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Original Article

HBV gene mutations in six multidrug-resistant chronic hepatitis B patients in China

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Abstract: The application of many nucleoside analogs has produced multidrug-resistant mutant strains of hepatitis B virus (HBV). This study aimed to analyze the association between HBV gene mutations in chronic hepatitis B patients and antiviral multidrug resistance. The whole HBV genome in serum samples of six cases of clinical multidrug-resistant patients was amplified using polymerase chain reaction (PCR), then purified and cloned into the pMD18T plasmid to construct 19 clones of recombinant pMD18T-HBV plasmids for whole genome sequencing. The mutations in the P region and other regions in the HBV genome were analyzed. We found that the 19 clones of HBV from these six cases were all of the C genotype. The major common mutation site in the P region was rtM204V/I, and the accompanied common sites were rtQ333K, rtH337N, and rtD392S. The common mutations in the S region of the 19 clones were T56N, K57Q, D62A, and V157A. The common mutation sites in the X region were A41S, G85A, and L92V. There was no common mutation site discovered in the C region. In conclusion, the detection of mutation sites in HBV will help reveal the mechanism underlying the multidrug-resistance of HBV and improve clinical antiviral therapy in chronic hepatitis B patients.

Keywords: Hepatitis B virus, gene mutation, nucleos(t)ide analog, multidrug resistance

Introduction

Hepatitis B virus (HBV) infection leads to chronic hepatitis, liver fibrosis, cirrhosis, or even the development of hepatocellular carcinoma. There are approximately 400 million people infected with HBV worldwide. Therefore, HBV treatment has become a global public health problem. With the application of anti-HBV nucleotide analogs, the diseases of some hepatitis B patients can be effectively controlled and treated. However, HBV replication in the body is prone to gene mutations that generate drug-resistant mutant strains, thus causing drug resistance to nucleotide analogs [1, 2]. In recent years, with the clinical application of many nucleoside analogs such as lamivudine, adefovir, entecavir, telbivudine, and tenofovir, HBV has produced multidrug-resistant mutant strains. Therefore, the situation of HBV drug resistance is severe [3].

In this study, we aimed to investigate the molecular mechanisms underlying the multidrug resistance of HBV in chronic hepatitis B (CHB) patients by performing whole genome sequencing of 19 strains of HBV clones from 6 Chinese CHB patients who had multidrug resistance during treatment and comparing the results with the genome of the wild type strain.

Patients and methods

Subjects

Six patients with chronic HBV infection were outpatients treated at the Department of Infectious Diseases at the Heilongjiang Provincial Hospital from July 2010 to July 2013. All patients were informed regarding the study, agreed on specimen collection, and signed informed consent forms.

All subjects were consistent with the diagnostic criteria for CHB: current HBsAg- and/or HBV DNA-positive patients with a history of hepatitis B or exhibiting a positive HBsAg test for over six months and persistent or recurrent elevated

Table 1. Clinical data of six CHB patients

Patient	Sex	Age	Treatment	ALT/AST (IU/ml)	Viral load (IU/ml)	HBsAg HBsAb	HBeAg HBeAb	Total HBcAb
P1	M	46	L/L+A/E+A	50/31	6.47×10^4	+/-	+/-	+
P2	M	28	L/L+A/E+A	28/26	9.58×10^3	+/-	+/-	+
P3	M	58	E/L+A/T+A	188/93	4.77×10^5	+/-	+/-	+
P4	M	38	A/A+L/A+T/A+E	44/36	1.75×10^5	+/-	-/+	+
P5	F	26	L/E/A+E	19/33	6.45×10^3	+/-	+/-	+
P6	M	50	E/E+A/T+A/E	30/19	2.17×10^8	+/-	+/-	+

Note: L: lamivudine; A: adefovir; E: entecavir. T: telbivudine.

alanine transaminase (ALT). In addition, patients received long-term nucleotide analogs for anti-HBV treatment such as lamivudine, adefovir, and entecavir, and had clinical drug resistance. The other diseases (such as cerebral infarction, hypertension, diabetes, cancer, and autoimmune diseases) were excluded. The clinical data of six multidrug-resistant patients are listed in **Table 1**.

Detection of transaminase levels

Detection of the serum levels of alanine transaminase (ALT) and aspartate transaminase (AST) was performed using the instrument and reagents from Beckman Coulter (USA) according to the manual. The reference ranges were ALT: 5 U/L-35 U/L and AST: 8 U/L-40 U/L. Values of ALT and AST higher than the reference values in two measurements were considered clinically significant.

Detection of HBV-related antigens by ELISA

The levels of HBV-related antigens in the serum were measured by ELISA using diagnostic kits for HBsAg, HBsAb, HBeAg, HBeAb, and HBcAb (Shanghai Shiye Kehua Company, China). In accordance with the manufacturer's instructions, a ratio of sample/negative (S/N) ≥ 2.1 was considered a positive response to the related antigen.

Detection of HBV DNA by real-time PCR

The level of HBV DNA molecules in the serum was detected with the Quantitative HBV PCR Fluorogence Diagnostic Kit (Roche). An HBV DNA level ≥ 5.0×10^2 IU/mL was considered a positive response according to the instructions.

Extraction of serum HBV DNA

Isolation and detection of HBV DNA from serum DNA extraction was performed using the AxyPrep Viral DNA/RNA Miniprep kits (Axygen Biotechnology, Hang-Zhou, China) according to the manufacturer's instructions.

Construction of recombinant pMD18T-HBV plasmids

The HBV full-length genome of isolated HBV DNA was amplified from the plasmids pMD-18T-HBV by PCR using sense primer 5' CCGGAAAGCTTGACTTTTTCACCTCTGCCTAATCA-3', and antisense primer 5' CGAAGAGCTCTTCAAAAAGTTGCATGGTGCTGG-3'. Amplification was performed for 35 cycles using the Platinum Pfx DNA Polymerase. The PCR hot-start procedure was as follows: 94°C for 2 min, 94°C for 30 s, 60°C for 30 s, and 72°C for 4 min. The HindIII/EcoRI digested PCR products were ligated into HindIII/EcoRI digested pcDNA3.1 (+) vector using T4 DNA ligase. The recombinant plasmids were then transformed into *Escherichia coli* JM109 and confirmed by restriction endonuclease digestion and DNA sequence analysis. The sequences were aligned using the Gene Runner version 3.05 (Hastings Software, Inc., Hastings, NY, USA).

Nucleic acid sequence analysis

For each sample, five clones were sequenced by an automated DNA sequencer (ABI 3100-Avant Genetic Analyze; Applied Biosystems, Carlsbad, CA). The sequences were aligned with reference HBV sequences (genotype C) using CLUSTAL W software implemented in DNA MAN version 5.2 (Lynnon Biosoft), and the alignment was confirmed by visual inspection. Uncommon mutations were identified via com-

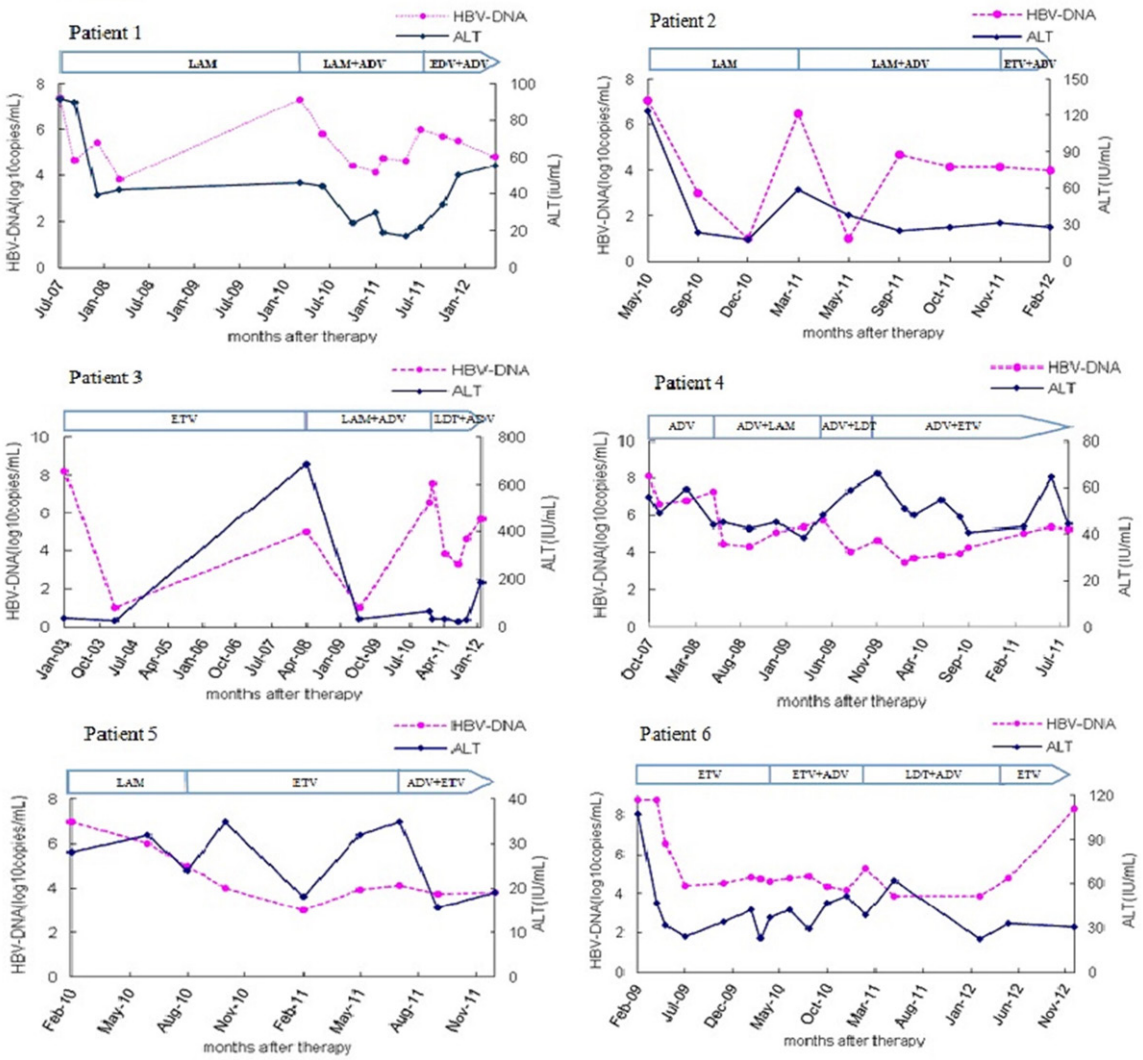

Figure 1. The monitoring of ALT and HBV DNA levels in six CHB patients during anti-viral drug treatment.

parison with all complete HBV sequences from Chinese HBsAg-positive patients accessible in GenBank. The HBV standard strain was Gen Bank AY123041.1.

Results

HBV treatment in six CHB patients and monitoring of ALT and HBV DNA

The average treatment course of HBV treatment in the six clinical multidrug-resistant CHB patients was 4.2 years. More than three nucleotide analogs were used in the anti-viral treatment including lamivudine, entecavir, and adefovir. Patients were given either sequential or combined treatment, but they showed poor clinical virological responses and multidrug resistance. The ALT and HBV DNA indictors did not show significant decrease. The details of anti-viral drug treatment in the six cases of CHB patients and the monitoring of ALT and HBV DNA are presented in **Figure 1**. The HBV DNA sequencing analysis was performed at the end-point of monitoring.

Mutations in the P region in 19 clones of HBV genomes from six patients

The detected HBV genotypes in the six multidrug-resistant CHB patients were all C genotype. The sequencing results revealed that the P regions in 19 clones had common mutation sites in multiple clones (**Table 2**). The common major drug-resistance site mutations, rtM204V/I, rtV173L, rtL180M, and rtL80I, were

Table 2. Mutations in the P region in 19 clones of HBV genomes from six patients

Genotype	Clone code	Polymerase		
		Mutant strains	Major mutation sites	Accompanied mutation sites
C	P1-1, P1-2, P1-3, P1-4, P1-5	5	rtV173L, rtL180M, rtM204V	rtQ118K, rtL269I, rtQ333K, rtH337N
C	P2-1, P2-2, P2-3	3	rtM204I	rtL269I, rtQ333K, rtH337N
C	P3-1, P3-2, P3-3, P3-4, P3-5	5	rtM204I, rtL80I	rtS317A, rtQ333K, rtH337N
C	P4-2, P4-3	2	rtM204I	rtL269I, rtQ333K, rtH337N
C	P5-4, P5-5	2		rtL269I, rtQ333K, rtH337N
C	P6-1, P6-2	2		rtL269I, rtQ333K, rtH337N

Table 3. Analysis of common mutation sites in all gene regions (P, S, X) in 19 clones of HBV from six patients

P region		S region		X region	
Mutation site	Number of mutant strains	Mutation site	Number of mutant strains	Mutation site	Number of mutant strains
rtQ333K	19 (100%)	T56N	19 (100%)	A41S	19 (100%)
rtH337N	19 (100%)	K57Q	19 (100%)	G85A	19 (100%)
rtD392S	19 (100%)	D62A	19 (100%)	L92V	19 (100%)
rtQ461R	17 (89.47%)	V157A	19 (100%)	F42S	14 (73.68%)
rtM204V/I	15 (78.95%)	A358V	15 (78.95%)		
rtL269I	14 (73.68%)	S177N	12 (63.16%)		
rtV375A	14 (73.68%)	A90V	12 (63.16%)		
rtS457H	14 (73.68%)				

detected in the 19 clones of HBV from the multidrug-resistant CHB patients. In addition, many other accompanied mutation sites such as rtQ333K, rtH337N, and rtD392S were detected in all 19 clones. The rtL269I mutation was detected in 14 clones.

Analysis of mutation sites in other gene regions (S, C, X) in 19 clones of HBV from six patients

Compared with the gene sequences of the standard strain, there were no large variations in the C gene region in the 19 clones from six multidrug-resistant CHB patients, and there was no common mutation site. Most clones had HBV common mutation sites in the S and X gene regions in HBV (**Table 3**). In addition to the P region that had major drug-resistance mutation sites, 19 clones from the six patients all had common mutation sites including rtQ333K, rtH337N, and rtD392S in the P region; T56N, K57Q, D62A, and V157A in the S region; and A41S, G85A, and L92V in the X region. Furthermore, mutation sites were present in the majority of clones including rtQ461R, rtM204V/I, rtV375A, and rtS457H in the P region; A358V, S177N, and A90V in the S region; and F42S in the X region.

Discussion

China is a highly prevalent region of HBV infection. There are approximately 30 million cases of CHB. The fundamental treatment of CHB is to inhibit the replication of HBV and clear HBV as much as possible [4, 5]. Although nucleos(t)ide analogs play a certain role in anti-hepatitis B treatment, long-term application of nucleos(t)ide analogs for anti-viral treatment will cause drug-resistant mutations in HBV and reduced anti-viral efficacy, thus causing disease recurrence. Therefore, investigating the drug-resistant mechanism of HBV is important for hepatitis B treatment. Currently, the five nucleos(t)ide analogs for clinical applications of anti-HBV treatment include lamivudine, telbivudine, adefovir, entecavir, and tenofovir. Studies in recent years have demonstrated that each nucleoside analog has major drug-resistant sites and accompanying drug-resistant sites. Mutations in these sites cause the development of clinical

drug resistance. The major drug-resistance site of lamivudine is rtM204I. This drug-resistant mutation is usually accompanied by compensatory mutation sites including rtL180M, L80I, and V173L. These compensatory mutation sites can enhance the replication of drug-resistant viral strains, leading to clinical drug resistance of lamivudine [6, 7]. The major drug-resistance sites of adefovir are rtN236T and/or rtA181T/V. An in vitro drug-sensitivity analysis revealed that the rtN236T mutation did not affect the sensitivity to lamivudine, telbivudine, and entecavir. However, the rtA181T mutation reduced the susceptibility to lamivudine by 10-fold, to adefovir by 2- to 8-fold, to tenofovir by 2- to 3-fold [8]. Entecavir has a high genetic barrier and a low drugresistance rate, which is associated with many drug-resistance sites. Drug resistance develops only when all these drug-resistance sites are present at the same time. Entecavir has many major drug-resistance sites including (rtI169T, rtL180M, rtS184F /A/I/L/G/C/M), (rtM204I/V and rtS202G/I), and (rtM250I/V). The drug-resistant mutations of entecavir could not reduce the efficacy of adefovir and tenofovir [9].

Multidrug resistance indicates that when drugs with different functional targets are applied for sequential or combined treatment, drug-resistant mutations of HBV occur in different drug-target sites, thus producing mutant viral strains with multidrug resistance [10]. Many studies have aimed to investigate whether the mechanism underlying the occurrence of multidrug resistance is the addition of drug-resistance sites for a single drug or is due to special drug-resistance sites. It was suggested that the combined mutations at the rtA181T, rtI233V, rtN236T, and rtM250L sites contributed to the development of multidrug resistance [11, 12]. As compensatory drug-resistance sites, rtI233V+M250L played important role in the development of multidrug resistance. Because the replication ability of the HBV drug-resistant strain was poorer than that of the wild type strain, mutations in compensatory drug-resistance sites could increase the replication ability of drug-resistant strains [13-15].

Based on the analysis of HBV DNA sequences from six clinical multidrug-resistant patients induced by sequential or combined treatment, this study showed that the major drug-resistance site rtM204V/I of lamivudine in the majority of the P regions of the HBV gene was detected in the six multidrug-resistant patients. However, the major drug-resistance sites of entecavir and adefovir were not detected. Indeed, the application of entecavir combined with adefovir for salvage treatment of poor virological responses had ineffective clinical treatment results, indicating that multidrug resistance occurred. Analysis of sequencing results of mutation sites in the P region of HBV genes in 19 strains from six patients showed common mutation sites, specifically, rtQ333K, rtH337N, and rtD392S. The rtH337N has been reported to be associated with clevudine resistance [16], whereas the rtQ333K and rtD392S mutation sites have not been reported. Although the major drug-resistance sites of entecavir and adefovir were not detected in HBV of the six patients in this study, it was possible that rtM204I combined with rtQ333K, rtH337N, and rtD392S yield the combined accompanied drug-resistance sites of multidrug resistance, thus causing clinical multidrug resistance in the six patients. Using sequential treatment with lamivudine, entecavir, and adefovir, Kurashige et al. discovered the rtL269I mutation [17]. Our study also detected this mutation in 14 strains, indicating that rtL269I is an important accompanying mutation site for multidrug resistance. Whether the combined mutations in these sites are combined auxiliary drug-resistance sites of multidrug resistance of lamivudine, entecavir, and adefovir still awaits further confirmation.

This study not only analyzed mutation sites in the P region of HBV but also compared and analyzed gene sequences of the S, C, and X regions in 19 clones of HBV from 6 cases of multidrug-resistant strains. The C region was more stable, had fewer mutation sites, and did not have common mutation sites. The S and X regions both had many common mutation sites. The T56N, K57Q, D62A, and V157A sites in the S region had the same mutations in all 19 strains. Mutations in the S region of HBV caused abnormal virus recognition during immune clearance of HBV in the body, thus causing the decrease in anti-HBV immunity. Whether these mutations also affect drug treatment effects requires further study. The X region was associated with the development of hepatocellular carcinoma [18, 19]. Mutations

in the A41S, G85A, and L92V sites in the X region all occurred in the 19 clones of the HBV genome from six patients. Whether these sites are accompanied sites of multidrug-resistance and whether they will increase the risk for hepatocellular carcinoma require long-tern follow-up studies in these six patients.

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Disclosure of conflict of interest

None.

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