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

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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.

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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.

Ready Reaction Kits, Version3.1; Foster City, CA) using an automated ABI DNA sequencer (Model 3730XL, Applied Biosystems). Primers used for sequencing are listed in Table 1.

2.6. Phylogenetic analysis

Sequences were assembled and analyzed by using BioEdit (version7.0.5; http://www.mbio.ncsu.edu/BioEdit/bioedit.html), with alignment performed using ClustalX (Thompson et al., 1997). HBV genotype was determined using phylogenetic analysis of surface antigen genes compared with reference HBV sequences from the NCBI database (http://www.ncbi.nlm.nih.gov/). To detect evidence of recombination, similarity scanning, boot scanning, and informative sites analysis were performed using SimPlot version 3.5.1 (http://sray.med.som.jhmi.edu/SCRoftware/SimPlot) (Lole et al., 1999). To compare the mutational patterns and genetic diversities of the clones, the nucleotide and amino acid sequences of the four HBV coding proteins of the clones were also aligned. Sequences with recombination or ambiguous nucleotides were removed from each alignment prior to evolutionary analysis. Phylogenetic analysis was performed using a Bayesian coalescent algorithm implemented in the Bayesian evolutionary analysis by sampling trees (BEAST) software package version1.4.8 (Drummond and Rambaut, 2007). Dated sequence sets were run using a relaxed lognormal clock with a Markov Chain Monte Carlo (MCMC) chain length of 12 million under the general time reversible (GTR) model of substitution. Output from BEAST was analyzed using the program TRACER (http://beast.bio.ed.ac.uk/Tracer). Nonsynonymous and synonymous distance was calculated by Mega version 4.1 using the modified Nei-Gojobori method with the Jukes-Cantor correction (Tamura et al., 2007). Sliding window analyses of nucleotide polymorphism were performed using DnaSP version 5.0 (Librado and Rozas, 2009) with a 100-nt-wide sliding window and 1-nt steps.

3. Results

3.1. Clinical characteristics of study patients

The clinical course of the study patients is shown in Fig. 1. Treated subjects 1, 2 and 5 received LAM as their initial antiviral therapy; another NUC was administered when virologic breakthrough occurred. All three patients showed virologic breakthrough to the rescue therapy. Treated subject 3 showed no response to LAM therapy, and a virologic breakthrough was observed during both the subsequent rescue therapies, one with ADV and one with LDT. Treated subject 4 received ADV-LDT sequential therapies and showed an elevated HBV DNA level to both of the NUCs. Serum samples were collected from the treated patients prior to NUC therapy and at the time of virologic breakthrough to each NUCs, three or four samples were studied for each patient.

The untreated patients showed normal ALT levels and positive-HBeAg on regular examinations for more than 2 years before ALT flare. For both of these patients, HBeAg/Anti-HBe status remained positive/negative during 2 years of follow-up after ALT flare. Two serum samples from each untreated patient were studied, one obtained during the asymptomatic period and one taken at ALT flare.

The characteristics of the serum samples obtained from the patients are listed in Table 2.

3.2. Intrapatient HBV full genome evolution

The phylogenetic reconstruction of the complete HBV genome for each individual is shown in Fig. 2. The HBV genomes from treated subjects 1, 2 and 3 showed a similar evolutionary pattern; viruses derived prior to therapy fell into distinct lineages, while those derived from virologic breakthrough clustered to separate clades according to time point and showed progressive divergent evolution. The topologies reconstructed from treated subject 4 showed less evidence of grouping according to time point. Viruses derived prior to therapy fell into distinct lineages. Two sequences from the second visit and one from the third formed a distinct clade, with the remainder forming another group. Treated subject 5 harbored a number of distinct viral populations; four sequences from the first visit formed a monophyletic clade, while one sequence from the first visit and three from the third formed another clade. All the viruses from the second visit and the remaining viruses of the third visit clustered to a primary clade. The phylogeny reconstructed from viral sequences obtained from untreated patient 1 showed progressive divergent evolution, and most of the viruses of the second serum sample, except for one, clustered to a clade. Viral sequences obtained from untreated patient 2 fell into three primary clades, and each clade contained sequences from both the first and the second time point.

3.3. Distribution of genetic variability along the full genome

Sliding-window analysis was performed to examine DNA polymorphism across the whole genome (Fig. 3). For each patient, the full genomes sequenced from each visit were studied. For treated patient 1, the more highly variable region was distributed evenly along the preS/S, X and preC/C genes; the level of variation decreased at LAM and LAM+LDT virologic breakthrough compared to the level prior to therapy. Treated patient 2 showed an increased level of variation in the RT region during NUC therapy. At LAM+ADV combination therapy breakthrough, the preC/C gene also showed a very high level of polymorphism. For treated patient 3, the level of variation within each region differed remarkably prior to therapy, with the highest variation occurring in the core gene and 3′ end of the P gene. However, the viral population presented clearly less variability in these two regions after ADV and LDT therapies. Treated patient 4 showed higher variability in the C gene prior to therapy, which decreased during NUC therapies. In contrast, the RT and preS region exhibited increased variability. For treated patient 5, the most variable region was the RT region; increased variability in the preC gene and decreased variability in the C gene was observed during antiviral therapy. Untreated patients 1 and 2 showed the same trend; in these patients, the polymorphism level in the preC gene and at the 5′ end of the C gene increased remarkably when ALT flare occurred.

To determine whether these nucleotide substitutions resulted in amino acid changes, we examined synonymous and non-synonymous divergence of sequences obtained at each visit by the subjects. Sequences of the HBV surface, core, X genes and the RT region were examined (Fig. 4). In treated patient 1, non-synonymous divergence of all four regions decreased at LAM virologic breakthrough and increased after LDT add-on therapy. Synonymous divergence of the RT region showed the same trend as that of non-synonymous divergence. For treated patient 2, synonymous and non-synonymous divergence of the four regions (with the exception of synonymous divergence of the X gene) decreased to its nadir at LAM breakthrough but increased during ADV and LAM+ADV rescue therapies. Treated patient 3 showed progressively decreased synonymous and non-synonymous divergence in each region during antiviral therapy. For treated patient 4, synonymous and non-synonymous divergence of the preS/S and RT regions increased both at ADV and at LDT breakthrough. The preC/C gene presented higher synonymous and non-synonymous divergence level at ADV breakthrough but decreased to nadir at rescue LDT therapy. The X gene showed an opposite changing trend in synonymous and non-synonymous divergence at ADV breakthrough but

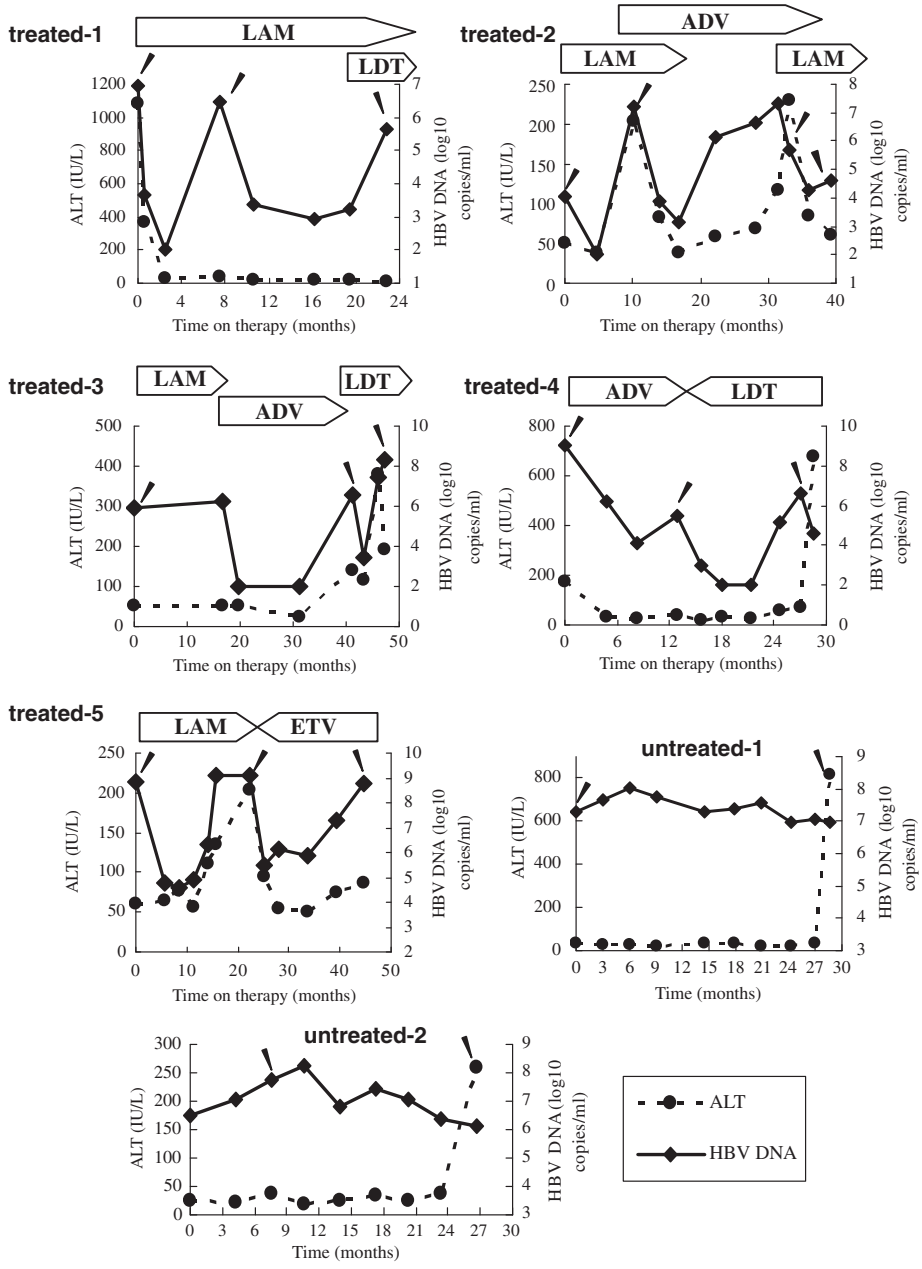

Fig. 1. Clinical course of the study subjects. HBV DNA levels in solid line and ALT levels in dashed line are plotted against time in months. Antiviral therapies are shown above each graph. Arrows indicate the timing at which the studied serum samples were taken. ALT, alanine amino transferase; LAM, lamivudine; ETV, entecavir; ADV, adefovir dipivoxil; LDT, telbivudine.

also decreased to a nadir at LDT breakthrough. For treated patient 5, non-synonymous divergence of the four genes decreased at LAM breakthrough, but increased at ETV breakthrough, while the synonymous divergence change in these regions showed a more complicated trend. Finally, in both untreated patients, the synonymous and non-synonymous divergence levels of the preC/C gene were higher at ALT flare than during the asymptomatic period.

3.4. Mutational patterns of the HBV full genome

The mutational pattern observed in precore, core promoter, RT domain and epitopes of the core protein and preS/S gene of treated subjects are listed in Table 3. Four of the five treated patients (1, 2, 3 and 5) showed NUC-resistant mutations (treated patient 4 did not show ADV-resistant mutation), and multi-drug resistant mutations were detected in patients 2, 3 and 5. No patients showed mutations in the "a" determinant (s124–148) prior to antiviral therapy; however, four subjects (1, 2, 4 and 5) showed amino acid changes in this region during follow-up after therapy. All treated subjects showed amino acid changes within the known T-cell or B-cell epitopes in the preS/S or core antigen, and most of these were accompanied by mutations in the RT region. In treated patients 1 and 5, cP135Q and cT147A were detected and were accompanied by an rtM204V/I drug-resistant mutation. Treated patients 3 and 5 presented amino acid changes within epitopes of the preS or core antigen prior to therapy, but these reverted to wild-type after antiviral therapy. Substitutions in the core promoter or preC region, accompanied by RT mutations, were also detected in patients 1, 3 and 5. However, these nucleotide substitutions were not usually accompanied by changes in HBeAg/anti-HBe status.

Table 2
Summary of patient serum data.

Subject	Gender/age (years)/ HBV genotype	Sera isolation date	No. of sequences	HBV DNA (copies/ml)	ALT (IU/L)	HBeAg/anti-HBe
Treated-1	M/32/B	2006-08-28	5	9.16E+06	1080	+/−
		2007-04-15	5	3.07E+06	39	+/−
		2008-07-21	9	4.44E+05	5	+/−
Treated-2	M/41/C	2005-04-07	6	1.11E+04	50	+/−
		2006-02-09	10	1.70E+07	203	+/−
		2008-01-14	5	4.94E+05	229	+/−
		2008-07-15	9	4.37E+04	61	+/−
Treated-3	M/48/B	2004-11-02	6	7.99E+05	54	−/+
		2008-03-18	8	3.92E+06	139	+/−
		2008-09-22	7	2.14E+08	193	−/+
Treated-4	M/34/C	2006-10-01	7	1.09E+09	173	+/−
		2007-10-23	9	3.11E+05	39	+/−
		2008-12-23	9	3.76E+06	68	+/−
Treated-5	M/32/B	2003-03-01	5	7.25E+08	60	+/−
		2005-01-02	5	1.23E+09	204	+/+
		2006-11-23	9	6.36E+08	87	+/−
Untreated-1	F/28/C	2004-10-17	8	1.93E+07	32	+/−
		2007-03-08	8	9.24E+06	814	+/−
Untreated-2	M/26/B	2005-12-12	7	5.51E+07	38	+/−
		2007-07-17	8	1.32E+06	260	+/−

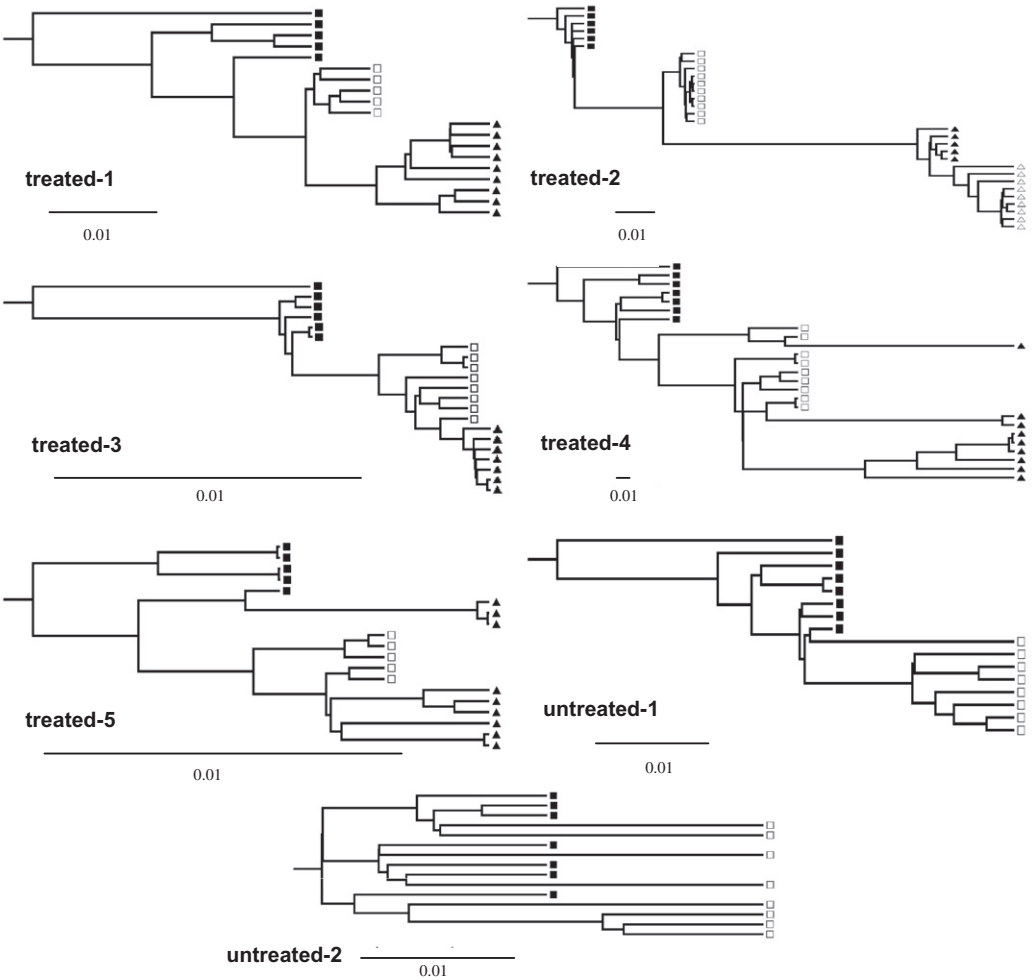

Fig. 2. Bayesian phylogenetic trees of the study subjects. Dated sequence sets were run using a relaxed lognormal clock with a MCMC chain length of 12 million under the general time reversible (GTR) model of substitution. Branch lengths are in accordance with the scale bar. ■: first visit; □: second visit; ▲: third visit; △: fourth visit.

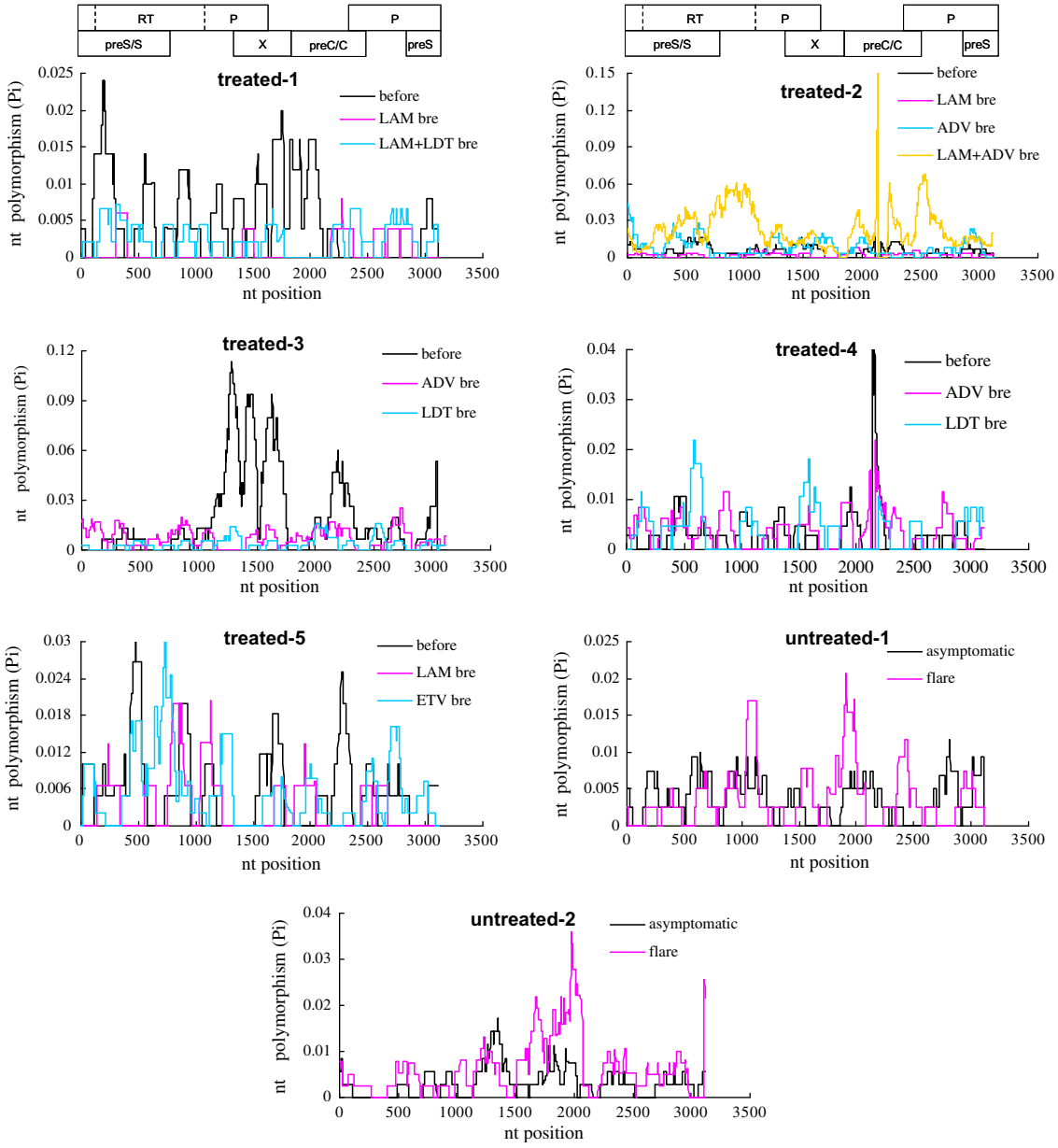

Fig. 3. Nucleotide (nt) polymorphism along the HBV genome of the study subjects. The frequency of polymorphic sites in the whole sites in 100-nt-wide windows of the full genomes sequenced at every time point of each patient is presented. Before: prior to therapy; bre: breakthrough.

In untreated subjects, most mutations were located in the core promoter and preC/C gene. A deletion pattern of nt1758–1777 was detected in untreated patient 1 at ALT flare and a cluster of amino acid changes in core protein were detected in both subjects (data not shown). Co-variation mutations of cY38H, cL60V, a stop codon mutation of cQ182 and A1846T substitution were detected at ALT flare in both untreated patients; in untreated patient 2, these co-variation sites were accompanied by amino acid change of sT140I in the B-cell epitope and substitution of G1896A.

4. Discussion

This report describes the evolution of the HBV full genome sequences in CHB patients receiving sequential NUC therapies during chronic infection. Two untreated patients with acute flares were also included in the study.

The divergence of HBV quasispecies in treated patients is interesting. For treated patients 1, 2 and 5 who received LAM initial therapy, divergence in four ORFs all decreased at LAM breakthrough but became elevated after rescue therapies. This pattern of clustering to a new clade on viral breakthrough followed by subsequent divergence is a classic example of genetic drift where the founder effect that has been observed when a population is decimated and then reemerges. Treated patients 3 and 4 showed distinct divergence change during ADV-LDT sequential therapies. The divergence in four ORFs all decreased at ADV breakthrough, with emergence of ADV-resistant mutations in treated patient 3. Treated patient 4 showed increased divergence at ADV breakthrough without detectable ADV-resistant mutations. The divergence change of the latter two subjects also showed different patterns at breakthrough on LDT rescue therapy.

The requirements of the internal population structure of a viral quasispecies to adapt to a new or changing environment are expected to depend on the nature of the selective constraint confronted by the viral population. Adaptation to a single antiviral inhibitor, which very often represents a highly specific selective

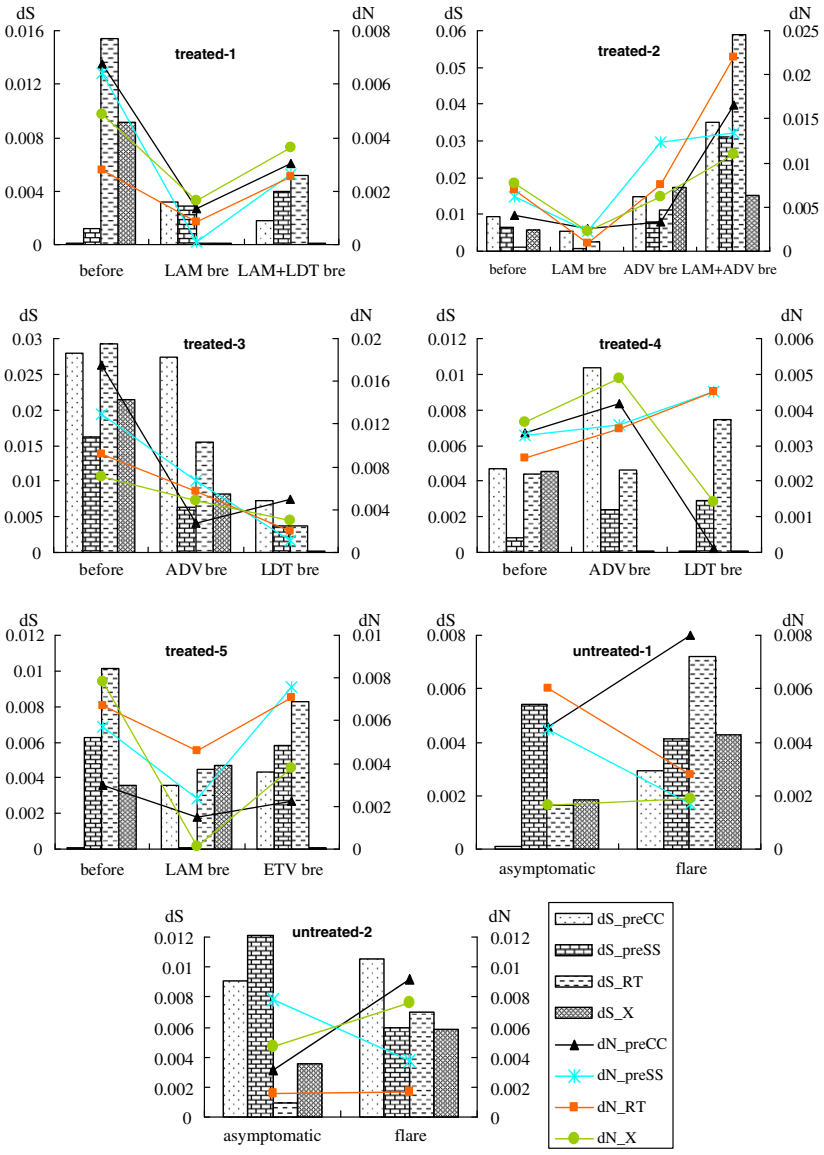

Fig. 4. Synonymous (dS) and nonsynonymous (dN) divergence of four HBV ORFs at each visit of the study subjects. Synonymous and nonsynonymous divergence was calculated from the clones obtained from each specimen using the method of Modified Nei-Gojobori with the Jukes-Cantor correction. before: prior to therapy; bre, breakthrough.

pressure directed to a defined viral target, may be compatible with a range of mutation rates and mutant spectrum complexities similar to or even below the levels commonly seen in viral populations. A lower than standard (typical of wild-type virus) mutation rate may be sufficient when one (or very few) mutation(s) can cause a decrease in the sensitivity of the virus to the inhibitor (Domingo and Gomez, 2007). This theory explains the phenomenon observed in treated patients 1, 2 and 5, in whom the diversity of the HBV genome decreased to its nadir at LAM resistance. HBV resistance to other antiviral drugs involves different dynamics and molecular mechanisms. Resistance to ETV often requires "secondary" mutations combined with the "primary" LAM-resistance mutations. HBV resistance to ADV is even more complicated. The two ADV-resistance mutations of rtN236T and rtA181V are associated with different patterns of HBV DNA kinetics in different patients (Angus et al., 2003; Villeneuve et al., 2003). Our study suggests that HBV quasispecies may follow a distinct evolutionary pattern in patients undergoing different NUC therapies.

In the two naïve patients with acute flares, we observed increased divergence in the preC/C region at ALT flare compared to that in the asymptomatic period. We did not observe this phenomenon in treated patients, although patients 2, 3, 4 and 5 also presented ALT flare during antiviral therapy. This indicates that the underlying mechanisms of ALT flare are different between treated and untreated patients. The factors that trigger the hepatitis flare remains unclear. A prospective study (Liu et al., 2003) suggested that hepatitis flares are not usually caused by an alteration of HBV genome, but rather by inadequate host immune control over the preexisting viral strain. To clarify the mechanisms of hepatitis flare in detail, a study of viral factors and host immune factors is required.

A notable finding of this study is that the polymorphism and divergence of HBV coding regions differed remarkably even within a single patient, and these changing patterns also differed between subjects. HBV has overlapping ORFs and this provides a constraint on divergence. PreC/C gene has a large portion of non-overlapping region; this region shows an approximately two-fold greater evolutionary divergence compared to regions where there is overlap (Zhou and Holmes, 2007). Diverse viral sequences might be a marker of a more intense immune response, more rapid viral

Table 3
Mutational patterns in precore, core promoter, core protein, surface protein and RT domain of treated patients.

Subject	HBe Ag/Ab	No. clones	preC	Core promotor			Core					preS			Surface							RT domain																					
			1896G	1613G	1677G	1762/64A/G	97I	114T	130P	135P	147T	40N	74L	121Q	40N	47T	126T	129Q	138C	140T	145G	80L	106S	122I	125Q	134N	137S	173V	176S	164L	180L	181A	184T	204M	225T	229L	236N	319Q	326T					
Treated-1																																												
Prior to therapy	+/−	5/5	A			T/A																																						
LAM breakthrough	+/−	5/5								Q	A											I										I											
LAM+LDT	+/−	8/9								Q	A											I										I											
breakthrough	+/−	1/9								Q	A							H				I										I											
Treated-2																																												
Prior to therapy	+/−	6/6				T/A										K								K										S				I				
LAM breakthrough	+/−	10/10				T/A										K								K								I		S		F			I			
ADV breakthrough	+/−	4/5				T/A										K					R			K										S				I				
		1/5				T/A										K								K										S				I				
LAM+ADV	+/−	2/9				T/A																								M/P	T/VV		V			T							
breakthrough		7/9				T/A															A		L						M														
Treated-3																																												
Prior to therapy	−/+	2/6										Y		K																													
		4/6					L		T						S																												
ADV breakthrough	+/−	3/8				T/A	L	V	T						S															S						T						
		3/8				T/A	L	V	T						S															S													
		2/8				T/A	L	V	T						S																													
LDT breakthrough	−/+	7/7				T/A	L/P	V	T				M		S															M	V		V										
Treated-4																																												
Prior to therapy	+/−	7/7				T/A																																						
ADV breakthrough	+/−	8/9				T/A																																						
		1/9				T/A											A		R			S																						
LDT breakthrough	+/−	1/9				T/A																						A																
		3/9				T/A						D																	N											R			
		5/9				T/A																																						
Treated-5																																												
Prior to therapy	+/−	2/5		A		T/A			T																																		
		3/5		A					T																																		
LAM breakthrough	+/+	2/5	A		A					Q	A												C			S		A			M			V								
		3/5	A		A					Q	A												C			S					M			V								
ETV breakthrough	+/−	1/9	A		A					Q	A												C			S					M		L	V								
		3/9	A							Q	A									I														I		V						
		5/9	A		A					Q	A												C			S					M			V								

turnover, and evidence of positive selection pressure. Our study demonstrates that different regions of the HBV genome confront distinct evolutionary forces during chronic infection.

Prior to antiviral therapy, no mutations were detected in the "a" determinant of the HBV surface protein in any of the five treated patients; however, four patients (1, 2, 4 and 5) showed amino acid changes in this region during follow-up after therapy. The HBV surface proteins containing the vaccine escape mutations in the "a" determinant demonstrated markedly reduced binding to anti-HBs antibody (Torresi et al., 2002). Our findings indicate that mutants that possess antigenically distinct HBV surface proteins may arise during sequential NUC therapies.

In this study, we found that amino acid changes in the T- or B-cell epitopes of surface antigen and core protein may accompany RT mutations during virologic breakthrough in NUC-treated patients. T-cell response to HBV core antigen is important in determining either the persistence or the clearance of HBV (Salfeld et al., 1989; Ferrari et al., 1990; Tsai et al., 1992). HBV core gene mutations could allow HBV to escape immune surveillance via loss or change in immuno-dominant epitopes (Chuang et al., 1993). The HBV preS region is highly immunogenic, containing both sequential and conformational epitopes with abundant T- and B-cell epitopes (Ferrari et al., 1992; Hong et al., 2004; Park et al., 2000; Maeng et al., 2000; Kuroki et al., 1990). The preS region is essential for viral assembly (Bruss and Thomssen, 1994) and is believed to play a major role in mediating virus attachment and entry into hepatocytes (Blanchet and Sureau, 2007). A previous study found that combined mutations in the preS/S and core promoter/precore regions of HBV increase the risk of hepatocellular carcinoma (Chen et al., 2008). In LAM-ADV treated patients, Chen et al. (2010) found that amino acid changes within the known epitopes of HBV surface and core antigens may emerge at LAM and/or ADV resistance. Our study, which used full length HBV clones generated from sera samples, allowed us to obtain information regarding the relative changes in HBV RT regions and in surface and core antigens. We found that all treated subjects showed amino acid changes within the known T-cell or B-cell epitopes in the preS/S or core gene and that these changes were accompanied by mutations in RT region. Some patients also showed co-variations in core promoter or precore regions, accompanied by RT mutations. Thus, our analyses indicate that NUC-resistant mutations can cause amino acid changes within epitopes of other HBV regions and that the characteristics of HBV immunogenicity may change when NUC resistance emerges. Determination of whether these specific mutations are randomly or regularly associated with the resistant mutations will require more research.

In conclusion, our study suggests that HBV quasispecies undergo a distinct evolutionary pattern in treated and untreated patients and that different regions of the HBV genome experience distinct evolutionary forces during chronic infection. Our analysis also indicates that the NUC-resistant mutations can cause amino acid changes within epitopes of the HBV preS/S or core antigen and that characteristics of HBV immunogenicity may change when NUC resistance emerges.

Conflict of interest statement

None declared.

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References

Angus, P., Vaughan, R., Xiong, S., Yang, H., Delaney, W., Gibbs, C., Brosgart, C., Colledge, D., Edwards, R., Ayres, A., Bartholomeusz, A., Locarnini, S., 2003. Resistance to adefovir dipivoxil therapy associated with the selection of a novel mutation in the HBV polymerase. Gastroenterology 125, 292–297.

Blanchet, M., Sureau, C., 2007. Infectivity determinants of the hepatitis B virus pre-S domain are confined to the N-terminal 75 amino acid residues. J. Virol. 81, 5841–5849.

Bruss, V., Thomssen, R., 1994. Mapping a region of the large envelope protein required for hepatitis B virion maturation. J. Virol. 68, 1643–1650.

Chen, C.H., Changchien, C.S., Lee, C.M., Hung, C.H., Hu, T.H., Wang, J.H., Wang, J.C., Lu, S.N., 2008. Combined mutations in pre-s/surface and core promoter/precore regions of hepatitis B virus increase the risk of hepatocellular carcinoma: a case-control study. J. Infect. Dis. 198, 1634–1642.

Chen, C.H., Lee, C.M., Tung, W.C., Wang, J.H., Hung, C.H., Hu, T.H., Wang, J.C., Lu, S.N., Changchien, C.S., 2010. Evolution of full-length HBV sequences in chronic hepatitis B patients with sequential lamivudine and adefovir dipivoxil resistance. J. Hepatol. 52, 478–485.

Chisari, F.V., Ferrari, C., 1995. Hepatitis B virus immunopathogenesis. Annu. Rev. Immunol. 13, 29–60.

Chuang, W.L., Omata, M., Ehata, T., Yokosuka, O., Ito, Y., Imazeki, F., Lu, S.N., Chang, W.Y., Ohto, M., 1993. Precore mutations and core clustering mutations in chronic hepatitis B virus infection. Gastroenterology 104, 263–271.

Domingo, E., Gomez, J., 2007. Quasispecies and its impact on viral hepatitis. Virus Res. 127, 131–150.

Drummond, A., Rambaut, A., 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol. Biol. 7, 214.

Ehata, T., Omata, M., Chuang, W.L., Yokosuka, O., Ito, Y., Hosoda, K., Ohto, M., 1993. Mutations in core nucleotide sequence of hepatitis B virus correlate with fulminant and severe hepatitis. J. Clin. Invest. 91, 1206–1213.

Enomoto, M., Tamori, A., Kohmoto, M.T., Morikawa, H., Habu, D., Sakaguchi, H., Takeda, T., Seki, S., Kawada, N., Shiomi, S., Nishiguchi, S., 2007. Mutational patterns of hepatitis B virus genome and clinical outcomes after emergence of drug-resistant variants during lamivudine therapy: analyses of the polymerase gene and full-length sequences. J. Med. Virol. 79, 1664–1670.

Feng, B., Wei, L., Chen, M., Wang, L., 2008. Dynamic changes of hepatitis B virus polymerase gene including YMDD motif in lamivudine-treated patients with chronic hepatitis B. Microbiol. Res. 163, 487–492.

Ferrari, C., Penna, A., Bertoletti, A., Valli, A., Antoni, A.D., Giuberti, T., Cavalli, A., Petit, M.A., Fiaccadori, F., 1990. Cellular immune response to hepatitis B virus-encoded antigens in acute and chronic hepatitis B virus infection. J. Immunol. 145, 3442–3449.

Ferrari, C., Cavalli, A., Penna, A., Valli, A., Bertoletti, A., Pedretti, G., Pilli, M., Vitali, P., Neri, T.M., Giuberti, T., 1992. Fine specificity of the human T-cell response to the hepatitis B virus preS1 antigen. Gastroenterology 103, 255–263.

Guo, J.J., Li, Q.L., Shi, X.F., Zhang, D.Z., Zeng, A.Z., Feng, T., Huang, A.L., 2009. Dynamics of hepatitis B virus resistance to entecavir in a nucleoside/nucleotide-naïve patient. Antiviral Res. 81, 180–183.

Hong, H.J., Ryu, C.J., Hur, H., Kim, S., Oh, H.K., Oh, M.S., Park, S.Y., 2004. In vivo neutralization of hepatitis B virus infection by an anti-preS1 humanized antibody in chimpanzees. Virology 318, 134–141.

Horiike, N., Duong, T.N., Michitaka, K., Joko, K., Hiasa, Y., Konishi, I., Yano, M., Onji, M., 2007. Characteristics of lamivudine-resistant hepatitis B virus (HBV) strains with and without breakthrough hepatitis in patients with chronic hepatitis B evaluated by serial HBV full-genome sequences. J. Med. Virol. 79, 911–918.

Ji, F., Zhou, L., Ma, S., Li, F., Ding, H., Zeng, C., 2009. Dynamic changes of HBV quasispecies and deletion patterns in a chronic hepatitis B patient. J. Med. Virol. 81, 1551–1559.

Kim, H., Jee, Y.M., Song, B.C., Hyun, J.W., Mun, H.S., Kim, H.J., Oh, E.J., Yoon, J.H., Kim, Y.J., Lee, H.S., Hwang, E.S., Cha, C.Y., Kook, Y.H., Kim, B.J., 2007. Analysis of hepatitis B virus quasispecies distribution in a Korean chronic patient based on the full genome sequences. J. Med. Virol. 79, 212–219.

Kuroki, K., Floreani, M., Mimms, L.T., Ganem, D., 1990. Epitope mapping of the PreS1 domain of the hepatitis B virus large surface protein. Virology 176, 620–624.

Lee, W.M., 1997. Hepatitis B virus infection. N. Engl. J. Med. 337, 1733–1745.

Librado, P., Rozas, J., 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25, 1451–1452.

Liu, C.J., Chen, P.J., Lai, M.Y., Kao, J.H., Chang, C.F., Wu, H.L., Shau, W.Y., Chen, D.S., 2003. A prospective study characterizing full-length hepatitis B virus genomes during acute exacerbation. Gastroenterology 124, 80–90.

Lole, K.S., Bollinger, R.C., Paranjape, R.S., Gadkari, D., Kulkarni, S.S., Novak, N.G., Ingersoll, R., Sheppard, H.W., Ray, S.C., 1999. Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. J. Virol. 73, 152–160.

Maeng, C.Y., Ryu, C.J., Gripon, P., Guguen-Guillouzo, C., Hong, H.J., 2000. Fine mapping of virus-neutralizing epitopes on hepatitis B virus PreS1. Virology 270, 9–16.

Nowak, M.A., Bonhoeffer, S., Hill, A.M., Boehme, R., Thomas, H.C., McDade, H., 1996. Viral dynamics in hepatitis B virus infection. Proc. Natl. Acad. Sci. USA 93, 4398–4402.

Pallier, C., Castera, L., Soulier, A., Hezode, C., Nordmann, P., Dhumeaux, D., Pawlotsky, J.M., 2006. Dynamics of hepatitis B virus resistance to lamivudine. J. Virol. 80, 643–653.

Park, J.H., Cho, E.W., Lee, Y.J., Shin, S.Y., Kim, K.L., 2000. Determination of the protective effects of neutralizing anti-hepatitis B virus (HBV) immunoglobulins by epitope mapping with recombinant HBV surface-antigen proteins. Microbiol. Immunol. 44, 703–710.

Pawlotsky, J.M., 2005. The concept of hepatitis B virus mutant escape. J. Clin. Virol. 34 (Suppl. 1), S125–S129.

Salfeld, J., Pfaff, E., Noah, M., Schaller, H., 1989. Antigenic determinants and functional domains in core antigen and e antigen from hepatitis B virus. J. Virol. 63, 798–808.

Tai, P.C., Banik, D., Lin, G.I., Pai, S., Pai, K., Lin, M.H., Yuoh, G., Che, S., Hsu, S.H., Chen, T.C., Kuo, T.T., Lee, C.S., Yang, C.S., Shih, C., 1997. Novel and frequent mutations of hepatitis B virus coincide with a major histocompatibility complex class I-restricted T-cell epitope of the surface antigen. J. Virol. 71, 4852–4856.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL_X windows interface. flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.

Torresi, J., Earnest-Silveira, L., Deliyannis, G., Edgtton, K., Zhuang, H., Locarnini, S.A., Fyfe, J., Sozzi, T., Jackson, D.C., 2002. Reduced antigenicity of the hepatitis B virus HBsAg protein arising as a consequence of sequence changes in the overlapping polymerase gene that are selected by lamivudine therapy. Virology 293, 305–313.

Tsai, S.L., Chen, P.J., Lai, M.Y., Yang, P.M., Sung, J.L., Huang, J.H., Hwang, L.H., Chang, T.H., Chen, D.S., 1992. Acute exacerbations of chronic type B hepatitis are accompanied by increased T cell responses to hepatitis B core and e antigens. Implications for hepatitis B e antigen seroconversion. J. Clin. Invest. 89, 87–96.

Villeneuve, J.P., Durantel, D., Durantel, S., Westland, C., Xiong, S., Brosgart, C.L., Gibbs, C.S., Parvaz, P., Werle, B., Trepo, C., Zoulim, F., 2003. Selection of a hepatitis B virus strain resistant to adefovir in a liver transplantation patient. J. Hepatol. 39, 1085–1089.

Villet, S., Ollivet, A., Pichoud, C., Barraud, L., Villeneuve, J.P., Trépo, C., Zoulim, F., 2007. Stepwise process for the development of entecavir resistance in a chronic hepatitis B virus infected patient. J. Hepatol. 46, 531–538.

Yim, H.J., Hussain, M., Liu, Y., Wong, S.N., Fung, S.K., Lok, A.S., 2006. Evolution of multi-drug resistant hepatitis B virus during sequential therapy. Hepatology 44, 703–712.

Zhou, Y., Holmes, E.C., 2007. Bayesian estimates of the evolutionary rate and age of hepatitis B virus. J. Mol. Evol. 65, 197–205.