Evolutionary patterns of hepatitis B virus quasispecies under different selective pressures: correlation with antiviral efficacy

Feng Liu,1 Li Chen,1,2 De-Min Yu,1 Lin Deng,1 Rong Chen,1 Yin Jiang,3 Liang Chen,3 Su-Yuan Huang,1 Jia-Lun Yu,1 Qi-Ming Gong,1 Xin-Xin Zhang1

ABSTRACT

Objective To investigate the evolution of hepatitis B virus (HBV) quasispecies (QS) within the reverse transcriptase (RT) region during the early stage of entecavir treatment and its impact on virological response, and to compare evolutionary patterns under different selective pressures.

Methods 31 patients with chronic hepatitis B receiving entecavir (17 responders and 14 partial responders according to the HBV DNA levels at week 48) and 25 patients receiving lamivudine (14 responders and 11 non-responders) as controls were included. An average of 26 clones (2892 total from both groups) spanning the RT region per sample was sequenced.

Results QS complexity and diversity, in addition to alanine aminotransferase and HBV DNA levels, were comparable between responders and partial responders at baseline. However, QS complexity in responders at week 4 was statistically lower than that in partial responders at the nucleotide level (0.6494 vs 0.7723, p = 0.039). Net changes in diversity as well as the viral nucleotide substitution rate of responders were higher than those of partial responders, and both correlated with virological responses at both week 48 and the final visit (mean: 28 months). A preliminary model of QS evolution variables predicted 16 of 17 responders and 13 of 14 partial responders in the entecavir group. Despite significant differences between responders to entecavir and responders to lamivudine at week 4, the characteristics of QS were quite similar between partial responders to entecavir and non-responders to lamivudine.

Conclusions The evolutionary patterns of HBV RT QS differ between responders and partial responders during the early stage of entecavir treatment. Characteristics of HBV QS evolution during the first 4 weeks contribute to the prediction of long-term virological responses. The similar patterns of HBV RT QS in partial responders and non-responders receiving different nucleoside analogues may imply a novel mechanism of drug resistance, which warrants further investigation.

More than 350 million people worldwide are chronically infected with hepatitis B virus (HBV); HBV infection accounts for >1 million deaths annually from related cirrhosis, liver failure and hepatocellular carcinoma (HCC).1 The goal of treatment for chronic hepatitis B (CHB) is to improve both quality of life and survival by preventing disease progression to cirrhosis, decompensated cirrhosis, end-stage liver disease, HCC and death.2 This goal can be achieved by suppressing HBV replication in a sustained manner, resulting in significant improvements in virology, biochemistry and histology. Entecavir (ETV), a nucleoside analogue (NA) approved for treatment of CHB, has been documented as a powerful inhibitor of HBV replication both in vitro and in vivo.3–4 Notably, the resistance rate to ETV treatment is as low as 1.2% within 5 or 6 years in NA-naïve patients.5–6 However, not all patients had complete virological and biochemical responses even without the development of resistance mutations. Some patients may acquire a partial virological response, defined by Keeffe et al7 or the European Association for the Study of the Liver guideline for CHB8 as detectable HBV DNA levels (by PCR) after either
How might it impact on clinical practice in the foreseeable future?

As the evolutionary patterns of HBV QS differ between patients with different responses to nucleoside analogues, HBV QS complexity and diversity may serve as parameters to monitor antiviral treatment in addition to HBV DNA levels. The evolutionary pattern during early stages of antiviral treatment may also predict the virological response, which makes it possible to modify antiviral treatment in advance.

With the rapid development of next-generation sequencing techniques such as Roche 454, determination of QS complexity and diversity can be much easier and can be normalised for diagnosis and monitoring of antiviral therapy.

The potential new mechanism of antiviral resistance may suggest a possible new strategy of antiviral therapy.

a 24 or 48 week treatment, respectively. For those patients, further interventions may be needed as previously recommended.2

As documented in many studies, HBV exists as quasispecies (QS), owing to a lack of proofreading capacity during reverse transcription and a high replication rate.3 6 QS implies a spectrum of mutants that possess different fitness levels in certain environments. Mutants with higher fitness levels may predominate by competitive replication, although predominant mutants may differ in changing environments.4 The evolution of QS is guided by the fitness gradient of the environment. Other studies of HBV QS, including those performed using ultra-deep pyrosequencing, have focused on the evolution of specific resistance mutations instead of the entire reverse transcriptase (RT) region in which the context of other sequences was neglected. In our previous study, we found that the HBV QS complexity and diversity within the entire RT region in responders were lower than those in non-responders during the early stage of treatment with lamivudine.5 Nevertheless, whether HBV QS evolution shows similar patterns during treatment with other NAs and the differences in HBV QS evolution under different selective pressures are not well defined.

The aim of this study was to investigate the evolution of HBV QS within the RT region during the early stage of ETV treatment and its role in antiviral responses and drug resistance, and, moreover, to compare evolutionary patterns of HBV QS under different selective pressures.

**PATIENTS AND METHODS**

**Patients**

Consecutive patients with CHB who received ETV mono-therapy at our medical centres from February 2007 to March 2009 were included in the present study. There were 60 patients retrospectively selected for screening; 15 patients were excluded because of history of other NA treatments or immunodepressants, 12 patients were excluded because of undetectable HBV DNA levels at week 4, and 4 patients were excluded because of poor compliance (defined as at least one interruption of three continuous doses or seven cumulative doses every 12 weeks).

Written informed consent was obtained from all patients, and the study protocol was approved by the Ethical Committee of Ruijin Hospital in accordance with the Declaration of Helsinki. Patients enrolled in this study met the following inclusion criteria: 18–65 years of age, presence of hepatitis B surface antigen (HBsAg) at least 6 months prior to treatment, elevated serum alanine aminotransferase (ALT) and HBV DNA levels >10^5 copies/ml on two occasions prior to treatment, and no signs of decompensated liver disease (e.g. variceal bleeding, ascites or encephalopathy). Patients were required to be naive to NAs—that is, no history of receiving NAs was found prior to ETV treatment in any enrolled patient.

The exclusion criteria were the presence of antibody to HIV, hepatitis C virus (HCV) or hepatitis D virus and liver disease due to other causes (e.g. autoimmune liver disease, alcoholic hepatitis and drug-induced hepatitis). Patients with poor compliance were also excluded.

Patients were given 0.5 mg/day ETV (Bristol-Myers Squibb, Shanghai, China) for at least 48 weeks and followed up at weeks 4 and 12 and thereafter at 3 month intervals. Antiviral efficacy was evaluated at both week 48 and the final visit according to serum HBV DNA levels by PCR (Cobas Amplicor, Roche Diagnostics, Basel, Switzerland; low limit of quantification: 500 copies/ml; also used in our previous study on lamivudine8). Virological response was defined as undetectable HBV DNA levels at week 48, while partial response was defined as HBV DNA levels of at least 500 copies/ml at week 48 but a decrease of >1 log_{10} copy/ml compared with baseline.

To compare the differences in HBV QS evolution under different selective pressures, a cohort of 25 patients with CHB receiving lamivudine treatment (14 responders and 11 non-responders) included in our previous study was studied as a control. For those patients, the virological responses were also evaluated at week 48. The inclusion and exclusion criteria of that study were the same as those of the present study and were described in detail previously.9

**Liver biochemistry, HBV serology and HBV DNA tests**

Liver biochemical parameters were tested at each visit using an automated chemistry analyser (Beckman Coulter, Fullerton, California, USA). HBV serological markers were determined by chemiluminescent microparticle immunoassay using the Abbot Architect immunoassay system (Abbott Laboratories, Abbott Park, Illinois, USA). The HBV DNA levels were measured by PCR using the Cobas Amplicor HBV Monitor Test (Roche Diagnostics) with a low limit of quantification of 300 copies/ml.

**Molecular cloning and sequencing**

HBV genomes were extracted from 200 μl serum samples at both baseline and week 4 using the QIAamp blood mini-kit (QIAGEN, Hilden, Germany). For samples with low HBV DNA titre (<10^6 copies/ml), the QIAamp ultrasens virus kit (QIAGEN) was used to extract HBV genomes from 1 ml of serum. The HBV RT region was amplified using PCR; nested PCR was preferred if serum HBV DNA levels were <10 000 copies/ml, as previously described.9 AccuPrime Pfx SuperMix (Invitrogen, Carlsbad, California, USA) was used to ensure the high-fidelity PCR. PCR products of 1096 bp were purified using the QIAquick Gel extraction kit (QIAGEN), cloned into the pGEM-T vector after the addition of adenylate tails (Promega, Madison, Wisconsin, USA) and transformed into TOP 10 Escherichia coli competent cells (Invitrogen) growing on ampicillin plates. An average of 26 (range: 22–50) clones per sample were sequenced randomly using an ABI 3730 automated sequencer (Applied Biosystems, Foster City, California, USA). Totals of 1694 and 1198 clones were sequenced in the ETV and lamivudine groups, respectively.
### Table 1: Demographic and clinical features

<table>
<thead>
<tr>
<th></th>
<th>Responders (n=17)</th>
<th>Partial responders (n=14)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>38.4±2.8</td>
<td>38.9±2.9</td>
<td>0.892</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>14/3</td>
<td>12/2</td>
<td>1.000</td>
</tr>
<tr>
<td>HBeAg (+/−)</td>
<td>17/0</td>
<td>14/0</td>
<td></td>
</tr>
<tr>
<td>Genotype (B/C)</td>
<td>4/13</td>
<td>8/6</td>
<td>0.075</td>
</tr>
<tr>
<td>ALT (IU/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>122 (52−315)</td>
<td>106 (61−319)</td>
<td>0.634</td>
</tr>
<tr>
<td>Week 4</td>
<td>72 (17−431)</td>
<td>73.5 (27−215)</td>
<td>0.889</td>
</tr>
<tr>
<td>HBV DNA (log_{10} copies/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>8.32±0.19</td>
<td>8.44±0.20</td>
<td>0.532</td>
</tr>
<tr>
<td>Week 4</td>
<td>4.12±0.15</td>
<td>5.07±0.19</td>
<td>0.004</td>
</tr>
<tr>
<td>Follow-up time (months)</td>
<td>29.5 (12−37)</td>
<td>29.0 (16−38)</td>
<td>0.937</td>
</tr>
</tbody>
</table>

ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus.

### Sequence analysis

Multiple alignments were carried out on all sequences to remove gaps using CLUSTAL X, version 2.0.11 RDP3 software was used to detect recombinant sequences, which were excluded.12 Genotypes of each sequence were determined using the HBV STAR program online.13

Viral QS heterogeneity was evaluated with two parameters: complexity and diversity. QS complexity, which means the number of variants identified in a single sample, was measured using normalised Shannon entropy (Sn) as previously described.14 Sn was calculated at both the nucleotide and amino acid levels according to the following formula:

\[ Sn = -\sum (p_i \ln p_i) / \ln N \]

where \( p_i \) represents the frequency of each clone in the QS population and \( N \) represents the total number of clones. QS diversity was evaluated by three parameters: the mean genetic distance (\( d \), also called Hamming distance), the number of synonymous substitutions per synonymous site (\( dS \)) and the number of non-synonymous substitutions per non-synonymous site (\( dN \)).

The evolutionary rate during the first 4 weeks of ETV treatment was calculated using PEBBLE 1.0 software and the serial sample unweighted pair group method with arithmetic mean (sUPGMA) algorithm under the appropriate substitution model for each patient, which was also estimated by ModelTest.21

### Statistical analysis

Results are expressed as either the mean±SE or the median and range. Variables between the responder and partial responder groups were compared using the Student t test, the Mann–Whitney test and the Fisher exact test. Frequency variables were compared by the Fisher exact test. Correlations between virological response and group variables were analysed using Spearman rank correlation. Logistic regression was performed to develop the predictive model. Results were considered statistically significant at \( p <0.05 \).

### RESULTS

#### Demographic, clinical and laboratory data

Thirty-one patients with CHB were enrolled in the present study. They had a median age of 38.6 years (range: 20–65 years), there were 26 males and 5 females, and all were hepatitis B e antigen (HBeAg) positive and followed up for a median duration of 29 months (mean: 28 months). Based on serum HBV DNA levels at week 48, the 31 patients were classified into the responder (n=17) and the partial responder groups (n=14). The demographic and clinical features at both baseline and week 4 are shown in table 1. Age, gender, genotype, HBeAg status, ALT levels and HBV DNA levels in responders and partial responders were comparable at baseline (p >0.05). ALT levels in both groups of HBV QS during the first 4 weeks of ETV treatment and was calculated using MEGA 4.0 software.

PHYML version 3.0 was used to reconstruct phylogenetic trees for baseline and week 4 using the maximum likelihood method under the general time reversible + proportion of invariant sites + shape parameter of the \( \gamma \) distribution (GTR + I + \( \Gamma \)) model, which was estimated by ModelTest 3.7 in advance, as previously described.19 20 Relative rates of nucleotide substitution (six categories), the proportion of invariant sites (I) and the shape parameter of the \( \gamma \) distribution (\( \Gamma \)) were also estimated using ModelTest. The viral evolutionary rate during the first 4 weeks was calculated using PEBBLE 1.0 software and the serial sample unweighted pair group method with arithmetic mean (sUPGMA) algorithm under the appropriate substitution model for each patient, which was also estimated by ModelTest.21

#### Figure 1

Hepatitis B virus (HBV) DNA kinetics of responders (A) and partial responders (B) during entecavir treatment. HBV DNA levels of all responders were undetectable until the final visit (mean: 28 months), while those of partial responders were still detectable at the final visit, although lower than at both baseline and week 48.
showed no differences at week 4, but HBV DNA levels of responders were lower than those of partial responders (4.12 ± 0.60 log_{10} copies/ml vs 5.07 ± 0.19 log_{10} copies/ml, \(p = 0.004\)). As all responders at week 48 maintained undetectable HBV DNA levels until the final visit, the long-term virological responses in this cohort of patients were the same as at week 48.

**HBV DNA kinetics and resistance-related mutations**

The kinetics of HBV DNA levels in both groups are shown in figure 1. Serum HBV DNA levels decreased dramatically during the first 4 weeks and gradually thereafter. HBV DNA levels of all responders were undetectable until the final visit (a mean treatment duration of 28.7 months), and three responders experienced HBeAg seroconversion (2 patients at week 72 and 1 patient at week 120). However, the HBV DNA levels of most partial responders fluctuated widely after a rapid decrease at 4 weeks. Seven patients developed virological breakthroughs (defined as an increase in HBV DNA levels of >1 log_{10} copy/ml compared with the nadir) before week 48. All partial responders had detectable HBV DNA levels until the final visit. HBV DNA levels of most partial responders (12/14) ranged from 10^3 to 10^4 copies/ml at the final visit, and the highest level was 19,400 copies/ml. None of the partial responders experienced HBeAg seroconversion. Although there were seven patients who developed virological breakthroughs before week 48, no resistance-related mutations were detected using direct sequencing of PCR products during follow-up. Additionally, no mutants harbouring triple mutations (rt180, rt204 and any of rt184, rt202 or rt250) were found by cloning analysis at both baseline and week 4.

![Figure 2](image)

**Figure 2** Hepatitis B virus (HBV) quasispecies (QS) complexity at both baseline and week 4 (A and B). Despite comparable complexities of both groups at baseline (\(p > 0.05\)), QS complexity in responders at week 4 was statistically lower than that in partial responders at the nucleotide level (\(p = 0.039\)). Additionally, the complexity of responders at week 4 was lower than that at baseline (\(p = 0.002\)). However, the complexities of both groups at the amino acid level showed no differences (\(p > 0.05\)).
Despite the occurrence of virological breakthroughs, no biochemical breakthroughs were observed during follow-up. At week 48 the median ALT level was 26 IU/ml (range: 12–70 IU/ml) and at the final visit it was 22 IU/ml (range: 11–50 IU/ml), and no signs of decompensated liver disease were found during follow-up.

**HBV QS complexity and diversity at both baseline and week 4**

At baseline, the QS complexities of responders were comparable with those of partial responders, at both the nucleotide and amino acid levels (p >0.05). However, at week 4, the QS complexity of responders was lower than that of partial responders at the nucleotide level (0.6494 vs 0.7723, p=0.039) but was similar at the amino acid level (0.4524 vs 0.5083, p=0.408; table 2 and figure 2).

QS diversity was also calculated for each patient at both baseline and week 4. The mean genetic distance (d), the number of synonymous substitutions per synonymous site (dS) and the number of non-synonymous substitutions per non-synonymous site (dN) did not differ statistically between responders and partial responders at both baseline and week 4, at either the nucleotide or the amino acid level (p >0.05).

**Phylogenetic analysis and viral nucleotide substitution rate**

The phylogenetic trees were reconstructed under the maximum likelihood algorithm for both baseline and week 4. An RT region of HBV genotype D (accession no. AB033559) served as the outgroup sequence. The phylogenetic trees are shown in figure 3. Sequences from a single patient clustered together as expected. The mean branch lengths of both groups at baseline, however, could not be calculated concisely and appear similar. Nevertheless, at week 4, the mean branch lengths of responders appeared shorter than those of partial responders, particularly in genotype B patients.

![Figure 3](image_url)
Viral nucleotide substitution rates during the first 4 weeks of ETV treatment were calculated for each patient. The median nucleotide substitution rate of responders (3.80 $\pm$ 10$^{-4}$/C0$^4$ (1 $\pm$ 10$^{-5}$/C0$^8$e$^{1.55}$ $\pm$ 10$^{-4}$/C0$^3$)) substitution/site/day) was higher than that of partial responders (2.13 $\pm$ 10$^{-5}$/C0$^5$ (1 $\pm$ 10$^{-5}$/C0$^8$e$^{7.66}$ $\pm$ 10$^{-4}$/C0$^4$)) substitution/site/day, p = 0.012).

Evolutionary patterns of HBV QS and HBV DNA levels in responders and partial responders
To investigate the evolutionary patterns of HBV QS, the average changes in QS complexity and diversity between both baseline and week 4 were calculated; in addition, the net changes in QS diversity, which reflect diversity changes in the context of sequence differences between the two QS populations, were also calculated.

The average changes in QS complexity showed distinct patterns of the responders and partial responders (figure 4). QS complexity of responders represented a decreased trend (a negative value), while that of partial responders represented an increased trend (a positive value). The average changes in QS complexity of responders were lower than those of partial responders, although the changes did not reach statistical significance at the amino acid level (p = 0.009 at the nucleotide level and 0.155 at the amino acid level).

The average changes in diversity, including d, dS, and dN, are shown in figure 5A. Analogous to the similar QS diversities at both baseline and week 4, the average changes in QS diversity of both groups were comparable (p > 0.05). However, net changes in QS diversity, which indicate the extent of clustered evolution under selective pressure, were statistically different between responders and partial responders (p < 0.01). The net changes in d, dS, and dN of the responders group were higher than those of the partial responder group, suggesting that the QS of the responders had a more significant change in heterogeneity (figure 5B).

High net changes in HBV QS diversities correlate with virological responses at both week 48 and the final visit
Spearman rank correlation analysis revealed a positive correlation between net changes in QS diversities and virological responses at both week 48 and the final visit, while average changes in QS diversity had no correlation with virological responses. Average changes in QS complexity (nucleotide level) correlated with virological responses at both week 48 and the final visit (table 5). Furthermore, the viral nucleotide substitution rate during the first 4 weeks correlated with virological responses at both week 48 and the final visit (r = -0.453, p = 0.010).

To explore a potential model to distinguish partial responders from responders, a dot histogram of selected variables was plotted in figure 6. Net changes in dS between baseline and week 4 were able to identify 11 of 14 partial responders and only 16 of 17 responders. HBV DNA levels at week 4, which may serve to predict long-term outcomes after lamivudine treatment as...
DISCUSSION

The characteristics of HBV QS evolution during the early stage of ETV treatment were shown to be quite different between responders and partial responders in the present study. The QS complexity of responders was significantly lower than that of the partial responders at week 4, although they are comparable at baseline. Additionally, net changes in QS diversities represented by genetic distance, dS and dN of responders were higher than those of the partial responders. The substitution rate of lamivudine (data shown in the supplementary file). The changes in HBV QS of the various groups are shown in table 4.

Despite the comparable QS complexities and diversities between the ETV and lamivudine groups at baseline, responders receiving lamivudine showed quite a different evolutionary pattern from responders receiving ETV. Both HBV QS complexity and diversity of responders in the lamivudine group were lower than those in the ETV group (p<0.01).

However, similar evolutionary patterns were found in partial responders receiving ETV and non-responders receiving lamivudine. HBV QS complexity and diversity of partial responders in the ETV group were comparable with those of non-responders in the lamivudine group at baseline and remained similar to those of patients in the lamivudine group after a 4 week antiviral treatment (p>0.05).

### Table 3  Correlation between quasispecies changes and virological response at week 48 and at the final visit

<table>
<thead>
<tr>
<th>Virological response</th>
<th>Average changes of complexity</th>
<th>Average changes of diversity</th>
<th>Net changes of diversity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nt (dnt)</td>
<td>aa (d(aa))</td>
<td>dS</td>
</tr>
<tr>
<td>Week 48 and final visit</td>
<td>r = 0.493*</td>
<td>p = 0.005</td>
<td>dN = 0.500</td>
</tr>
</tbody>
</table>

*Correlation is significant at the 0.01 level two-tailed.

aa, amino acid; d, mean genetic distance; dN, the number of synonymous substitutions per synonymous site; dS, the number of synonymous substitutions per synonymous site; nt, nucleotide.

Figure 6  Dot histogram of selected variables, representing quasispecies (QS) evolutionary patterns. Complexity at the nucleotide level at week 4 (C(nt)4W), average changes in QS complexity at the nucleotide level (ΔC(nt)) and net changes in number of synonymous substitutions per synonymous site (ΔS) between baseline and week 4 (NetΔS) were plotted. Hepatitis B virus DNA levels at week 4 (HBV DNA4W) were also plotted for comparison. NetΔS had a higher specificity (16/17, 94.1%), while HBV DNA4W had a higher sensitivity (13/14, 92.8%), and thus complementary to each other.

Hepatitis B virus DNA levels at week 4 (HBV DNA4W) were also plotted for comparison. NetΔS had a higher specificity (16/17, 94.1%), while HBV DNA4W had a higher sensitivity (13/14, 92.8%), and thus complementary to each other.
responders was also higher than that of the partial responders. Moreover, HBV QS complexity at the nucleotide level decreased during the first 4 weeks in responders, while it increased in partial responders. Spearman rank correlation analysis indicated that the net changes in QS diversities during the first 4 weeks correlated with virological responses at both 48 weeks and the final visit (mean: 28 months).

In contrast to the rare reports on HBV QS evolution during NA treatment, the correlation between HCV QS heterogeneity and antiviral response has been well established in the last decade. Hino et al reported the lower complexity of the hyper-variable region 1 QS predicted response to interferon treatment in a prospective study involving 55 patients with chronic hepatitis C. Similar results were reported in several studies thereafter. However, Fan et al recently reported higher dN and complexities of HCV QS at baseline associated with early virological responses in 153 patients receiving interferon and ribavirin. Besides the different sample sizes involved in those studies, the techniques adopted to assess QS complexity and diversity may be responsible for the contradictory results. Gel-based methods, such as single-stranded conformation polymorphism and heteroduplex complexity assay, can determine only the number of different mutants, while cloning/sequencing can determine both the number and the frequency of the mutants, and the corresponding parameter, Sn, is better than the number of mutants for describing QS complexity. For this reason, cloning/sequencing is thought to be the gold standard technique to assess viral complexity and diversity. In addition, ultra-deep pyrosequencing was not preferred in the present study because of its short read length (~300 bp, much shorter than our target sequence of 1032 bp). However, further investigation based on this technique is being performed in our group to explore its potential application in the clinical setting.

Similar but not identical patterns of change were found between the ETV and lamivudine groups. Both the complexity and diversity of HBV QS within the RT region were comparable between responders and partial responders/non-responders at baseline in our study, suggesting that the QS characteristics of patients with CHB at baseline were unrelated to virological responses in this cohort of patients, which were quite different from those of patients with chronic HCV infection receiving interferon and ribavirin. These differences may due to the biological features of HBV and HCV, the status of the diseases and the pharmaceutical actions of the different treatments.

Responders receiving either ETV or lamivudine showed decreased QS complexity during the first 4 weeks of treatment, and the decrements were statistically significant compared with the partial responders. This pattern of change resulted from selective pressures of host immunity and antiviral drugs; the latter played a key role in the early stage of antiviral treatment. For responders, HBV QS were purified by the selective pressure that was the main driving force of evolution. In this case, HBV QS became less complicated with weaker adaptability, which was represented by lower HBV DNA levels at week 4. Another reason for the decreased QS complexity is the duality of HBV genomic forms—that is, covalently closed circular DNA (cccDNA) and relaxed circular DNA (rcDNA). The rcDNA QS in serum derived from cccDNA QS in the nuclei of infected cells and the new mutations generated during replication increase the complexity of rcDNA QS. The cccDNA QS are relatively stable and cannot be affected seriously in 4 weeks. Since HBV replication was inhibited more severely in responders, fewer generated mutations resulted in lower QS complexity.

However, the diversity of responders to ETV was higher than that of responders to lamivudine. The following reasons may account for this difference. First, ETV has been documented to be more potent against HBV than lamivudine; greater potency means higher selective pressure for HBV QS evolution, leading to a higher evolution rate. Secondly, ETV has a higher genetic barrier to drug resistance that can be calculated by the number of primary resistance mutations. Thus, more HBV QS mutants were blocked by the higher barrier in responders, resulting in higher QS complexity and diversity. Therefore, the diversity and evolution rate of responders to ETV were higher than those of responders to lamivudine.

Despite differences between responders receiving ETV and those receiving lamivudine, partial responders and non-responders at week 48 were so similar that no differences were found in any variable. It was noted that the complexities of partial responders and non-responders increased during the first 4 weeks of treatment. Higher complexity means a broader and more complex mutant spectrum. Viral QS are interactive variants rather than a collection of diverse mutants, which together contribute to the characteristics of the population. In this view, viral populations rather than individuals are the targets of evolutionary selection. Recently, increasing evidence indicated that the interplay between different variants within QS could facilitate entry and replication of the virus population. Thus, high QS complexity provides not only a larger reservoir of variants but also more space or complementary interactions among variants, contributing to higher QS adaptability. Consequently, QS with higher fitness under antiviral pressure are more likely to overtake genetic barriers and develop resistance mutants. As for predicting virological responses to NAs, Yuen et al reported a clinical study of 80 patients with CHB receiving lamivudine that indicated that HBV DNA levels at week 4 of lamivudine treatment could predict a 5 year virological response. This conclusion was validated in our study, in both the lamivudine and ETV groups. In addition, we found that the evolutionary pattern of HBV QS (represented by net changes in QS diversity) was a good parameter to distinguish partial responders, working as well as or even better than HBV DNA...

### Table 4 Characteristics of hepatitis B virus quasispecies in different groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Entecavir Responders</th>
<th>Lamivudine Responders</th>
<th>Partial/non-responders</th>
<th>Entecavir</th>
<th>Lamivudine</th>
<th>Partial/non-responders</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complexity</td>
<td></td>
<td></td>
<td></td>
<td>p Value</td>
<td></td>
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<tr>
<td>(nucleotide level)</td>
<td></td>
<td></td>
<td></td>
<td>Baseline</td>
<td>0.7953</td>
<td>0.7087</td>
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<td></td>
<td>Week 4</td>
<td>0.6494</td>
<td>0.3703</td>
<td>&lt;0.001</td>
<td>0.7723</td>
<td>0.6385</td>
</tr>
<tr>
<td>Complexity</td>
<td></td>
<td></td>
<td></td>
<td>Baseline</td>
<td>0.5182</td>
<td>0.5676</td>
</tr>
<tr>
<td>(amino acid level)</td>
<td>Week 4</td>
<td>0.4524</td>
<td>0.1773</td>
<td>0.001</td>
<td>0.5083</td>
<td>0.5456</td>
</tr>
<tr>
<td>d (10^-3 substitutions) (nucleotide level)</td>
<td>Baseline</td>
<td>3.4575</td>
<td>4.1660</td>
<td>0.544</td>
<td>2.9356</td>
<td>3.4090</td>
</tr>
<tr>
<td></td>
<td>Week 4</td>
<td>10.1659</td>
<td>8.8215</td>
<td>&lt;0.001</td>
<td>4.1992</td>
<td>4.6800</td>
</tr>
<tr>
<td>d (10^-3 substitutions) (amino acid level)</td>
<td>Baseline</td>
<td>3.6597</td>
<td>4.4220</td>
<td>0.279</td>
<td>3.4928</td>
<td>5.1690</td>
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<td></td>
<td>Week 4</td>
<td>8.8610</td>
<td>8.8401</td>
<td>&lt;0.001</td>
<td>4.0958</td>
<td>5.8600</td>
</tr>
<tr>
<td>dS (10^-3 substitutions/site)</td>
<td>Baseline</td>
<td>7.1330</td>
<td>7.7840</td>
<td>0.739</td>
<td>5.8483</td>
<td>6.4700</td>
</tr>
<tr>
<td></td>
<td>Week 4</td>
<td>24.2769</td>
<td>1.0300</td>
<td>&lt;0.001</td>
<td>9.1750</td>
<td>9.3340</td>
</tr>
<tr>
<td>dN (10^-3 substitutions/site)</td>
<td>Baseline</td>
<td>1.6915</td>
<td>2.1060</td>
<td>0.399</td>
<td>1.6187</td>
<td>2.1600</td>
</tr>
<tr>
<td></td>
<td>Week 4</td>
<td>4.3096</td>
<td>0.4750</td>
<td>&lt;0.001</td>
<td>1.9042</td>
<td>2.4800</td>
</tr>
</tbody>
</table>

*d*, mean genetic distance; *dS*, the number of non-synonymous substitutions per non-synonymous site; *dN*, the number of synonymous substitutions per synonymous site.
levels. Furthermore, this parameter could identify partial responders who were falsely identified by HBV DNA levels at week 4. A model involving HBV DNA levels at week 4, nucleotide substitution rate and the average change in QS complexity was established to predict virological responses at week 48 and long-term outcome. With higher sensitivity and specificity (both >90%), this preliminary model appears superior to any single variable and was validated in the lamivudine treatment group. The model warrants further modification and validation in a larger cohort before application in clinical practice.

In conclusion, the dynamic changes in HBV QS within the RT region are quite different between responders and partial responders during the early stage of ETV treatment, although the extent is less than for those receiving lamivudine. Net responders during the early stage of ETV treatment, although receiving different NAs may imply a novel mechanism of drug resistance that warrants further investigation.

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Evolutionary patterns of hepatitis B virus quasispecies under different selective pressures: correlation with antiviral efficacy

Feng Liu, Li Chen, De-Min Yu, et al.

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