Accumulation of mutations in reverse transcriptase of hepatitis B virus is associated with liver disease severity in treatment-naïve Chinese patients with chronic hepatitis B

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Abstract

Background. Mutations in reverse transcriptase (RT) of the hepatitis B virus (HBV) are demonstrated to be strongly associated with nucleos(t)ide analog resistance, which is supposed to be the biggest obstacle during the long-term anti-viral treatment of chronic hepatitis B. However, the presence of RT mutations in treatment-naïve chronic hepatitis B patients and its clinical significance are not well known.

Objectives. To investigate the significance of mutations in reverse transcriptase of the hepatitis B virus in treatment-naïve Chinese patients with chronic hepatitis B.

Material and methods. In this study, 288 treatment-naïve chronic HBV patients were recruited and the RT region was sequenced. The results showed that 71 patients (24.65%) were found with RT mutations, within which there were no well-defined primary nucleotide analog-resistant mutations.

Results. There were a total of 28 mutant sites, which formed 3 dominant mutant clusters: rt124-139, rt191-212 and rt225-229. Among these 71 patients, 63.38% (45/71) of patients had a single mutation while 19.72% (14/71), 12.68% (9/71) and 4.23% (3/71) of patients had 2, 3 or 4 mutations, respectively. Patients with RT mutations showed significantly decreased serum baseline HBV DNA loads ($p = 0.0363$) and blood platelet count ($p = 0.0181$) than patients without RT mutations. Patients with multiple mutant sites (≥ 2) had significantly decreased baseline HBV DNA loads ($p = 0.0004$) and blood platelet count ($p = 0.0011$) than patients with single mutant site. Moreover, the number of RT mutant sites is significantly associated with severity of liver fibrosis ($p = 0.0128$).

Conclusions. This study demonstrated that there was a prevalence of RT mutations in treatment-naïve chronic hepatitis B patients, which reflects a tougher liver environment for the virus and deeper liver injury for the host. Accumulation of RT mutations was associated with liver disease severity in treatment-naïve chronic hepatitis B patients.

Key words: hepatitis B virus, mutation, treatment-naïve, reverse transcriptase

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Hepatitis B virus (HBV) infection is the most common etiologic agent of acute and chronic liver disease in China.¹ Individuals infected with HBV in infancy or childhood often develop into chronic hepatitis, eventually progressing from liver fibrosis to cirrhosis or hepatocellular carcinoma.² HBV is an enveloped, partially double-stranded DNA virus containing a genome of 3200 nucleotides encoding four open reading frames (ORFs): pre-S/S, pre-C/C, HBx and polymerase. The polymerase gene includes four domains such as the terminal protein, spacer, ribonuclease H and reverse transcriptase (RT). The RT replicates the HBV genome through its DNA polymerase activity using RNA intermediates as a template.³ Because the RT does not possess proof reading activity during viral replication, the error rate of HBV genome synthesis has been mounted as 10^{-7} per nucleotide, which is 10-fold higher than other DNA viruses.⁴ The high rate of HBV mutation results in lots of genomic variants and survival of the fittest during the selection of liver environment, anti-viral drugs and host immune defense.

Mutations in the RT region were thoroughly reported in recent data for their nucleos(t)ide analog-resistant activity. For instance, rtM204I is a classical mutation reducing susceptibility to monotherapy by lamivudine (LMV) or telbivudine (LdT); the rtI169T mutation was reported as an entecavir (ETV)-resistant amino acid substitution and rtA181V showed its resistance to adefovir dipivoxil (ADV).^{5–8} In addition, gene mutations A799G, A987G, and T1055A in the RT region have been reported consistently associated with hepatocellular carcinoma (HCC) and these mutations were always detectable 4–5 years prior to HCC diagnosis.⁹ Moreover, since the envelope (S) gene is completely overlapped by the RT gene, mutations in the RT region may produce changes in its overlapping S gene, causing amino acid substitution or stop codon in the S protein. For instance, rtM204I and rtL180M/M204I produce I195M and W196S in the S protein, leading to an altered structural integrity of S protein and consequently reduced its binding affinity to anti-HBs antibody.¹⁰

Compared with single amino acid mutation, a combination of 2 or more mutations is complicated and always linked to the development of HCC. A combination of G1613A + C1653T or any pre-S mutation + C1653T + T1753V significantly associates with hepatocellular carcinoma in genotype C HBV infected patients.^{11,12} Among the 8 key mutations comprising G1613A, C1653T, T1753V, A1762T, G1764A, A1846T, G1896A and G1899A in the X/preC region, a combination of ≥ 6 mutations shows increased risk of HCC in genotype C2 HBV infected Korean patients.¹³ However, the combination of mutations in the RT region and its clinical significance have seldom been discussed.

In this study, we sequenced the RT gene of HBV in 288 treatment-naïve chronic hepatitis B patients and 71 patients (24.65%) were found with RT mutations. The pattern of mutations and nucleotide analog-resistant muta-

tions as well as the combination of these mutations were analyzed.

Material and methods

Patients and blood samples

A total of 288 treatment-naïve chronic HBV patients were enrolled at the First Affiliated Hospital of Xinxiang Medical University (Henan, China) from July 2009 to May 2014. All patients were diagnosed as chronic hepatitis B based on the criteria suggested by the Chinese Medical Association for Liver Diseases in 2005.¹⁴ The average duration time of HBV infection since first diagnosed as serum HBsAg positive was 28.71 ± 10.24 years. They were all confirmed LMV, ADV, ETV, LdT and interferon (IFN) treatment-naïve. Patients co-infected with the hepatitis A/C/D virus or human immunodeficiency virus, or other concomitant liver disease such as autoimmune liver disease, primary biliary cirrhosis, alcohol or drug abuse were excluded. All patients had written consent on entry into the trials and agreed to authorize the hospital to deal with their blood and tissue samples for research purposes. The study was approved by the Ethics Committee of the First Affiliated Hospital of Xinxiang Medical University. Patients' sera were collected and stored at -80°C .

Diagnostic tests

Liver function tests and serum HBV markers were conventionally conducted in the clinical lab of the First Affiliated Hospital of Xinxiang Medical University. Serum hepatitis B s antigen (HBsAg), anti-HBs, hepatitis B e antigen (HBeAg), anti-HBe and anti-HBc were determined on the wholly automatic immune fluorescence analyzer Abbott Type I2000 (Abbott Laboratories, USA) using the original, attached commercial kits. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin (TBIL) were assayed by the wholly automatic biochemical analyzer DXC800 (Beckman Coulter, USA). Blood platelet count was determined on the fully automatic hematology analyzer LH780 (Beckman Coulter, USA). Serum HBV DNA loads were quantified with fluorescence quantitative PCR assay (Da'An GENE, Guangzhou, China) performed on an ABI 7500 (Applied Biosystems, USA). The detection sensitivity was as low as 500 copies/mL.

Amplification and sequencing of HBV RT region

HBV DNA was extracted from 500 μL sera of the patients according to the protocol of the QIAamp DNA Blood Kit (Qiagen, Germany). The HBV RT gene was amplified by nested PCR as previously described.¹⁵

The primers used for the outer round PCR system were 5-AGTCAGGAAGACAGCCTACTCC-3 (nt 3146-3167) and 5-AGGTGAAGCGAAGTGCACAC-3 (nt 1577-1596); the primers used for the inner round PCR system were 5-TTCCTGCTGGTGGCTCCAGTTC-3 (nt 54-75) and 5-TTCCGCAGTATGGATCGGCAG-3 (nt 1258-1278). PCR conditions were 94°C for 3 min; 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1.5 min; then 72°C for 10 min. The PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen, Germany) and directly sequenced (Sangon Bioengineering, Shanghai, China). Nucleotide sequences were analyzed using DNASTar 5.0 and MEGA 4.0 software. Mutations of the HBV RT gene were determined by sequence alignment with the reference stains in GenBank.¹⁶

Fibrosis staging

Liver biopsies were obtained using a 17G core aspiration needle (Hepafix, Germany), with a biopsy length of 2~5 cm. The biopsy specimens were fixed, paraffin-embedded, cut into 3~5 µm thick sections, and stained with haematoxylin and eosin (H&E) and Masson's trichrome. The degree of liver fibrosis was evaluated by experienced hepato-pathologists, who were blinded to the clinical data of the patients. Staging of liver fibrosis was performed semi-quantitatively according to the published grading and staging system: F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis and few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis.¹⁷

Statistical analysis

Statistical analyses were performed using SPSS16.0 software (SPSS, Chicago, USA). The one-way ANOVA t-test or Pearson's χ^2 test were used appropriately for continuous variables and categorical variables. The serum level of HBV DNA loads were expressed on a logarithmic scale. A p-value of less than 0.05 was considered to be statistically significant.

Results

Patient characteristics

The HBV RT gene was sequenced in all 288 treatment-naïve chronic hepatitis B patients. The HBeAg positive rate was 71.18% (205/288), and all of them were genotype B or C with the HBV B/C ratio of 38.19%/61.81% (110/178). Multiple comparisons of the main characteristics according to serum HBeAg positivity are shown in Table 1. HBeAg-negative patients were significantly older than the HBeAg-positive group, while no significant differences in gender, genotype, ALT, AST and TBIL were found between the HBeAg-positive and HBeAg-negative group.

Table 1. Characteristics of 288 treatment-naïve chronic hepatitis B patients

Characteristics	HBeAg+ CHB (N = 205)	HBeAg- CHB (N = 83)	p-value
Age (years)	42.12 ±14.90	46.56 ±13.61	0.0169
Gender (male/female)	131/74	56/27	0.5655
Genotype (type B/type C)	79/126	31/52	0.8510
HBV DNA (log copies/mL)	6.67 ±1.42	5.49 ±1.33	<0.0001
ALT (U/L)	660.26 ±782.94	504.30 ±546.09	0.0984
AST (U/L)	353.41 ±376.74	281.61 ±261.42	0.1135
TBIL (µmol/L)	74.76 ±102.23	73.83 ±100.86	0.9441
Platelet (×10 ⁹ /L)	169.44 ±159.85	122.31 ±55.58	0.0093

HBeAg – hepatitis B e antigen; HBV – hepatitis B virus; ALT – alanine aminotransferase; TBIL – total bilirubin.

HBeAg-negative patients had much lower serum viral loads than HBeAg-positive patients (HBeAg⁺ vs HBeAg⁻: $10^{6.67}$ vs $10^{5.49}$, $p < 0.0001$), which is consistent with published data.¹⁸ Interestingly, HBeAg-negative patients also showed a significantly lower level of blood platelet than HBeAg-positive patients ($p = 0.0093$).

Characterization of mutations in HBV RT gene from treatment-naïve CHB patients

Among 288 treatment-naïve chronic hepatitis B patients, HBV RT mutations were found in a total of 71 patients (24.65%). The distribution of amino acid mutant sites within the RT gene identified in this study is shown in Fig. 1. There was a total of 28 mutant sites, which formed 3 dominant mutant clusters: rt124-139, rt191-212 and rt225-229, indicating different susceptibility to mutations under a natural history of HBV replication. Although there were 3 patients that had mutations at the rt181 site (A181S), no well-defined primary nucleotide analog-resistant mutations (i.e. I169T, A181T/V, T184A/C/F/G/I/L/M/S, A194T,

Fig. 1. Amino acid substitutions at 28 positions of HBV reverse transcriptase analyzed in this study

Fig. 2. Frequency of accumulation of amino acid substitutions in 71 patients with RT mutations

S202C/G/I, M204I/V/S, N236T, M250I/L/V) were found among these treatment-naïve patients.^{19,20} When analyzing the number of mutations in these 71 patients with a mutant RT gene, we found that 63.38% (45/71) patients had a single mutation while 19.72% (14/71), 12.68% (9/71) and 4.23% (3/71) of patients had 2, 3 or 4 mutations, respectively (Fig. 2).

Correlation between HBV RT mutations and clinical features

To investigate the clinical significance of HBV RT mutations in treatment-naïve chronic hepatitis B patients, the clinical characteristics were compared between the 217 patients without RT mutations and the 71 patients with RT mutations. No significant differences were found in age, gender, HBV genotype, HBeAg status, ALT, AST and TBIL between patients with and without RT mutations (Table 2). However, patients with RT mutations showed significantly decreased serum baseline HBV DNA loads ($p = 0.0363$), indicating a much tougher environment for viral survival or replication. Furthermore, there was a much lower blood platelet count in patients with RT mutations ($p = 0.0181$), demonstrating that there may be much deeper liver injury in these patients.

Correlation between clinical features and number of RT mutations

Among these 71 patients with RT mutations, 45 patients (63.38%) had a single mutant site and 26 patients (36.62%) had 2 or more mutant sites. A comparison of clinical characteristics between these 2 groups is summarized in Table 3. Although there were no significant differences in age, gender, HBV genotype, HBeAg status, ALT, AST and TBIL, the patients with multiple mutant sites (≥ 2) had significantly decreased baseline HBV DNA loads ($p = 0.0004$) and lower blood platelet count ($p = 0.0011$), compared to patients

Table 2. Comparison of main characteristics of patients with and without HBV RT mutations

Characteristics	Group with mutations (n = 71)	Group without mutations (n = 217)	p-value
Age (years)	45.17 $\pm$ 11.72	42.83 $\pm$ 14.91	0.2289
Gender (male/female)	48/23	139/78	0.5863
Genotype (type B/ type C)	26/45	84/133	0.7531
HBeAg (positive/negative)	51/20	154/63	0.8891
HBV DNA (Log copies/mL)	6.02 $\pm$ 1.28	6.43 $\pm$ 1.47	0.0363
ALT (U/L)	561.36 $\pm$ 613.59	632.97 $\pm$ 794.18	0.4878
AST (U/L)	312.34 $\pm$ 298.72	339.38 $\pm$ 382.13	0.5868
TBIL (μ mol/L)	69.61 $\pm$ 98.86	76.09 $\pm$ 104.07	0.6452
Platelet (109/L)	125.14 $\pm$ 46.39	165.91 $\pm$ 141.87	0.0181

HBeAg – hepatitis B e antigen; HBV – hepatitis B virus; ALT – alanine aminotransferase; TBIL – total bilirubin.

with a single mutant site. It demonstrated that there may be much deeper injury in the liver of patients with multiple RT mutations and the liver environment of these patients was not good enough for viral survival or replication.

Multiple RT mutations are associated with more severe liver fibrosis

To investigate the relationship between RT mutations, especially multiple RT mutations, with liver disease se-

Table 3. Comparison of main characteristics of patients with different number of RT mutations

Characteristics	Single mutant site (n = 45)	Mutant sites (≥ 2) (n = 26)	p-value
Age (years)	45.17 $\pm$ 11.72	42.83 $\pm$ 14.91	0.2289
Gender (male/female)	48/23	139/78	0.5863
Genotype (type B/ type C)	26/45	84/133	0.7531
HBeAg (positive/negative)	51/20	154/63	0.8891
HBV DNA (Log copies/mL)	6.02 $\pm$ 1.28	6.43 $\pm$ 1.47	0.0363
ALT (U/L)	561.36 $\pm$ 613.59	632.97 $\pm$ 794.18	0.4878
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HBeAg – hepatitis B e antigen; HBV – hepatitis B virus; ALT – alanine aminotransferase; TBIL – total bilirubin.

Fig. 3. Distribution of liver fibrosis in 164 treatment-naïve chronic hepatitis B patients who had fibrosis staging performed based on the number of HBV RT mutations

F0 – no fibrosis; F1 – portal fibrosis without septa; F2 – portal fibrosis and few septa; F3 – numerous septa without cirrhosis; F4 – cirrhosis.

verity in treatment-naïve chronic hepatitis B patients, a total of 164 individuals had liver biopsies performed: 116 patients without RT mutations, 31 patients with a single RT mutation, and 17 patients with multiple RT mutations. Liver fibrosis was evaluated semi-quantitatively: F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis and few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis.¹⁷ The distribution of liver fibrosis staging is showed in Fig. 3: the number of RT mutations is significantly associated with the severity of liver fibrosis ($p = 0.0128$). 24.14% (28/116) of patients without RT mutations, 6.45% (2/31) of patients with a single RT mutation, and 5.88% (1/17) of patients with multiple RT mutations were diagnosed as F0 fibrosis (no fibrosis). 12.93% (15/116) of patients without RT mutations, 29.03% (9/31) of patients with a single RT mutation, and 47.06% (8/17) of patients with multiple RT mutations had histological evidence of cirrhosis (F4 fibrosis).

Discussion

Reverse transcriptase (RT) preforms the major enzymatic activity for viral replication and the main target of anti-HBV drugs such as nucleos(t)ide analogs (NAs). NAs are reverse transcriptase inhibitors which mimic the natural nucleosides and incorporate into the DNA chain so as to inhibit viral replication.²¹ Treatment of NAs is known as an effective way to restrain HBV replication and restore liver function. However, nucleoside analog-resistant mutations, which always occur at the RT region, are the biggest obstacle during NAs treatment. Moreover, nucleoside analog-resistant mutations were even found in treatment-naïve chronic hepatitis B patients, so it is suggested that patients should be examined for RT mutations before NAs treatment.²² In this study, a total of 288 treatment-

naïve chronic hepatitis patients were sequenced for the RT gene, and 71 patients (24.65%) were found with mutations. Among these mutations, no well-characterized primary or secondary/compensatory nucleoside analog-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) were found according to the classification summarized by previous reports.^{19,20} However, many mutations found in these treatment-naïve patients were putative nucleoside analog-resistant mutations, which may potentially associate with NAs resistance or compensatory replication capacity. For instance, rtV191I, rtV207I and rtL229V have been reported resistant to lamivudine, and rtA181S, rtV214A and rtE218D have been shown potentially related to adefovir resistance.^{23–28} In any case, this data suggests that, rather than primary or secondary/compensatory nucleoside analog-resistant mutations, putative nucleoside analog-resistant mutations showed high prevalence in untreated Chinese chronic hepatitis B patients. The biological and clinical significance of these putative nucleoside analog-resistant mutations need to be further investigated.

The HBV RT gene consists of 6 functional domains (F, A, B, C, D and E) and 5 connecting interdomains (F–A, A–B, B–C, C–D and D–E).²⁹ In this study, we found 28 mutant sites in treatment-naïve patients, and these mutations didn't distribute evenly in the RT region. There were 3 dominant mutation clusters rt124–139, rt191–212 and rt225–229, located at the A–B interdomain, B–C interdomain/C domain and C–D interdomain, respectively. These mutation hotspots showed much a higher frequency of mutations than other sites, indicating that interdomains in the RT region are more susceptible to mutation under natural interaction between HBV replication and host immune defense.

As HBV infection usually starts in early childhood and lasts for a lifetime, the possibility of these patients getting HBV infection from their relatives who ever received formal antiviral treatment is very small, because during their childhood, LMV, ADV, ETV, LdT and IFN treatment were not popular in China. (The first anti-HBV drug LMV was approved by the FDA on 1998.) Since all the patients in this study were treatment-naïve and these RT mutations were selected under liver inflammation induced by the virus and host immune response, we compared the clinical data of patients with and without RT mutations and found that no significant differences were shown in age, gender, HBV genotype, HBeAg status, ALT, AST and TBIL. However, patients with RT mutations had significantly decreased serum baseline HBV DNA loads and much lower blood platelet. The decreased baseline HBV DNA loads reflected a tougher liver environment for viral survival and replication. Blood platelet count is an important index for liver fibrosis, and the much lower platelet count in patients with RT mutations indicated that there were fewer undamaged hepatocytes which can produce enough

thrombopoietin or there was more severe hypersplenism resulting in platelet activation and depletion.^{30,31} In addition, decreased serum baseline HBV DNA loads and lower blood platelet were also found in patients with multiple mutant sites (≥ 2), compared to patients with a single mutant site. Therefore, the significance of RT mutations in treatment-naïve chronic hepatitis B patients is a reflection of a much tougher liver environment for both virus and host.

Compared to serum markers of liver function such as ALT, AST, TBIL and blood platelet count or other non-invasive measures such as aspartate aminotransferase-to-platelet ratio index (APRI), liver biopsy is considered to be the gold standard to evaluate the severity of liver fibrosis.^{32,33} In this study, 164 chronic hepatitis B patients underwent liver biopsies before treatment. Liver fibrosis in patients with RT mutations was more severe than in patients without RT mutations, and liver fibrosis in patients with multiple mutant sites (≥ 2) was more severe than in patients with a single mutant site. This is consistent with the result of a decreased blood platelet count in patients with RT mutations, because patients with liver fibrosis are always associated with dramatically decreased blood platelet count.³⁴ The mechanistic explanation for the relationship between RT mutations and liver fibrosis is presently unclear. It could be speculated that the appearance of RT mutations was a sign of the interaction history between the HBV and host immune response in the liver environment. The appearance and accumulation of RT mutations reflected much longer or more severe liver inflammation, which plays a central role in the liver fibrosis of chronic hepatitis B patients.³⁵ This was the overall effect of natural occurring RT mutations on untreated patients, and the biological or clinical significance of individual mutations (especially those dominant mutations) needs to be further explored.

Based on this data, we demonstrated that the appearance and accumulation of RT mutations in treatment-naïve chronic hepatitis B patients was associated with decreased baseline HBV DNA loads and blood platelet count as well as more severe liver fibrosis. These results reinforce the linkage between the viral mutation and clinical progression of chronic hepatitis, and emphasize that the natural accumulation of RT mutations is a process of viral survival and chronic liver fibrosis.

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