Original Article

The rtA181 Mutation Produces Similar Clinical and Cellular Characteristics in Patients Infected with Hepatitis B Virus Genotypes B and C

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Objective To investigate the association between HBV genotypes and characteristics of rtA181 mutation.

Methods Total of 85 chronic hepatitis B (CHB) patients who appeared rtA181 mutation after nucleos(t)ide analogs (NAs) therapy were enrolled in this study. Levels of serum ALT, AST, HBV DNA and HBsAg titers were monitored during therapy. HBV reverse transcriptase genes were amplified and sequenced to identify genotypes and resistance mutations. Virions and HBsAg in HepG2 cell with rtA181 mutation were also compared between genotypes B and C.

Results The majority of sera contained HBV genotypes B (15.7%) and C (84.3%). There were no significant difference of rtA181 mutant patterns between genotypes ($P > 0.05$). After emergence of rtA181 mutation, serum ALT, AST, HBV DNA levels and HBsAg titers were decreased than that at baseline ($P < 0.05$), while these characteristics were not different between genotypes B and C ($P > 0.05$). In cellular experiment, there were no significant differences between genotypes B and C not only in HBV virions but also in HBsAg titres ($P > 0.05$).

Conclusions No differences of clinical characteristics and cellular results were found in rtA181 mutation of HBV genotypes B and C.

Key words: Hepatitis B virus; Nucleos(t)ide analogs; Mutation; Genotype

Hepatitis B virus (HBV) infection is a major health problem in the world that caused over 240 million people suffering from chronic infection. Every year, 600 000 people died from HBV related liver failure, liver cirrhosis and hepatocellular carcinoma.¹ Several nucleos(t)ide analogs (NAs) are commonly used to suppress HBV replication so as to prevent the progression of liver diseases. In contrast to therapeutic benefits, long term NAs treatment increased the emergence of drug-resistant mutations in the 1212 HBV reverse transcriptase (RT) domain, leading to the treatment failure. The rtA181 mutation is a special multi-drug resistance that not only decreases the efficacy to lamivudine (LAM),^{2,3} adefovir dipivoxil (ADV)⁴ and telbivudine

(LDT),⁵ but also impacts tenofovir disoproxil (TDF) sensitivity.⁶ Additionally, in vitro studies have found the rtA181 mutation affect virus replication,² protein secretion⁷ and tumorigenesis.^{8,9}

HBV is classified into at least nine genotypes based on nucleotide sequence divergence among strains of greater than 8%.^{10,11} Reports in recent years have shown that HBV genotypes are potential factors that influence the severity of liver disease and treatment effectiveness.¹² Compared to patients with genotype C, patients with genotype B have earlier HBeAg seroconversion,¹³ lower risk of reactivation and progression to cirrhosis.¹⁴ Patients with genotype A have more favourable response to interferon- α compared to patients with genotype D.¹⁵ It remains controversial whether there is an association between HBV genotypes and response to

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NAs therapy. A study from Hong Kong showed that there was no difference in the antiviral response and the rate of development of resistant mutations with genotypes B and C after treatment with lamivudine for one year. Another study from China reported that YMDD (tyrosine-methionine-aspartate-aspartate) mutant pattern was not different between patients with genotypes B and C.¹⁶ On the contrary, Pan et al found that the YMDD mutation patterns and serum HBV DNA levels differ between lamivudine-resistant HBV genotypes B and C.¹⁷ To date, there is a few data about the characteristics of rtA181 mutation in HBV genotypes B and C.

In this study, we investigated the mutation patterns and clinical characteristics in rtA181 mutation patients, and detected virus particle and HBsAg in HepG2 cells encoding rtA181 mutation, with the aim to explore whether there were any differences in characteristics associated with the rtA181 mutation in HBV genotypes B and C.

MATERIALS AND METHODS

Patients

Total of 85 patients with chronic HBV infection who visited Nanjing PLA 81st Hospital Liver Clinic Center from January 2010 to January 2012 were enrolled in the study. They were treated with NAs and scheduled follow up every 3-6 months. They exhibited viral breakthrough during antiviral treatment and were identified rtA181T or rtA181V mutations. Patients who received IFN- α treatment or co-infected with hepatitis C and hepatitis D virus, or human immunodeficiency virus were excluded from this study. The diagnostic criteria were based on the Management Scheme of Diagnostic and Therapy Criteria of Viral Hepatitis issued by the Chinese Society of Infectious Diseases.

Assay for liver function, serological marker and quantification of HBV DNA

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured by the Olympus AU5400 Chemistry System (Beckman Coulter, Switzerland). HBsAg/anti-HBs, HBeAg/

anti-HBe and anti-HBc were detected by enzyme linked immunosorbent assay (Kehua Bio-Engineering Co., Shanghai, China) or chemiluminescent assay (Elecsys 2010, Roche Diagnostics, Basel). HBV DNA was quantified by real-time PCR with a LightCycler detection system (ABI 7300 Realtime PCR system, USA) and with a commercial detection kit (Kehua Bio-Engineering). The lower limit of detection for this assay was 500 copies/ml.

Analysis of HBV genotyping and drug resistance

Serum HBV DNA was extracted from 200 μ l serum, as described in the Qiagen DNA blood kit (Qiagen, Germany). The fragments (nt130-nt1129), encompassing the reverse transcriptase (RT) domain, were amplified by nested PCR. The first pair of primers for the PCR were 5'-AAGCTCTGCTAGATCCCAGAGT-3' (sense: nt18-nt40) and 5'-TTTCGCTCCAGACCGGCTGC-3' (antisense: nt1320-nt130). The second pair of primers for the PCR included 5'-GCGGGGTTTTCTTGTTGAC-3' (sense: nt56-nt75) and 5'-AGTATGGATCGGCAGAGGAG-3' (antisense: nt1272-nt1253). The amplification reactions consisted of denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s, and a final extension at 72°C for 5 min. PCR products were directly sequenced on an automated DNA sequencer (ABI 3730XL, USA) using the second sense primer. Substitutions at positions rt80, rt173, rt180, rt181, rt184, rt202, rt204, rt236, and rt250 were taken as resistance mutations for analysis.¹⁸ HBV genotypes classification were based on phylogenetic analysis of the 1 225-bp-long S/Pol gene sequence as described previously.¹⁹

Construction of 1.24-fold genome HBV vectors

In order to investigate the effect of rtA181T/V mutations on HBV biological activity at cellular level between genotypes B and C, Ba_JPN58 and C_JPNAT HBV plasmid kindly provided by Sugiyama,²⁰ were used as wild type templates to construct rtA181T and rtA181V mutations. Site directed mutagenesis (Stratagene, La Jolla, CA) were carried out to introduce mutations, respectively. The mutagenesis

primers were 181T-sense (5'-GCCCGTTTCTCCTGA CTCAGTTTACTAGTGCC-3') and 181T-anti (5'-GGC ACTAGTAACTGAGTCAGGAGAAACGGGC-3'), 181V-sense (5'-GCCCGTTTCTCCTGGTTCAGTTT ACTAGTGCC-3') and 181V-anti (5'-GGCACTAGTA AACTGAACCAGGAGAAACGGGC-3').

Cell culture and transfection experiments

HepG2 human hepatoma cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco) as described before.²¹ After 24 h of culture, cells were transiently transfected with 5 µg of HBV construct using FuGENE HD Transfection (Roche, Switzerland). Transfection efficiency was measured by cotransfection with 0.1 µg pSEAp and determination of alkaline phosphatase activity in cell supernatants using SEAP Reporter Assay Kit (Roche, Switzerland). Cell and supernatants were harvested for further analysis 3 day after transfection. All experiments were performed in triplicates.

Relative replication and HBsAg expression yield assays

For intracellular HBV virions and proteins, cells were washed twice with phosphate-buffered saline (PBS, Gibco) and treated with 200 µl lysis buffer (50 mM Tris[pH 7.5], 1% NP-40, 1 mM EDTA, 150 mM NaCl, 8% sucrose, Beyotime Biotechnology, China) at 37°C for 10 min. After centrifugation at 12 000 g for 5 min, cleared supernatants were collected and added ddH₂O up to 1000 µl. For extracellular HBV virions and proteins, cell culture supernatants were used directly. HBV DNA from virus particles was quantified by real-time PCR after the following process. 20 µl 10 × Reaction Buffer (100 mM Tris[pH 7.5], 25 mM MgCl₂, 1 mM CaCl₂), 2 µl of 20 mg/ml DNase I, and 1 µl 10 mg/ml RNase A (Sigma, Germany) were added to the 180 µl solution and incubated at 37°C for 2 h, followed by inactivation reaction at 70°C for 10 min with EDTA at a final concentration of 10 mM. HBsAg were determined by commercial assay kit mentioned above. The replication and HBsAg expression yield for each mutant were standardized relative to the wild type, defined as 1.0.

Statistical analysis

Quantitative data were expressed as means ± standard deviations and analyzed by independent samples *t* test or *U* test. Count data constitute were analyzed by χ^2 test. Statistical analysis was carried out with SPSS 13.0 software. Significance was set at $P < 0.05$.

RESULTS

Clinical characteristics of patients

Total of 85 patients appeared rtA181T/V mutations after receiving NAs treatment for 10 months or more. Of them, 78 (91.8%) were males and 7 (7.2%) females, their mean age was 41 ± 10 years old. Concerning genotyping, genotype C was the largest group (70 patients, 84.3%), followed by genotype B (13 patients, 15.7%). Other genotypes were not detected in our study. After emergence of rtA181 mutant, the ALT and AST levels were statistically different from that at baseline (81.8 ± 49.1 U/L vs. 175.0 ± 130.2 U/L, $P < 0.05$; 70.6 ± 42.8 U/L vs. 125.0 ± 99.5 U/L, $P < 0.05$, respectively). HBV DNA levels were lower than that at baseline (4.10 ± 1.18 log₁₀ copies/ml vs. 5.97 ± 1.26 log₁₀ copies/ml, $P < 0.05$), and HBsAg titers were also different (3.50 ± 0.45 log₁₀ COI vs. 3.66 ± 0.36 log₁₀ COI, $P < 0.05$). However, from comparison of clinical characteristics of the patients between genotype B and C, no obvious difference was observed in mean age, sex ratio, ALT levels, AST levels, HBV DNA levels, HBsAg titers and HBeAg positive rate ($P > 0.05$). The data were shown in Table 1.

Relationship between genotype and pattern of rtA181 mutation

Two patients were showed the presence of mixture mutant of rtA181T and rtA181V. Other patients represent the similar frequency of mutations with rtA181T ($n = 44$) compared with those with rtA181V ($n = 39$). The occurrence of all rtA181T mutation was lower in genotype B (30.8%) than in genotype C (55.6%), meanwhile the occurrence of all rtA181V was higher in genotype B (69.2%) than in genotype C (41.7%). However, these difference did not reach statistical significance ($P > 0.05$). Other mutations, combined with rtN236T or M204I/V mutation

Table 1. Characteristics of HBV rtA181 mutant patients

Characteristics	Genotype B	Genotype C	<i>P</i> value
Mean age (years)	42 ± 8	41 ± 11	0.765
Sex (male/female)	13/0	65/7	0.532
ALT (U/L)	95.3 ± 64.3	79.3 ± 47.0	0.290
AST (U/L)	73.3 ± 44.1	70.1 ± 42.9	0.808
HBV DNA (log ₁₀ copies/ml)	4.23 ± 1.77	4.07 ± 1.05	0.764
HBsAg (log ₁₀ COI)	3.72 ± 0.52	3.46 ± 0.43	0.065
HBeAg (positive/negative)	10/3	52/20	0.725

Table 2. HBV mutation patterns of rtA181 between genotypes B and C

HBV mutation patterns	Genotype B (n = 13)	Genotype C (n = 72)	<i>P</i> value
All rtA181T mutation	4 (30.8%)	40 (55.6%)	0.100
rtA181T	2	23	0.228
rtA181T + N236T	1	9	0.620
rtA181T + M204I/V	1	7	0.818
rtA181T + M204I/V + N236T	0	1	0.669
All rtA181V mutation	9 (69.2%)	30 (41.7%)	0.066
rtA181V	4	14	0.358
rtA181V + N236T	5	12	0.071
rtA181V + M204I/V	0	4	0.384
rtA181T + rtA181V	0 (0%)	2 (2.7%)	0.543

were also found no significant difference between genotypes B and C ($P > 0.05$). The data were shown in Table 2.

Relative replication and HBsAg expression yield of HBV rtA181 mutation in cells between genotypes B and C

To address the clinical results, in vitro cell replication and secretion experiments were performed. A slight defect in HBsAg and moderate defect in virions secretion were observed in HBV encoding rtA181V. There were no significant differences in the relatively yield of HBsAg and HBV virions in rtA181V mutation compared genotypes B with C ($78.4\% \pm 2.2\%$ vs.

$\pm 7.23\%$, $P > 0.05$, respectively). HBsAg and virions were secreted at extremely low levels (less than 1% of wild type) from cells transfected with HBV encoding rtA181T. So they were not available to be compared in statistics analysis. Intracellular HBsAg and HBV virions relatively yield of rtA181T mutation was increased to 50% and 20%, respectively. The HBsAg of rtA181V mutation quite approached to wild type, and HBV virions were still decreased at half of wild type levels. Compared genotypes B with C, there were no significant differences in the relatively yield of HBsAg and HBV DNA inside cells ($P > 0.05$). The data were shown in Figure 1 and Figure 2.

Figure 1. The relatively replication in genotypes B and C.

Notes: HBV DNA was measured 3 days after transient transfection with HBV constructs indicated. Relatively yield was normalized to wild type level. Mean value (from 3 independent experiments) was compared between genotypes B and C. No significant difference was observed ($P > 0.05$). For extracellular HBV DNA, rtA181T clone was less than 1% of wild type, they were not available to be compared.

Figure 2. The relatively level of HBsAg in genotypes B and C.

Notes: There was no significant difference between genotypes B and C ($P > 0.05$). For extracellular HBsAg, rtA181T clone was less than 1% of wild type, they were not available to be compared.

DISCUSSION

The various HBV genotypes have different geographical distributions worldwide.²² In China, genotypes B and C are the two most commonly found genotypes, that contributing 90% of the population of chronic hepatitis B patients. The clinical importance of genotype related differences in CHB patients remains incompletely understood. In LAM associated rtM204 mutation patients, some studies have confirmed that there was no difference in the antiviral response and the rate of development mutations in patients between different genotypes.^{16,23-25} In rtA181 resistant patients, Li et al showed that rtA181V mutation was more often seen in HBV genotype C2,²⁶ while others argued that rtA181T/V occurrence was not associated with genotypes.^{27,28} Based on these, we sought to examine whether there were any differences in genotypes B and C by assessing clinical and cellular characteristics. Unfortunately, we could not demonstrate any difference between genotypes B and C in terms of rtA181 mutant patterns, biochemical severity, HBsAg titers, HBV DNA levels, HBeAg status and mutations patterns in this study. Additionally, in vitro cell transfection study confirmed our clinical results. There were no differences in the replication and HBsAg expression of plasmids carrying rtA181T/V mutation between genotypes B and C.

In patients treated with interferon, HBV genotype probably is an important factor to predict antiviral response. Genotype B has a more favourable response compared to genotype C, and genotype A is prior to genotype D. This favourable response may not apply in oral NAs therapy. The underlying mechanism may lies in the different action modes of the two antiviral drugs. NAs act through suppression the virus replication, while interferon acts through boosting the immune response against the virus. HBV genotypes lead to different virulence and pathogenicity,²⁹ which may induce different immune response in the host. Therefore, patients may have a better effect to interferon that stimulated a more pronounced immune response. As the response to NAs may not act via the immune system, the patients would not have the

different treatment effect with different genotypes.

In agreement with previous studies, our results showed that genotypes B and C were the major genotypes in China.^{26, 28} In spite of emergence of rtA181 mutant, the patients' liver function, HBV DNA levels and HBsAg titers were lower than that at baseline, which suggested the antiviral drug still show effect to rtA181 mutant, or the mutant virus defective. These explained that after appearance of rtA181 mutant, many patients who continued previously treatment could still improve biochemical and virological results. This finding was similar to other studies in LAM resistance.¹⁶

In vitro studies have found that the rtA181T mutation could affect the S gene aa172 from tryptophan to termination codon, resulting in loss of the C-terminal hydrophobic area in HBsAg, inhibiting the secretion of HBsAg from cell, leading to a decline in HBV DNA replication activity.⁷ Our findings confirmed that little HBsAg or virions were secreted from cells transfected with rtA181T mutation not only in genotype B but also in genotype C. HBV encoding rtA181V has a moderate secretion defect, suggested that rtA181V mutation affect the surface protein exchange and have little influence on virus secretion.²⁷ Through this study, no differences of clinical characteristics and cellular results were found in rtA181 mutation of HBV genotypes B and C. Determination of HBV genotypes was probably not an important factor to predict antiviral responses in genotypes B and C.

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