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# Evolutionary pattern of full hepatitis B virus genome during sequential nucleos(t)ide analog therapy

Ying-Zi Tang, Lin Liu, Mei-Min Pan, Yu-Ming Wang *, Guo-Hong Deng *

*Institute for Infectious Diseases, Southwest Hospital, Third Military Medical University, Chongqing 400038, PR China*

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ABSTRACT

The evolutionary and mutational pattern of full hepatitis B virus (HBV) quasispecies during sequential nucleos(t)ide analog (NUC) therapy remains unclear. In this study, full-length HBV clones were generated from serial serum samples of five chronic hepatitis B patients who received sequential NUC therapies (treated patients) and two untreated patients with acute flares. The evolutionary and mutational patterns of full HBV quasispecies were studied. In the three treated patients who received lamivudine as initial antiviral therapy, nucleotide polymorphism and nonsynonymous divergence all decreased at lamivudine breakthrough but increased after rescue therapies. Conversely, two other treated patients showed a distinct change in divergence during adefovir–telbivudine sequential therapies. Untreated subjects exhibited increased polymorphism and divergence in the preC/C region at *ALT flare*. Four of the treated patients presented amino acid changes in the "a" determinant during NUC therapy. All of the treated subjects showed amino acid changes within the known T-cell or B-cell epitopes in the surface or core antigen, most of which were accompanied by mutations in reverse transcriptase (RT) region. Co-variations in the core promoter, the preC region and in the known epitopes of the preS gene accompanied by RT mutations, were common. In untreated patients, most of these co-variations located in the preC/C gene. In conclusion, the distribution of genetic variability of HBV shows remarkably different *patterns* between the treated and untreated subjects and the quasispecies divergence of different regions of HBV may vary remarkably even within a single host.



## 1. Introduction

Chronic hepatitis B virus (HBV) infection is a major public health problem worldwide (Lee, 1997). In recent years, treatment of chronic hepatitis B (CHB) has been improved with the availability of nucleoside/nucleotide analogs (NUCs) such as lamivudine (LAM), adefovir dipivoxil (ADV), telbivudine (LDT), and entecavir (ETV). NUCs target the HBV reverse transcriptase (RT), thus inhibiting viral replication and leading to virologic, biochemical, and histological improvement in most patients. However, the emergence of drug-resistant mutations has become an increasing problem during the treatment with NUCs. Drug resistance has been associated with the emergence of polymerase gene mutations and is often followed by viral breakthrough, subsequent increase in alanine amino transferase (ALT) levels, and, in some circumstances, liver failure.

Most studies of HBV drug resistance have focused on the analysis of the A to D domain of the HBV DNA polymerase gene after detection of antiviral-resistant HBV mutants and the drug-resistant mutational pattern that occurs in this region has been well characterized. The HBV genome contains four partially overlapping open reading frames (ORFs) [Surface (S), Polymerase (P), Core (C), and X proteins (X)], with no noncoding regions and about 50% of its genome involved in two overlapping ORFs. Mutations in the RT domain can affect the amino acid sequence of the surface protein, especially the "a" determinant or T-cell epitope, leading to alterations of immunogenicity (Tai et al., 1997; Chisari and Ferrari, 1995). HBV-related hepatitis activity is HLA class I restricted and T-cell mediated (Chisari and Ferrari, 1995), and previous studies have found that the immune-escape variants probably appeared after antiviral-resistant variants emerge; this might be responsible for the exacerbation of chronic hepatitis B hepatitis (Ehata et al.,

*Abbreviations:* CHB, chronic hepatitis B; HBV, hepatitis B virus; NUC, nucleos(t)ide analog; RT, reverse transcriptase; LAM, lamivudine; ADV, adefovir dipivoxil; LDT, telbivudine; ETV, entecavir; ALT, alanine amino transferase; ORF, open reading frame; HBeAg, hepatitis B e antigen; anti-HBe, antibody to HBeAg; HBsAg, hepatitis B surface antigen; PCR, polymerase chain reaction; preC, precore; BCP, basic core promoter.

* Corresponding authors. Tel.: +86 23 68754858; fax: +86 23 65334998 (Y.-M. Wang); tel.: +86 23 68754289; fax: +86 23 65334998 (G.-H. Deng).

*E-mail addresses:* wym417@163.com (Y.-M. Wang), ghdengsnp@hotmail.com (G.-H. Deng).

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1993; Liu et al., 2003). Based on these findings, dynamic changes within other regions (especially in the immune-targeted surface and core antigen) might also be informative, and investigation of the mutational pattern of the HBV full genome is necessary.

Although HBV is a DNA virus, it replicates through RNA intermediates which require reverse transcriptase. Because the proof-reading function of the reverse transcriptase is insufficient, mutations occur at a higher rate than in other DNA viruses (Nowak et al., 1996). As a result, like other RNA viruses, HBV shows quasispecies distribution in infected patients. Each HBV-infected person harbors a group of viral quasispecies, or a swarm of genetically distinct but related variants. Evolution of the HBV genome has proven to be an informative marker of host–virus interaction. Quasispecies distributions of HBV facilitate the selection of variants that possess survival advantages against host immune responses and antiviral therapeutic agents (Pawlotsky, 2005), thus its evolution and distribution should be monitored to allow chronic infection management.

Pallier et al. (2006) showed that quasispecies variants bearing drug-resistant mutations could be detected at several months before virologic breakthrough and gradually became predominant. The dynamic evolution of HBV quasispecies during the commonly used antiviral therapies have also been investigated in other studies (Ji et al., 2009; Feng et al., 2008; Villet et al., 2007; Guo et al., 2009; Yim et al., 2006). These studies focused on the HBV DNA polymerase gene and did not investigate the mutational pattern of the HBV full genome. Several recent studies have reported the mutational pattern of the HBV full genome. Enomoto et al. (2007) suggested that mutational patterns of HBV DNA at the time of emergence of YMDD variants were unrelated to clinical outcomes during lamivudine therapy. Horiike et al. (2007) found no significant difference of mutations between breakthrough hepatitis and non-breakthrough hepatitis patients. Chen et al. (2010) found in LAM/ADV-treated patients that amino acid changes within the known T-cell or B-cell epitopes of the HBV surface and core antigens might emerge at the LAM and/or ADV resistance. In most of these studies, the HBV full genome sequences were examined by direct sequencing, not by cloning. Thus, the evolutionary pattern of complete HBV quasispecies remains unclear.

In this study, we used full genome sequences generated from patient sera to assess the quasispecies evolution dynamics of five CHB patients with sequential NUC therapies, as well as of two untreated patients with acute flares. The aim of this study is to elucidate the mutational pattern and quasispecies evolution of full-length HBV sequences from CHB patients with sequential NUC therapies.

## 2. Patients and methods

### 2.1. Source of samples

Sera were obtained from seven patients who were persistently positive for hepatitis B surface antigen (HBsAg) and who were followed at the Institute for Infectious Diseases, Southwest Hospital of the Third Military Medical University. The seven patients included five patients with NUCs therapy and two untreated patients with acute flare. The untreated patients showed ALT levels that were within the normal range on regular examinations performed every 3–6 months for more than 2 years before ALT flare occurred. None of the patients had a history of hepatitis C virus or hepatitis D virus co-infection. As for the causes of hepatitis, alcoholic liver disease and autoimmune hepatitis were ruled out in our patients. All serum samples were collected and stored at −80 °C until testing.

### 2.2. HBV markers and HBV DNA detection

HBsAg, HBeAg and anti-HBe were detected by enzyme-linked immunosorbent assay (ELISA; Abbott Laboratory, North Chicago, IL). Transaminase levels were determined in a clinical laboratory, and HBV DNA was measured using the COBAS Amplicor monitor test (Roche Molecular Systems, Branchburg, NJ), in accordance with the Manufacturer's instructions.

### 2.3. DNA extraction

Two hundred microliters of serum were incubated at 65 °C for 3 h with 600 μl of TES buffer (10 mM Tris–HCl, pH 8.0, 5 mM EDTA, 0.5% SDS, and 50 mg of proteinase K). DNA was extracted using phenol/chloroform/isoamylalcohol (25:24:1) and precipitated with isopropylalcohol. DNA pellets were solubilized with 30 μl TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0); and 4 μl aliquots of purified DNA were used as PCR templates.

### 2.4. Amplification of the HBV full genome

PCR was performed according to the method described previously (Kim et al., 2007) with modifications. First-round PCR was performed using the sense primer P1 (5′-TTTTTCACCTCTGCCTAR(A/G)TCATCTC-3′ from 1821 nt to 1845 nt) and the antisense primer P2 (5′-AAAAAGTTGCATGGTGY(C/T)TGGTGM(A/C)AC-3′ from 1825 nt to 1,01 nt). Second-round PCR was performed using the sense primer P3 (5′-TTCACCTCTGCCTAR(A/G)TCATCTC-3′ from 1824 nt to 1845 nt) and the antisense primer P4 (5′-AAAGTTGCATGGTGY(C/T)TGGTGM(A/C)AC-3′ from 1823 nt to 1801 nt). PCR was performed using LA Taq (TaKaRa Bio Inc., Ohtsu, Japan) for 40 cycles in the first PCR and 35 cycles in the second PCR. The conditions of first and second PCR were an initial denaturation at 94 °C for 2 min, and 94 °C for 25 s, 58 °C for 40 s, 68 °C for 3 min 30 s for elongation step, followed by a final extension at 68 °C for 10 min in a thermal cycler.

### 2.5. Cloning and sequencing of the HBV full genome

In order to facilitate the cloning of the full HBV genome, PCR products were gel purified and cloned by utilizing a TOPO-XL-PCR cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocols. Thirty colonies were randomly picked for each specimen and cultured in 1000 μl of Luria–Bertani broth with 50 μg/ml kanamycin. All 30 clones were stored in 20% glycerol at −80 °C. All necessary precautions were taken to prevent cross-contamination and negative controls were included in each assay. The sequencing reaction was performed according to the manufacturer's instruction (ABI Prism BigDye Terminator Cycle Sequencing

**Table 1**
Position and sequences of the primers used for sequencing.

| Primer | Nucleotide sequence (5′–3′) | Position on HBV genome |
|---|---|---|
| P1F | AGATCTCCTCGACACCGCCT | 1984–2003 |
| P2R | GCGAGGGAGTTCTTCTTCTA | 2387–2368 |
| P3F | CCTGTCTTACTTTTGGRAGAG | 2217–2237 |
| P4R | CCMGTAAAGTTTCCCACCTT | 2488–2469 |
| P5F | AGCATTCGGGCCAGGGTTCA | 3030–3049 |
| P6R | ACAAGAAAAACCCCGCCTGT | 218–199 |
| P7F | TCCTGCTGGTGGCTCCAGTT | 55–74 |
| P8R | AGTGACTGGAGATTTGGGA | 335–317 |
| P9F | TGCCTTTRTATGCATGTATAC | 1055–1076 |
| P10R | GGTTCCACGCATGCGCYGAT | 1245–1226 |
| P11F | TGTGCTGCCAACTGGATCCT | 1387–1406 |

R = G or A; M = A or C; Y = T or C; primer M13+ and M13− were also used for sequencing.