtA181V/T/S/G, rtT184A/I, rtL199V, rtV207F/I/L/M, rtS213T, rt214A/E/I, rtS219A, and rtL220I occurred more frequently in patients in (LC and HCC) group than those in (CC+CHB) group, but there was no difference in the frequency of rtM204V/I between these two groups (Figure 3B). Since gender, genotype, age, and HBeAg status, which were risk factors for ALD,<sup>28-31</sup> were significantly different between the (CC+CHB) and (LC+HCC) groups (Table 2), we used the two-logistic regression to analyze rtL180M, rtA181V/T/S/G, rtT184A/I, rtL199V, rtV207F/I/L/M, rtS213T, rtV214A/E/I, rtS219A, and rtL220I, adjusted by gender, genotype, age, and HBeAg status. The results showed that gender, age, and rtV214A/E/I were independent risk factors for ALD (Figure 3C). The mutations at rt214 site were potential risk factors in the virological breakthrough of NAS-treated patients,<sup>32</sup> but its correlation with the disease progression was not reported in the previous studies. Further cell and animal experiments were needed to be carried out to prove the pathogenic mechanism of rtV214A/E/I.

In summary, this study comprehensively compared the mutant characteristics of HBV RT region between different genotypes, different levels of viral loadings and different liver diseases. Our results demonstrated the impacts of genotypes and liver diseases on mutation across RT region and proved that the alteration of amino acid residues at rt214 was an independent risk factor for ALD, providing

FIGURE 3 Correlations between RT mutations and liver disease progression were analyzed. (A) The distributions of mutations across RT region were observed among patients in the (CC+CHB) group or (LC+HCC) group, respectively. (B) The mutation frequencies were significantly different at rt180, rt181, rt184, rt199, rt207, rt213, rt214, rt219, and rt220 between patients in the (CC+CHB) group and those in the (LC+HCC) group. (C) The logistic regression analysis was performed, incorporating various factors, including mutations with significant difference, gender, HBV genotype, age, and HBeAg status. The results were presented in the form of forest plots





a potential virological marker for the liver disease progression. In the future, further cell and animal experiments would be performed to prove the mechanism underlying the declining replication of HBV conferred by rtQ/L267H/R and the progression of liver disease caused by rtV214A/E/I.

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#### CONFLICT OF INTEREST

No potential conflict of interest was reported by the authors.

#### DATA AVAILABILITY STATEMENT

Requests for data should be directed to the corresponding author Yin Zhang ([fmuzyin@163.com](mailto:fmuzyin@163.com)).

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#### SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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