Comparison of serum hepatitis B virus RNA levels and quasispecies evolution patterns between entecavir and pegylated-interferon mono-treatment in chronic hepatitis B patients

Xiao-qi Yu, Ming-jie Wang, De-min Yu, Pei-zhan Chen, Ming-yu Zhu, Wei Huang, Yue Han, Qi-ming Gong, Xin-xin Zhang

a Research Laboratory of Clinical Virology, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China
b Clinical Research Center, Ruijin Hospital North, Shanghai Jiaotong University School of Medicine, Shanghai, China
c Department of Gastroenterology, Ruijin Hospital North, Shanghai Jiaotong University School of Medicine, Shanghai, China
d Department of Infectious Diseases, Institute of Infectious & Respiratory Diseases, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China

Running Title: HBV RNA levels and QS during antiviral treatment

# Address correspondence to Pr. Xin-xin Zhang, zhangxinxin@rjh.com.cn and Qi-ming Gong, gongqm@hotmail.com.
22 Corresponding authors:

23 Prof. Xin-xin Zhang

24 Research Laboratory of Clinical Virology, Ruijin Hospital, Shanghai Jiaotong University

25 School of Medicine

26 No.197 Ruijin Er Road, Shanghai 200025, China

27 Or Prof. Qi-ming Gong

28 Department of Infectious Diseases, Ruijin Hospital, Shanghai Jiaotong University School of Medicine

30 List of Abbreviations:

31 ALT, alanine aminotransferase; HBV, hepatitis B virus; HBV pgRNA: HBV pregenomic RNA; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; QS, quasispecies; NA, nucleos(t)ide analogue; ETV, entecavir; Peg-IFN, pegylated-interferon.
Abstract:

Hepatitis B virus RNA (HBV RNA) may independently predict virological and serological response. This study aimed to compare dynamic changes in serum HBV RNA levels and its quasispecies evolution patterns between entecavir and pegylated-interferon mono-treatment in chronic hepatitis B patients and to determine its clinical significance during treatment. A TaqMan real-time PCR was used for quantitative analysis. HBV RNA levels were retrospectively determined in serial serum samples from 178 chronic hepatitis B patients who received either entecavir or pegylated-interferon treatment. Both serum HBV DNA and RNA quasispecies were analyzed via next-generation sequencing. Receiver operating characteristics (ROC) analysis was performed to evaluate the prediction value in hepatitis B e antigen (HBeAg) seroconversion of individual biomarkers. Pegylated-interferon treatment induced a stronger decline in HBV RNA levels than entecavir treatment. Serum HBV RNA levels were lower in patients with subsequent HBeAg seroconversion. At baseline, the level of HBV RNA was better than other indicators in predicting HBeAg seroconversion. Moreover, the predictive value of serum HBV RNA levels was better in the entecavir group. Baseline HBV RNA exhibited a significantly higher genetic diversity than HBV DNA, and had a significant decline after 4 weeks of entecavir treatment. Higher baseline genetic diversity may result in a better outcome in pegylated-interferon treated patients. Serum HBV RNA levels showed different decline kinetics, and its quasispecies showed different evolution patterns in entecavir and pegylated-interferon mono-treatment. Taken together, serum HBV RNA may serve as a promising biomarker of HBeAg seroconversion in patients during antiviral treatment.
Keywords: hepatitis B virus (HBV), quasispecies (QS), nucleos(t)ide analogue treatment, entecavir (ETV), Pegylated-interferon (Peg-IFN).

Introduction:

Hepatitis B virus (HBV) infection is a global health problem (1) and can cause liver cirrhosis or hepatocellular carcinoma (HCC) (2). Because of the persistence of covalently closed circular DNA (cccDNA) as HBV transcriptional template (3), the majority of chronic hepatitis B patients need lifelong therapy to prevent the recurrence of disease activity (4). Despite long-term antiviral treatment, only a few patients can achieve the loss of hepatitis B surface antigen (HBsAg), which is considered as functional cure (5); therefore, non-cirrhotic patients who achieve hepatitis B e antigen (HBeAg) seroconversion that marks a partial immune control and long-term virological suppression may allow treatment withdrawal (6).

Currently, there are two kinds of antiviral treatment to prevent disease progression: nucleos(t)ide analogue (NA) and pegylated-interferon (Peg-IFN) (7). Peg-IFN is an immunomodulator and has a weak direct antiviral effect. In contrast, NA blocks reverse transcription, resulting in rapid and effective suppression of HBV DNA to levels below detection, but does not affect the HBV pregenomic RNA (pgRNA) formation (8).

Host and viral markers including alanine aminotransferase (ALT), HBV DNA, HBsAg, and HBeAg have been confirmed to be associated with response (9), but still have limitations in identification accuracy and prediction value. HBV RNA is a replicative intermediate and may serve as a marker of HBV infection. Recent studies show that HBV...
RNA detected in serum is mainly encapsidated pregenomic RNA and has many potential clinical significance (10), such as predicting treatment response and guiding discontinuation (11). Quasispecies (QS) analysis also indicates that serum HBV RNA is genetically homogenous to intrahepatic HBV RNA (12). However, the kinetics of its decline and the evolution patterns of HBV RNA QS induced by two different kinds of treatment are still not very clear. In this study, we aimed to compare the decline mode of serum of HBV RNA levels and its relation to HBeAg seroconversion, and to investigate the QS evolution patterns of both HBV RNA and HBV DNA quasispecies during the early stage of entecavir (ETV) and Peg-IFN treatment groups.

Materials and Methods:

CHB patients receiving long-term antiviral treatment

178 CHB patients were retrospectively enrolled from Ruijin Hospital (Shanghai, China) between 2008 and 2018, among which 122 were treated with entecavir (ETV) as mono-treatment for > 1 year and 56 received 1-year Peg-IFN treatment. HBeAg positive patients were divided into two outcome subgroups depending on whether they achieved HBeAg seroconversion within 48 weeks of treatment. Serum samples were collected on the day of therapy initiation (baseline), and on week 4, week 12, week 24, week 48. All samples were stored in -20°C until assayed. The study was approved by the Ethics Committee of Ruijin Hospital in accordance with the Helsinki Declaration.

Standard laboratory assessments

Blood biochemical parameters, including ALT, were measured using an automated chemistry analyzer (Beckman Coulter). Serum HBV DNA levels were quantified using
real-time PCR (Shanghai KEHUA Bio-engineering Co., LTD.) with a lower limit of detection of 500 IU/mL. Serum quantitative HBsAg levels and presence of HBeAg and anti-HBe were measured using the Abbott Architect immunoassay system (Abbott Laboratories). HBV genotypes were determined by direct sequencing of the preS/S gene and nucleotide divergence analysis with the reference sequences in GenBank (NCBI) data.

**Extraction of viral nucleic acids**

Total nucleic acids were isolated from 140 μL serum using the TIANamp virus DNA/RNA kit (Tiangen), following the manufacturer’s protocol and obtained in 60μL DEPC-treated water. An internal control was added to each sample as a control for nucleic acid extraction to exclude false-negative results. Isolated RNA was then treated with DNase I (Ambion).

**Quantification of HBV RNA in serum**

To ensure that no HBV DNA was measured, qPCR was performed in parallel with a digestion control. DNase-treated RNA was reverse transcribed and then amplified using a TaqMan real-time PCR technique with specific HBV primers according to the manufacturer’s instructions (PerkinElmer). The sequences of primers are as follows (Fig. S2): HBV RNA-FW: 5’-AGACCACCAATGCCCTGCT-3’ and HBV RNA-RV: 5’-AGGCGAGGAGTCTCTTCTCTA-3’. The experiment was performed using the following protocol: one cycle at 37°C for 2 min, 50°C for 5 min, 42°C for 20 min and 94°C for 10 min, 40 cycles at 94°C for 15 sec and 62°C for 45 sec. The lower limit of detection (LLD) is 200 (2.3 log_{10}) copies/mL. For statistical analysis, quantification results below the LLD were adjusted to 2.3 log_{10} copies/mL.
Next-generation sequencing

DNase-treated RNA was reverse transcribed using PrimeScript RT Master Mix (TAKARA BIO INC). The target region of HBV RNA and HBV DNA were amplified using barcoded pair of primers (Fig. S2): HBV QS-FW: 5'-AGACGAAGGTCTCAATCGCC-3' (nt 2393 to 2412) and HBV QS-RV: 5'-GTTCCCAAGAATATGGTGACC-3' (nt 2814 to 2835). The PCR reaction mixture (50 μL) contained 25 μL of PrimeSTAR HS Premix (TAKARA BIO INC), 1 μL of forward primer (10 μM), 1 μL of reverse primer (10 μM), 5 μL of DNA or cDNA template, and 18 μL double distilled water. The PCR cycling conditions were as follows: 95°C for 5 minutes, 35 cycles at 95°C for 15 seconds, 56°C for 30 seconds, and 72°C for 30 seconds, with a final extension of 72°C for 6 minutes. Deep sequencing of the PCR products was performed using an Illumina Miseq platform, according to the manufacturer’s PE 2 × 300 bp protocol.

QS sequence data analysis

The nature of the viral population was characterized by viral genetic complexity and diversity using data from the two methods. The QS complexity, known as the Shannon entropy (Sn), was defined as the proportions of different genome sequences in a mutant distribution. Possible values of Sn range from 0 (when all sequences are identical) to 1 (when each sequence is unique). Sn can be calculated with the formula: Sn = \(-\sum_{i}(p_i \ln p_i)/\ln N\), where N is the total number of clones and pi is the frequency of each clone in the viral QS population. The QS diversity, which was defined as the number of mutations that distinguished any two sequences from the population, was evaluated by the mean genetic distance (d, also called Hamming distance). Estimation of mean genetic
distance was conducted using the Tamura 3-parameter model. Before down-stream analysis, error correction was carried out by using previously published software (13, 14).

All parameters were calculated using MEGA 7.0 software.

**Statistical analyses**

HBV DNA, HBsAg, HBeAg, and HBV RNA data were log$_{10}$-transformed prior to analyses. The values were compared by Student’s t-tests or the Mann-Whitney test as appropriate. To assess the distribution of HBV genotypes in different patient groups, a chi-square test ($\chi^2$) was applied. Logistic regression was used to identify factors associated with HBeAg seroconversion. Receiver operating characteristics (ROC) analysis was performed to evaluate the prediction value in HBeAg seroconversion. The area under ROC curve (AUROC) was calculated to compare different variables. Optimal cut-off values were obtained from Youden’s index. The sensitivity, specificity, positive predictive value, and negative predictive value were calculated at the optimal cut-off value for each biomarker.

Graphs were plotted using GraphPad Prism 7.0. Statistical software used included SPSS 24.0 and MedCalc 15.2.2. A two-sided $P$ value of less than .05 was considered statistically significant.

**Data Availability**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Results**

**Demographics, clinical and laboratory data**

Study population characteristics are shown in Table 1. Overall, 178 CHB patients were
enrolled in this study. The mean age was 37.8 years, 127 were male and 51 were female patients. HBV genotype distribution was 59 (35%) for B and 110 (65%) for C. Among them 122 patients were treated with ETV (72 were HBeAg positive) and 56 were treated with Peg-IFN (44 were HBeAg positive). No difference were found in HBV genotype, the mean levels of ALT, HBsAg, HBeAg, HBV DNA, and HBV RNA between the two treatment groups at baseline.

Sequential changes in serum HBV RNA levels during ETV versus Peg-IFN treatment

Before treatment, serum HBV RNA levels, as well as HBV DNA and HBsAg levels showed no difference between ETV and Peg-IFN treated groups. During 48 weeks of treatment, serum HBV RNA could still be detected even when HBV DNA levels were below the LLD in some patients in both treatment groups (Fig. 1A). In the Peg-IFN treatment group, 65.5% patients had undetectable HBV DNA levels, and the proportion of patients with HBV RNA levels below the LLD was 31.0% at week 48. While in the ETV treatment group, 90.0% patients had undetectable HBV DNA levels, but only 18.0% patients had HBV RNA levels below the LLD at week 48.

ETV showed more powerful suppression on HBV DNA levels than Peg-IFN at all time points during treatment. However, the inhibition on HBV RNA levels is less efficient in ETV treated patients than in Peg-IFN treated patients; thus, HBV RNA levels remained higher than HBV DNA levels at all time points during ETV treatment, whereas HBV DNA levels could be either higher or lower than HBV RNA levels in Peg-IFN treated patients (Fig. 1B). Peg-IFN induced a stronger decrease in HBV RNA levels from baseline to week 4 (1.28 versus 0.54 copies/mL), to week 24 (2.42 versus 1.73 copies/mL), and to
week 48 (2.92 versus 2.11 copies/mL) in comparison with ETV mono-treatment (Fig. S1A). A significant difference was also found in the reduction of HBsAg levels between the two treatment groups at week 48 post-treatment. HBsAg levels decreased by only 0.42 log_{10} copies/mL from baseline in the ETV treatment group and by 0.89 log_{10} copies/mL in the Peg-IFN treatment group at week 48 (Fig. S1C).

**Serum HBV RNA levels in relation to HBeAg seroconversion**

The mean serum HBV RNA level was 6.41 ± 1.86 copies/mL at baseline, which was higher in HBeAg-positive patients by 7.28 ± 1.48 copies/mL than in HBeAg-negative patients by 4.75 ± 1.32 copies/mL (p < 0.01). HBV RNA levels declined continuously, and remained higher in HBeAg-positive patients than in HBeAg-negative patients at all subsequent time points in both treatment groups (p < 0.05). The proportions of patients who achieved HBeAg seroconversion were (11/72) 15.3% in the ETV treatment group and (16/44) 36.3% in the Peg-IFN treatment group, respectively.

No difference was found in baseline levels of HBV DNA and HBsAg in patients with or without subsequent HBeAg seroconversion (data not shown). ETV treated patients with subsequent HBeAg seroconversion had significantly lower HBV RNA levels at baseline than those who did not achieve HBeAg seroconversion (Fig. 2A), and a stronger decline was observed in the HBeAg seroconversion subgroup than in the no seroconversion subgroup at all time points. In the Peg-IFN treatment group, patients with subsequent HBeAg seroconversion also had lower HBV RNA levels at baseline and during treatment than those without subsequent seroconversion, but a significant difference was only observed at week 24 (Fig 2B). In HBeAg negative patients, a marked decline was also observed in HBV RNA levels during treatment.
Prediction of HBeAg seroconversion by serum HBV RNA levels

Lower levels of HBsAg, HBV DNA, and HBV RNA at baseline were correlated with HBeAg seroconversion in all 178 patients by univariable logistic regression, while age, gender, ALT level, and HBV genotype were not associated with HBeAg seroconversion. In multivariable analysis, only baseline HBV RNA levels was an independent predictor of HBeAg seroconversion (Table 2).

We further performed ROC analysis to evaluate the prediction value of factors associated with HBeAg seroconversion. At baseline, the AUROC of HBV RNA levels were highest in predicting HBeAg seroconversion in both ETV (Fig. 2C) and Peg-IFN (Fig. 2E) groups (AUROC=0.82 and 0.71, respectively). During treatment, serum HBV RNA levels at week 4 had the best accuracy for distinguishing HBeAg seroconversion from no HBeAg seroconversion patients in ETV treatment group (AUROC=0.88, Fig 2D), while in Peg-IFN treatment group, HBV RNA levels at week 24 best predict HBeAg seroconversion (AUROC=0.74, Fig. 2F). The optimal cut-off value of serum HBV RNA levels and the corresponding sensitivity, specificity, positive predictive values, and negative predictive values were calculated (Table 3). The predictive value of serum HBV RNA levels was better in the ETV treatment group than in the Peg-IFN treatment group.

Serum HBV RNA and DNA QS complexity and diversity

Because of the low viral load and other technical reasons, we finally obtained 122 high-quality sequencing data of paired HBV RNA and DNA samples at baseline (ETV: 87 and Peg-IFN: 35). To compare HBV RNA and HBV DNA QS characteristics, both QS complexity and viral genetic distance were calculated. HBV RNA QS complexity and diversity were significantly correlated (r=0.4183, p<0.01), neither complexity nor...
diversity is correlated to quantitative HBV RNA levels (Fig. S3). Overall, baseline quasispecies QS was different between HBV RNA and HBV DNA:

(a) Genetic complexity: No significant difference was found between HBV RNA and HBV DNA QS complexity at baseline (Fig. 3A and 3C). (b) Genetic diversity: The mean genetic distance of baseline HBV RNA was significantly higher than HBV DNA in both treatment groups (Fig. 3B and 3D).

**HBV RNA QS evolution patterns in ETV versus Peg-IFN treatment groups**

At baseline, HBV RNA QS complexity and mean distance showed no difference between ETV and Peg-IFN treatment groups. To investigate the evolution patterns of HBV QS, both QS complexity and viral genetic distance were also calculated at week 4. Overall, the virus quasispecies was successfully amplified and matched QS data were obtained in 48 patients at week 4. (ETV: 36 and Peg-IFN: 12):

(a) Genetic complexity: The QS complexity of HBV RNA represented a significant decreased trend after 4 weeks of treatment in both ETV and Peg-IFN treatment groups (Fig. 3E). (b) Genetic diversity: A significant decline in HBV RNA diversity was only observed in the ETV treatment group at week 4 (Fig. 3F). The diversity of HBV RNA was significantly lower than HBV DNA at week 4 in the ETV treatment group. However, HBV DNA diversity was lower than HBV RNA at week 4 in the Peg-IFN treatment group (Fig. 3D).

Patients in the Peg-IFN treatment group with positive HBV RNA QS amplification results at week 4 had significantly lower baseline mean genetic distance (Fig. 4A); meanwhile, they had significantly higher week 4 HBV RNA levels (Fig. 4B). Therefore, we analyzed the relationship between baseline mean genetic distance and HBV RNA
levels decline. Patients with HBV RNA levels decline greater than 1 log\textsubscript{10} copies/mL after 4 weeks of treatment had higher mean genetic distance, especially in the Peg-IFN treatment group (Fig. 4C). Thus, higher baseline mean genetic distance may indicate rapid HBV RNA levels decline > 1 log\textsubscript{10} copies/mL at week 4 post-treatment in the Peg-IFN treatment group (Fig 4D).

Discussion:

There are five different types of viral RNAs including the 3.5 kb pregenomic RNA (pgRNA) and precore RNA, 2.4 kb and 2.1 kb HBsAg RNAs, and 0.7 kb HBx RNA (15), however, the origin and the form of serum HBV RNA are still unclear, and it is generally accepted that HBV RNA can be released into serum, and the major form is enveloped 3.5 kb pregenomic RNA containing virions (11, 16). Due to the diversity of RNA forms, different methods are developed for HBV RNA quantification; hence, the sequence regions for HBV RNA amplification were also inconsistent (17). Some previous studies used a new rapid amplification of cDNA-ends with PCR (RACE-PCR) that target 3'-ends of HBx gene (18-20). In this study, we used a TaqMan real-time technique, which is also applied by some other research groups (11, 21-23), with specific primers target on the unique sequence of 3.5 kb long HBV RNA. Different amplification regions for full-length or truncated HBV RNA fragments may result in different quantification results, so further evidence is needed to prove the consistency among these methods.

Peg-IFN has both immunomodulatory and weak direct antiviral actions to control the virus, while NAs are direct inhibitors of HBV polymerase at the reverse transcription step of HBV replication (24). Previous studies showed the kinetics of HBV RNA levels in
NA-based (16) or Peg-IFN based treatment (20). In the present study, the kinetics of
HBV RNA was compared between the two kinds of mono-treatment with different
antiviral mechanisms. Because HBV RNA levels are related to the antiviral potency of
NAs (25), we only selected patients receiving ETV mono-treatment in the NA cohort to
eliminate the potential confounders. Our results found that serum HBV RNA levels can
still be detected even when HBV DNA levels were below the LLD, and this phenomenon
is particularly pronounced in the ETV group. ETV treatment induced a weaker decline in
HBV RNA level compared with Peg-IFN treatment, indicating that in contrast to its
strong suppression of HBV DNA levels, the effect of ETV treatment on HBV RNA
levels seems to be limited, since the generation of HBV RNA cannot be inhibited by NA
directly.

In accordance with recent findings, we observed a stronger decline in HBV RNA levels
in HBeAg seroconversion patients (18), and the decline mode in HBeAg negative
patients was similar to that in HBeAg seroconversion patients, suggesting that serum
HBV RNA may reflect the virological and immunological properties of the host. Serum
HBV pgRNA may also represent a marker for the persistence and transcription activity
of HBV cccDNA (26, 27), and due to the persistence of cccDNA, HBV pgRNA
containing virus can be continuously produced. NAs have no effect on the transcription
of HBV cccDNA, therefore, in HBeAg seroconversion patients treated with ETV, serum
HBV RNA levels were significantly lower at baseline and during treatment, indicating a
better virus control. In contrast, serum HBV RNA levels were not significantly different in
patients receiving Peg-IFN with or without subsequent HBeAg seroconversion. Since
Peg-IFN can activate the immune system and have a long-term antiviral effect, which
leads to the degradation of the viral RNA and the exhaustion of HBV cccDNA, Peg-IFN treatment may also reduce HBV RNA levels in non-responders to a certain extent; thus, differences between the two subgroups were not sufficient for statistical analysis. The proportion of patients achieving HBeAg seroconversion was limited even with Peg-IFN treatment (28), therefore, specific indicators or models to predict HBeAg seroconversion are desirable during antiviral treatment. By using antiviral drugs, especially NA with high antiviral activity, HBV replication can be significantly inhibited and serum HBV DNA levels can be rapidly decreased to below the LLD in the majority of patients. Serum HBsAg levels often remain unchanged and its production can be derived from either cccDNA or integrated DNA, so it could persist even after prolonged therapy (29); thus the usefulness of serum HBV DNA and HBsAg levels for prediction is diminished. Some recent findings revealed the utility of serum HBV RNA levels as a novel biomarker to monitor infection and antiviral response. Luo et al. reported that serum HBV RNA levels could predict HBeAg seroconversion during ETV treatment, but the sample size is relatively small (22). Jia et al. found that serum HBV RNA levels might be helpful for predicting HBeAg seroconversion in patients treated with Peg-IFN alone or in combination with adefovir (23). In the present study, we expanded the sample size and compared the prediction value of serum HBV RNA between ETV and Peg-IFN mono-treatment. The results revealed that HBV RNA levels were better predictors for HBeAg seroconversion than baseline HBV DNA and HBsAg levels. Corroborating the previous observations, the absolute HBV RNA levels were superior to the decline from baseline for predicting response (20). The AUROC scores were better in the entecavir treatment group. In this study, high negative predictive value (>90%) at the optimal
cut-off value indicated that HBV RNA levels could help identify non-responders and recommend the consideration of a combination treatment. Therefore, serum HBV RNA during treatment may serve as a potential new biomarker for monitoring antiviral treatment.

Next-generation sequencing technologies allow massive parallel amplification and detection of individual molecules so that they can be used in HBV QS studies (30). Previous studies have investigated the early changes in HBV DNA QS during lamivudine treatment (31) and ETV treatment (32), and found that pretreatment HBV DNA QS heterogeneity may also predict virological outcomes (33). However, the evolution pattern of HBV RNA quasispecies during treatment has not been well characterized, particularly in the early stage. Since the virus reservoir dynamically changes over time and might be constantly replenished by a small amount of ongoing viral replication even under antiviral pressure, it would be interesting to observe the changes in QS pattern of HBV virions from the peripheral blood.

Our results showed that baseline HBV RNA QS diversity was significantly higher than HBV DNA, which we speculate that the virus replicates and evolves actively, but only a portion of packaged pgRNA is reversed transcribed into HBV DNA and released into the peripheral blood. When under antiviral pressure, Peg-IFN treated patients showed quite different QS genetic characteristics from ETV treated patients. It appeared that HBV RNA QS heterogeneity was more likely to reduce during ETV treatment, and viral QS preserves better replicative fitness in the Peg-IFN group. Higher baseline QS diversity may result in better outcomes in Peg-IFN treated patients than in ETV treated patients.

The amplified fragment encodes terminal protein (TP), which serves as a primer when
during reverse transcription and guides the synthesis of minus-strand DNA, and it can also prevent the activation of interferon-induced genes in host cells and inhibit the effect on interferon (34), this may be related to the phenomenon in which the QS diversity of this fragment is more correlated with Peg-IFN treatment response.

In conclusion, we compared the different kinetics of serum HBV RNA levels in two treatment groups and observed a stronger correlation between HBV RNA levels and subsequent HBeAg seroconversion during treatment. Baseline HBV RNA quasispecies diversity is more relevant to the Peg-IFN treatment response, which leads to the hypothesis that higher HBV RNA QS diversity indicates a relatively efficient host immune response. Overall, these findings will help us to better understand the clinical significance of serum HBV RNA, and expand our knowledge of HBV RNA quasispecies evolution patterns in the early stage of ETV and Peg-IFN treatment. We propose that serum HBV RNA may serve as a potential biomarker for predicting HBeAg seroconversion during two different kinds of antiviral treatment.

Conflict of interest:
All other authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Acknowledgements:
This work was supported by grants from the National Natural Science foundation of China (81672069), National Science and Technology Major Project (2017ZX10202202;
2018ZX10302204-001-003), International Collaboration Project of Shanghai Scientific and Technology Commission (16410711900) and Shanghai Shen Kang Hospital Development Center (SHDC12016101).
References


Hepatology 61:66-76.


virus quasispecies during lamivudine treatment and the correlation with antiviral efficacy. J Hepatol 50:895-905.


Figure Legends:

Figure 1. Dynamic changes in serum HBV RNA, HBV DNA, and HBsAg levels. (A) Undetectable rates of serum HBV RNA and HBV DNA during antiviral treatment. Numbers represent the undetectable rate at each time point during treatment. (B) Sequential changes in serum HBV RNA, HBV DNA, and HBsAg levels during ETV or Peg-IFN treatment. The dotted horizontal line represents the lower limit of detection of HBV RNA (2.3 log_{10}) or HBV DNA (2.7 log_{10}). BL: baseline; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; ETV, entecavir; Peg-IFN, pegylated-interferon.

Figure 2. Levels of HBV RNA in relation to HBeAg seroconversion. Box plots of serum HBV RNA levels in patients receiving ETV (A) or Peg-IFN (B) treatment according to hepatitis B e antigen seroconversion (HBeAg SC). The number of patients observed at each time point is given below. The dotted horizontal line represents the lower limit of detection (2.3 log_{10}). Receiver operating characteristics (ROC) curves describe the performance of the prediction of serum baseline biomarkers and HBV RNA levels during treatment in achieving HBeAg seroconversion in ETV treated (C and D) and Peg-IFN treated (E and F) patients. Numbers represent the corresponding AUROC scores. BL: baseline, W4: week 4 post-treatment. W12: week 12 post-treatment. W24: week 24 post-treatment. RNA refers to serum HBV RNA levels. HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; ETV, entecavir; Peg-IFN, pegylated-interferon.

Figure 3. HBV RNA quasispecies evolution pattern. Comparison of hepatitis B virus
RNA and DNA quasispecies complexity and diversity at both baseline and week 4 in ETV treated (A and B) and Peg-IFN treated (C and D) patients. Dynamic changes in HBV RNA quasispecies genetic characteristics in complexity (E) and diversity (F) between baseline and week 4 in both ETV and Peg-IFN treatment groups. Each patient has paired samples at both baseline and week 4. The line of the box indicates the median.

HBV, hepatitis B virus; ETV, entecavir; Peg-IFN, pegylated-interferon.

Figure 4. Relationship between HBV RNA quasispecies and clinical outcome. (A) Baseline QS diversity between different groups. (B) Serum HBV RNA levels at week 4 between different groups. (C) Baseline QS diversity in patients with HBV RNA levels decline either greater than 1 log_{10} copies/mL or less than 1 log_{10} copies/mL after 4 weeks of treatment. (D) Receiver operating characteristics (ROC) curves of baseline genetic mean distance to predict rapid HBV RNA decline $> 1 \log_{10}$ IU/mL after 4 weeks of treatment. W4 QS (+): patients with positive HBV RNA QS amplification results at week 4; W4 QS (-): patients with week 4 negative HBV RNA QS amplification results. HBV, hepatitis B virus; QS, quasispecies.
Table 1. Patient baseline characteristics, by treatment groups and HBeAg status

Data are mean values ± standard deviation.

N.A., not applicable.

a 25 patients without ALT levels at baseline.

b nine HBeAg negative patients without genotype data.

<table>
<thead>
<tr>
<th></th>
<th>All patients (n=178)</th>
<th>(A) ETV treatment (n=122)</th>
<th>(B) Peg-IFN treatment (n=56)</th>
<th>P value (A) vs. (B)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HBeAg positive (n=72)</td>
<td>HBeAg negative (n=50)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>HBeAg positive (n=44)</td>
<td>HBeAg negative (n=12)</td>
<td></td>
</tr>
<tr>
<td>Male/female</td>
<td>127/51</td>
<td>54/18</td>
<td>35/15</td>
<td>27/17</td>
</tr>
<tr>
<td>Age, y</td>
<td>37.8±11.6</td>
<td>35.4±8.9</td>
<td>47.8±12.9</td>
<td>30.8±7.4</td>
</tr>
<tr>
<td>ALT, IU/mL</td>
<td>152.7±147.8a</td>
<td>174.1±189.7</td>
<td>110.8±85.6</td>
<td>172.5±121.9</td>
</tr>
<tr>
<td>HBV genotype b</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>59 (35)</td>
<td>22 (31)</td>
<td>18 (41)</td>
<td>14 (32)</td>
</tr>
<tr>
<td>C</td>
<td>110 (65)</td>
<td>50 (69)</td>
<td>26 (59)</td>
<td>30 (68)</td>
</tr>
<tr>
<td>HBsAg, log10 IU/mL</td>
<td>3.73±0.75</td>
<td>4.16±0.72</td>
<td>3.19±0.44</td>
<td>3.77±0.71</td>
</tr>
<tr>
<td>HBeAg, log10 S/CO</td>
<td>2.51±0.87</td>
<td>2.59±0.88</td>
<td>N.A.</td>
<td>2.38±0.83</td>
</tr>
<tr>
<td>HBV DNA, log10 copies/mL</td>
<td>6.61±1.37</td>
<td>7.36±1.00</td>
<td>5.54±1.17</td>
<td>6.97±1.03</td>
</tr>
<tr>
<td>HBV RNA, log10 copies/mL</td>
<td>6.41±1.86</td>
<td>7.64±1.37</td>
<td>4.79±1.25</td>
<td>6.78±1.39</td>
</tr>
</tbody>
</table>
Table 2. Logistic regression analysis of factors associated with HBeAg seroconversion

<table>
<thead>
<tr>
<th></th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95% CI)</td>
<td>p value</td>
<td>OR (95% CI)</td>
</tr>
<tr>
<td>Age</td>
<td>0.968 (0.92, 1.02)</td>
<td>0.227</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>1.862 (0.76, 4.58)</td>
<td>0.175</td>
<td></td>
</tr>
<tr>
<td>ALT</td>
<td>1.001 (0.99, 1.00)</td>
<td>0.349</td>
<td></td>
</tr>
<tr>
<td>HBV genotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.702 (0.28, 1.73)</td>
<td>0.443</td>
<td></td>
</tr>
<tr>
<td>HBsAg</td>
<td>0.450 (0.25, 0.82)</td>
<td>0.009</td>
<td>0.827 (0.33, 2.07)</td>
</tr>
<tr>
<td>HBV DNA</td>
<td>0.634 (0.43, 0.93)</td>
<td>0.023</td>
<td>1.34 (0.68, 2.63)</td>
</tr>
<tr>
<td>HBV RNA</td>
<td>0.524 (0.38, 0.73)</td>
<td>&lt;0.001</td>
<td>0.479 (0.30, 0.77)</td>
</tr>
</tbody>
</table>

OR: odds ratio; CI: confidence interval.
Table 3. Predictive performance of serum HBV RNA for HBeAg seroconversion in patients receiving ETV and Peg-IFN

<table>
<thead>
<tr>
<th></th>
<th>Cut-off</th>
<th>Se, %</th>
<th>Sp, %</th>
<th>PPV, %</th>
<th>NPV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ETV treated patients</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline RNA</td>
<td>6.67</td>
<td>72.73</td>
<td>82.25</td>
<td>47.1</td>
<td>94.5</td>
</tr>
<tr>
<td>Week 4 RNA</td>
<td>7.37</td>
<td>100.00</td>
<td>66.67</td>
<td>35.1</td>
<td>100.0</td>
</tr>
<tr>
<td>Week 12 RNA</td>
<td>5.84</td>
<td>100.00</td>
<td>62.71</td>
<td>32.6</td>
<td>100.0</td>
</tr>
<tr>
<td>Week 24 RNA</td>
<td>5.38</td>
<td>90.91</td>
<td>63.46</td>
<td>31.0</td>
<td>97.5</td>
</tr>
<tr>
<td><strong>Peg-IFN treated patients</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline RNA</td>
<td>7.23</td>
<td>93.75</td>
<td>57.14</td>
<td>55.6</td>
<td>94.1</td>
</tr>
<tr>
<td>Week 4 RNA</td>
<td>6.47</td>
<td>100.00</td>
<td>38.46</td>
<td>42.9</td>
<td>100.0</td>
</tr>
<tr>
<td>Week 12 RNA</td>
<td>5.72</td>
<td>92.31</td>
<td>40.91</td>
<td>48.0</td>
<td>90.0</td>
</tr>
<tr>
<td>Week 24 RNA</td>
<td>3.77</td>
<td>92.31</td>
<td>59.09</td>
<td>56.3</td>
<td>93.1</td>
</tr>
</tbody>
</table>

Se, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value.