Infection and Drug Resistance

Dovepress
open access to scientific and medical research

Open Access Full Text Article

ORIGINAL RESEARCH

Higher baseline viral diversity correlates with lower HBsAg decline following PEGylated interferon-alpha therapy in patients with HBeAg-positive chronic hepatitis B

This article was published in the following Dove Press journal:
Infection and Drug Resistance

Hu Li
Li Zhang
Hong Ren
Peng Hu

Department of Infectious Diseases, Key Laboratory of Molecular Biology for Infectious Diseases, Ministry of Education, Institute for Viral Hepatitis, The Second Affiliated Hospital, Chongqing Medical University, Chongqing, China

Background: Viral diversity seems to predict treatment outcomes in certain viral infections. The aim of this study was to evaluate the association between baseline intra-patient viral diversity and hepatitis B surface antigen (HBsAg) decline following PEGylated interferon-alpha (Peg-IFN-α) therapy.

Patients and methods: Twenty-six HBeAg-positive patients who were treated with Peg-IFN-α were enrolled. Nested polymerase chain reaction (PCR), cloning, and sequencing of the hepatitis B virus *S gene* were performed on baseline samples, and normalized Shannon entropy (Sn) was calculated as a measure of small hepatitis B surface protein (SHBs) diversity. Multiple regression analysis was used to estimate the association between baseline Sn and HBsAg decline.

Results: Of the 26 patients enrolled in the study, 65.4% were male and 61.5% were infected with hepatitis B virus genotype B. The median HBsAg level at baseline was 4.5 $\log_{10}$ IU/mL (interquartile range: 4.1–4.9) and declined to 3.0 $\log_{10}$ IU/mL (interquartile range: 1.7–3.9) after 48 weeks of Peg-IFN-α treatment. In models adjusted for baseline alanine aminotransferase (ALT) and HBsAg, the adjusted coefficients (95% CI) for ΔHBsAg and relative percentage HBsAg decrease were −1.3 (−2.5, −0.2) $\log_{10}$ IU/mL for higher SHBs diversity (Sn≥0.58) patients and −26.4% (−50.2%, −2.5%) for lower diversity (Sn<0.58) patients. Further analysis showed that the "a" determinant upstream flanking region and the first loop of the "a" determinant (nucleotides 341–359, 371–389, and 381–399) were the main sources of higher SHBs diversity.

Conclusion: Baseline intra-patient SHBs diversity was inverse to HBsAg decline in HBeAg-positive chronic hepatitis B (CHB) patients receiving Peg-IFN-α monotherapy. Also, more sequence variations within the "a" determinant upstream flanking region and the first loop of the "a" determinant were the main sources of the higher SHBs diversity.

Keywords: HBsAg decline, viral diversity, Shannon entropy, interferon, chronic hepatitis B

Introduction

Chronic hepatitis B (CHB) is a serious infectious disease affecting ~250,000,000 people and resulting in an estimated 800,000 deaths annually worldwide.[1] Current CHB treatment guidelines recommend nucleot(s)ide analogs (NAs) or PEGylated interferon (Peg-IFN) therapy for CHB patients; highlighted hepatitis B surface antigen (HBsAg) loss is the ideal end point of treatment.[2–4] However, the HBsAg loss is rare with either NAs or Peg-IFN therapy.

Significantly different treatment-induced HBsAg outcomes can be found in CHB patients with the same disease status.[5,6] A number of studies have demonstrated that

Correspondence: Peng Hu
Department of Infectious Diseases, Institute for Viral Hepatitis, Key Laboratory of Molecular Biology for Infectious Diseases, Ministry of Education, The Second Affiliated Hospital of Chongqing Medical University, 74# Linjiang Road, Yuzhong, Chongqing 400010, China
Tel +86 236 369 3289
Fax +86 236 370 3790
Email hp_cq@163.com

submit your manuscript | www.dovepress.com
Dovepress
http://dx.doi.org/10.2147/IDR.S163765

certain clinical and patient factors are associated with serum HBsAg decline during antiviral therapy, such as HBeAg status, early pronounced HBsAg decline (>1 $\log_{10}$ decline by week 12 or 24), high pretreatment serum interleukin-23 levels, and genotype.[7–9] Patients with genotypes A and D had more pronounced HBsAg decline and higher proportions of HBsAg loss than those with genotypes B and C during NA treatment.[6,10–13] In addition to these aforementioned factors, the nature of small hepatitis B surface protein (SHBs), which is a part of HBsAg, has a significant impact on serum HBsAg levels.[14,15] Xiang et al found that SHBs mutations were negatively correlated with serum HBsAg levels both in vivo and in vitro.[16] Recently, a global ultra-deep sequencing-based genotyping study revealed that mutations in SHBs have an extremely high prevalence and that the majority of these amino acid mutations are found in the hepatitis B virus (HBV) B and C genotype population.[17]

Viral diversity within patients can be assessed using the measurement normalized Shannon entropy (Sn), which takes into account the diversity and frequency of amino acid substitutions at different genomic positions.[18] Previous studies have reported that baseline viral diversity was associated with virological outcomes in hepatitis C virus (HCV),[18–22] human immunodeficiency virus,[23–25] human papillomavirus,[26] cytomegalovirus,[27] and hepatitis B e antigen seroconversion in CHB.[18,28] Although these studies differ in pathogenic agents, medications, and clinical end points, there appears to be a universal feature present in all of the study results: high baseline intra-patient viral diversity is associated with poor treatment outcomes. Therefore, a hypothesis that baseline SHBs diversity may affect HBsAg decline during Peg-IFN therapy was proposed.

To date, little is known about the relationship between baseline SHBs diversity and serum HBsAg level. Thus, the aim of this study was to investigate whether baseline SHBs diversity affects HBsAg decline following Peg-IFN-α monotherapy in HBeAg-positive CHB patients.

Patients and methods

Patients

This was a longitudinal study conducted using prospectively collected serum samples from 26 eligible patients who were HBeAg-positive and treated with Peg-IFN-α monotherapy (180 μg per week) for 48 weeks between May and December 2013 at the outpatient service of the Second Affiliated Hospital, Chongqing Medical University. The inclusion criteria were as follows: age 18–65 years; serum HBsAg-positive for at least 6 months; not been previously treated with NAs or interferon (including conventional or Peg-IFN-α) within 6 months prior to Peg-IFN-α therapy; and completed full therapy course. The exclusion criteria included coinfection with hepatitis A virus, HCV, hepatitis D virus, hepatitis E virus, or HIV; liver cirrhosis, hepatocellular carcinoma; or a history of antiviral therapy within 6 months. This study was approved by the ethical committee of the Second Affiliated Hospital of Chongqing Medical University, and written informed consent was obtained from each study participant.

Variables

HBsAg quantification

Serum HBsAg levels were quantified using the electrochemiluminescence immunoassay Elecsys® HBsAgII (Roche Diagnostic, Mannheim, Germany) according to the manufacturer's instructions. The dynamic range of quantification of this assay is 0.05–52,000 IU/mL. In highly concentrated samples above the upper limit, a further dilution step was necessary to achieve results within the measuring range; these results were later multiplied by the dilution factor. HBsAg loss was defined as HBsAg-positive at baseline and HBsAg-negative at the end of treatment (EOT).

HBV DNA load, HBV markers, and biochemical index

Serum HBV DNA levels were measured using the Roche COBAS® AmpliPrep/COBAS TaqMan HBV test v2.0 (Roche Molecular Diagnostics, Pleasanton, CA, USA). The results were expressed in international units per milliliter (IU/mL). The lower limit of detection of the assay is 20 IU/mL. Serum HBe status was determined using commercial enzyme immunoassays (Roche Diagnostics, Mannheim, Germany) and were expressed as ratios of signal OD to cutoff. Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase were detected using an Automatic Biochemistry Analyzer (Beckman LX-20, Beckman, Brea, CA, USA), with a normal range of 0–40 U/L and 0–50 U/L, respectively.

SHBs diversity

SHBs diversity was assessed at baseline using clone-based sequencing (25–30 clones per patient) and was determined by calculating the Sn using the following formula: $Sn = -\sum_i (pi \ln pi) / \ln N$, where pi is the frequency of each species, and N is the total number of clones analyzed within each subject. The process and performance of this assay was described in detail in our previous study.[29] Briefly, after the extraction of viral DNA from 200 μL of serum using the QIAamp Ultrasens Virus Kit (Qiagen NV, Venlo, the

Netherlands), a nested polymerase chain reaction (PCR) was used to amplify the entire *S gene* fragment. PCR products were then cloned into the pEASY-T5 zero vectors (TransGen Biotech, Beijing, China) and transformed into *Escherichia coli* JM109 competent cells growing on ampicillin plates. Finally, ~25–30 individual clones per sample were randomly chosen for further sequencing. The protein sequences were analyzed using BioEdit 7.2.5 (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and MEGA 7.0.[30]

Viral genotype

The HBV genotypes were determined using the National Center for Biotechnology Information (NCBI) viral genotyping tool (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi).

Study outcomes

The outcomes of this study were ΔHBsAg and relative percentage HBsAg decrease. ΔHBsAg was defined as the difference in HBsAg between baseline and EOT. Relative percentage HBsAg decrease was defined as ΔHBsAg divided by baseline HBsAg.

Statistical analysis

Data are presented as mean ± SD or median (interquartile range [IQR]) for continuous variables and proportions for categorical variables. Continuous variables were compared using Student's *t*-test. Categorical variables were compared using Fisher's exact test. Baseline SHBs diversity (Sn) was tested as both a continuous and a binary variable stratified by the turning point (0.58) obtained through piecewise regression. The kinetics of HBsAg decline was then assessed using mixed models with a random intercept and a random slope per subject and with a covariance structure depending on the different degree of baseline Sn (<0.58 or ≥0.58). To further explore the independent association of baseline Sn and HBsAg decline, multiple linear regression was used to estimate coefficients (95% CIs) with adjustment for baseline level of ALT and HBsAg. Statistical analyses were performed using EmpowerStats (http://www.empowerstats.com, X&Y Solutions, Inc., Boston, MA, USA) and R (http://www.R-project.org, The R Foundation). A 2-sided significance level of 0.05 was used to evaluate statistical significance.

Results

Patient characteristics

Twenty-six HBV-infected HBeAg-positive patients receiving a 48-week course of antiviral therapy with Peg-IFN-α were included in the study. Subjects' characteristics are presented in Table 1. Of the 26 patients evaluated, 17 (65.4%) were male and 9 were female, and the mean (SD) age and body mass index were 37.3 years (5.6) and 21.2 kg/m^2 (2.5), respectively. At baseline, the median HBV DNA level was 8.1 $\log_{10}$ IU/mL (IQR: 7.8–8.5 $\log_{10}$ IU/mL), and the median HBsAg level was 4.5 $\log_{10}$ IU/mL (IQR: 4.1–4.9 $\log_{10}$ IU/mL). At the EOT, the median HBsAg level was 3.0 $\log_{10}$ IU/mL (IQR: 1.7–3.9 $\log_{10}$ IU/mL). Of the entire study population at baseline, 14 patients (53.8%) achieved a >1 $\log_{10}$ IU/mL decline of HBsAg, and 2 (7.7%) achieved HBsAg loss at EOT. After 2 years of follow-up, 2 patients were lost to follow-up and 4 received new treatment regimens. The follow-up of remaining patients was well documented. At 2 years of follow-up, the median HBsAg level was 3.2 $\log_{10}$ IU/mL (IQR: 1.7–3.9 $\log_{10}$ IU/mL), the ΔHBsAg was 1.5 $\log_{10}$ IU/mL (IQR: 0.5–2.6 $\log_{10}$ IU/mL), and the relative percentage of HBsAg decrease at 2 years of follow-up was

Table 1 Demographic, biochemical and virological characteristics of the study population

Characteristics	Total (N=26)
Baseline	
Male, n (%)	17 (65.4)
Age, years, mean (SD)	27.2 (5.6)
BMI, kg/m^2, mean (SD)	21.2 (2.5)
ALT, U/L, median (IQR)	119 (88–167)
AST, U/L, median (IQR)	65 (45–97)
HBV DNA, $\log_{10}$ IU/mL, median (IQR)	8.1 (7.8–8.5)
HBsAg, $\log_{10}$ IU/mL, median (IQR)	4.5 (4.1–4.9)
Genotype	
B	16 (61.5)
C	10 (38.5)
Cirrhosis,[a] n (%)	0
Fibrosis-4 score, n (%)	
<1.45	21 (80.8)
1.45–3.25	5 (19.2)
End of treatment (EOT)	
HBsAg, $\log_{10}$ IU/mL, median (IQR)	3.0 (1.7–3.9)
ΔHBsAg,[b] $\log_{10}$ IU/mL, median (IQR)	1.9 (0.5–2.8)
Relative percentage HBsAg decrease,[c] %	40.0 (10.8–62.9)
HBsAg loss, n (%)	2 (7.7)
Follow-up[d] (2 years)	
HBsAg, $\log_{10}$ IU/mL, median (IQR)	3.2 (1.2–3.8)
ΔHBsAg,[b] $\log_{10}$ IU/mL, median (IQR)	1.5 (0.5–2.6)
Relative percentage HBsAg decrease,[c] %	32.1 (12.0–69.5)

Notes: [a]Cirrhosis was diagnosed by an ultrasound of the liver. [b]ΔHBsAg = HBsAg at baseline – HBsAg at EOT. [c]Relative percentage of HBsAg decrease = ΔHBsAg/HBsAg at baseline. [d]Twenty patients were included in analysis. The 2 patients who were lost of follow-up and 4 who received new treatment regimens were not included.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; IQR, interquartile range.

32.1% (IQR: 12.0%–69.5%). Of the entire study population at 2 years of follow-up, 2 patients achieved HBsAg loss and 1 obtained HBsAg seroconversion.

Non-linear negative relationship between baseline SHBs diversity (Sn) with HBsAg decline at EOT

There was a non-linear negative relationship between baseline SHBs diversity and ΔHBsAg at EOT (Figure 1A). Similar to ΔHBsAg, this relationship was also found between baseline Sn and the relative percentage of HBsAg decrease at EOT (Figure 1B). Next, we took advantage of piecewise regression to examine the threshold effect of baseline SHBs diversity on HBsAg decline. The HBsAg decline degree decreased with the baseline SHBs diversity levels up to the turning point (0.58) ($p<0.01$ for both ΔHBsAg and relative percentage HBsAg decrease). When the SHBs diversity level was ≥0.58, it was not associated with ΔHBsAg and relative percentage HBsAg decrease at EOT (both $p>0.05$). These results indicate that intra-patient SHBs diversity might have different effects on HBsAg decline in patients treated with Peg-IFN-α.

Kinetics of HBsAg decline differ between patients with higher and lower baseline SHBs diversity (Sn)

We further analyzed the change in HBsAg kinetics in patients with different SHBs diversity. The decline in serum HBsAg levels during 48 weeks of treatment with Peg-IFN-α in HBeAg-positive patients is displayed in Figure 2. Both the magnitude of HBsAg decline (mean decline 2.44 versus 1.03 $\log_{10}$ IU/mL, $p=0.02$, Figure 2A) and relative percentage of HBsAg decrease (mean decline 51.6% versus 23.6%, $p=0.03$, Figure 2B) were larger in the patients who had lower SHBs diversity (Sn<0.58) compared with patients who had higher diversity (Sn≥0.58) at EOT. During the Peg-IFN-α treatment, the kinetics of HBsAg decline was also significantly stronger in the lower diversity patients than in higher diversity patients ($p<0.001$ for comparison of the slope of HBsAg decline; Figure 2C). At 2 years of follow-up, the median of serum HBsAg level rose slightly in both groups with higher (n=9) and lower (n=11) baseline SHBs diversity. However, the magnitude of HBsAg decline (mean decline 2.14 versus 0.80 $\log_{10}$ IU/mL) and relative percentage of HBsAg decrease (mean decline 47.9% versus 18.7%) were still markedly stronger in the patients who had lower SHBs diversity compared with patients who had higher diversity.

Multiple linear regression reveals that baseline SHBs diversity affects HBsAg decline

In the crude model, the estimated coefficients of ΔHBsAg and relative percentage of HBsAg decrease comparing subjects with higher baseline Sn to those with lower Sn were −1.4 (−2.5, −0.3) and −28.0 (−51.5, −4.5), respectively (Table 2). The negative association was virtually unchanged in multiple linear regression models after adjusting for baseline ALT and baseline HBsAg (β=−1.3, 95% CI [−2.5 to −0.2], $p=0.04$ for ΔHBsAg; β=−26.4, 95% CI [−50.2 to −2.5], $p=0.04$ for

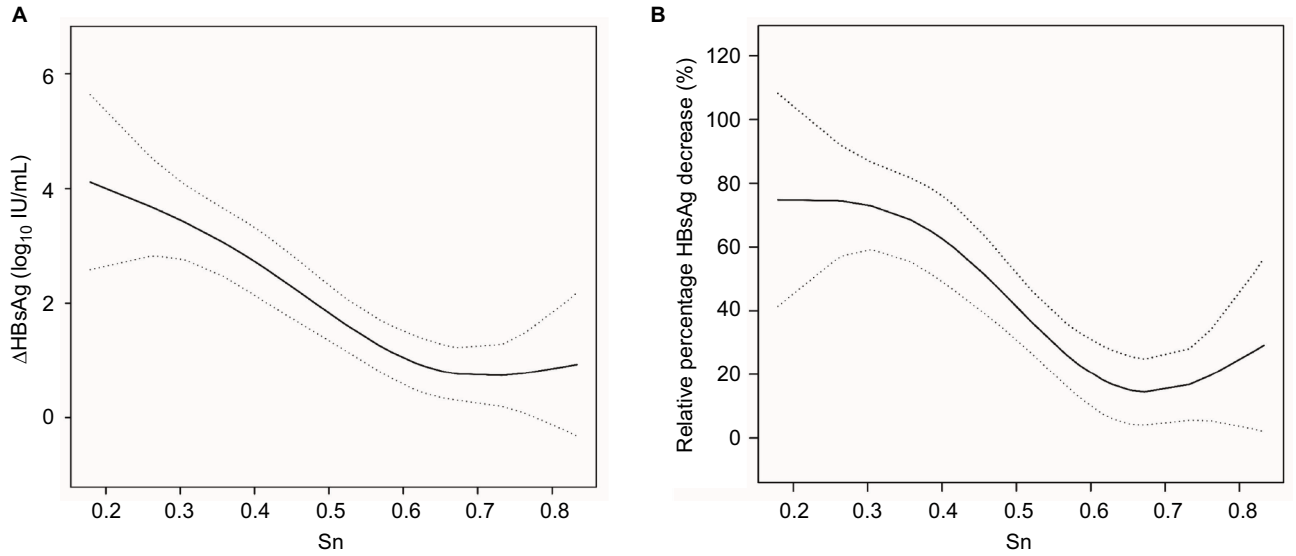

Figure 1 The non-linear negative relationship between baseline SHBs diversity (Sn) and ΔHBsAg (**A**), relative percentage of HBsAg decrease (**B**).
Notes: The black solid line represents the fitted line and the black dotted line represents the 95% CI of the fitted line.
Abbreviations: HBsAg, hepatitis B surface antigen; SHBs, small hepatitis B surface protein; Sn, normalized Shannon entropy.

relative percentage HBsAg decrease; Table 2). Our results suggest that baseline Sn appears to be an independent determinant of the decrease in HBsAg level following Peg-IFN-α therapy.

Sliding window analysis reveals multiple areas account for the differences in baseline Sn

As the aforementioned results demonstrated that SHBs diversity contributed to differences in HBsAg outcomes for Peg-IFN-α therapy, we carried out a sliding window analysis to determine if the Sn difference was driven by specific subregions (Figure 3). The sliding window analysis was used with a window size of 19 base pair (bp) nucleotide length segments migrating every 10 bp spanning the entire small *HBs gene* (678 bp) as described previously.[14] In this analysis, 70.6% (48/68) of the 19-bp segments across the small *HBs gene* had lower mean SHBs diversity in the lower Sn group (<0.58) compared with those in the higher Sn group (≥0.58). Interestingly, differences in mean Sn between the 2 groups exceeded the significant threshold in only 3 regions, nucleotides 341–359, 371–389, and 381–399, corresponding to the "a" determinant upstream flanking region and the first loop of the "a" determinant (Figure 3). In contrast, transmembrane regions (nucleotides 21–84, 240–291, 510–573, and 606–669) were relatively conservative in the 2 groups.

In addition, previous studies reported the significant correlation between amino acid substitutions in HBV surface protein and lower HBsAg synthesis and/or secretion both in vitro and in vivo.[16,31] We conducted a detailed and systematic inventory of the published literature (Table S1). Among these patients, at least 1 related mutation was detected in 16 (61.5%) patients (Figure 4; Table S2). In 14 of 16 (87.5%) patients, the related mutations occurred with an intra-patient prevalence of <35% (mean: 10.3%, range: 3.6%–33.3%; Figure 4), suggesting their fixation in the viral population as a non-predominant species. As expected, the influence on HBsAg decline was not significantly different (Figure 5A and B). The related mutation of sL98V and sT126S was detected in the remaining 2 patients who belonged to dif-

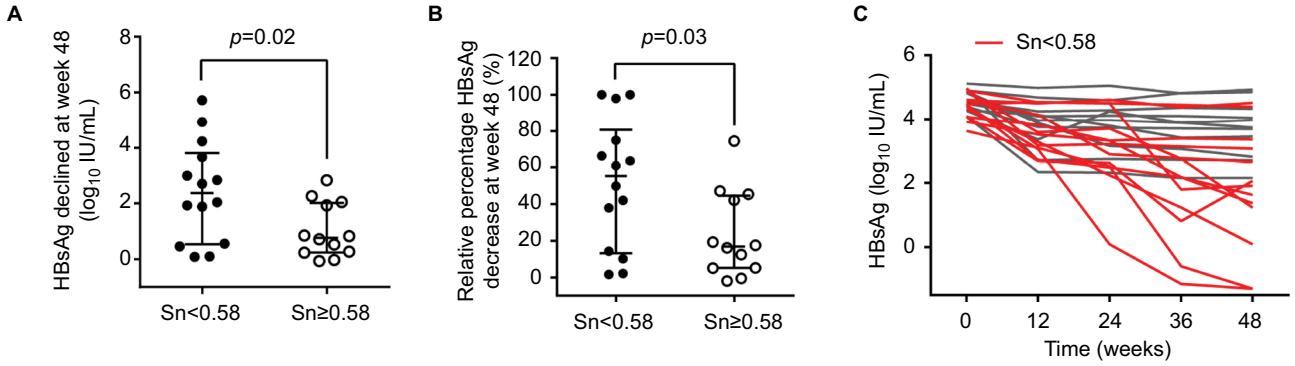

Figure 2 The change in HBsAg kinetics in patients with different degrees of SHBs diversity (Sn <0.58 versus ≥0.58).
Notes: Comparison of HBsAg decline (**A**) and relative percentage HBsAg decrease (**B**) between the Sn <0.58 (n=14) and ≥0.58 (n=12) groups at EOT. (**C**). The serum level of HBsAg in every patient in the Sn <0.58 (n=14) and the Sn ≥0.58 (n=12) groups during Peg-IFN-α treatment. Patients with Sn <0.58 tended to have a steeper HBsAg decline than patients with Sn ≥0.58 (p<0.001 for the comparison of the slope of HBsAg decline determined using a linear mixed model).
Abbreviations: EOT, end of treatment; HBsAg, hepatitis B surface antigen; Peg-IFN, PEGylated interferon; SHBs, small hepatitis B surface protein; Sn, normalized Shannon entropy.

Table 2 Multiple linear regression models examining the impact of baseline SHBs diversity (Sn) on HBsAg decline following Peg-IFN-α therapy

Variables	Crude model	Model I	Model II
ΔHBsAg			
Sn<0.58	Reference	Reference	Reference
Sn≥0.58	−1.4 (−2.5 to −0.3) 0.02	−1.4 (−2.5 to −0.2) 0.03	−1.3 (−2.5 to −0.2) 0.04
Relative percentage HBsAg decrease			
Sn<0.58	Reference	Reference	Reference
Sn≥0.58	−28.0 (−51.5 to −4.5) 0.03	−26.7 (−50.5 to −3.2) 0.04	−26.4 (−50.2 to −2.5) 0.04

Notes: Data are presented as β, 95% CI, and p-values. The crude model did not adjust for any variables; Model I adjusted for baseline ALT; and Model II adjusted for Model I + baseline HBsAg.
Abbreviations: ALT, alanine aminotransferase; HBsAg, hepatitis B surface antigen; Peg-IFN, PEGylated interferon; SHBs, small hepatitis B surface protein; Sn, normalized Shannon entropy.

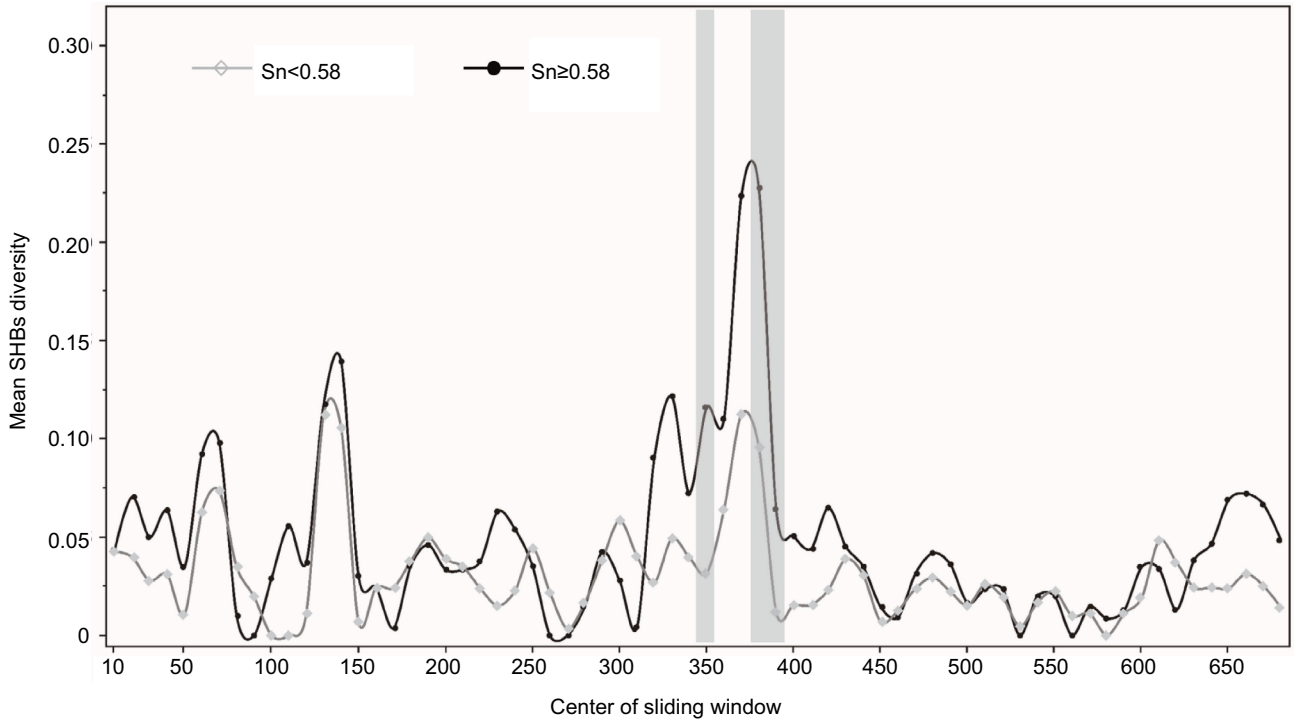

Figure 3 A sliding window analysis with overlapping 19-nucleotide segments migrating every 10 nucleotides for the small *HBs gene*.
Notes: The gray line represents the Sn <0.58 group, and the black line represents the Sn ≥0.58 group. Grayed out segments correspond to statistically significant differences.
Abbreviations: HBs, hepatitis B surface; SHBs, small hepatitis B surface protein; Sn, normalized Shannon entropy.

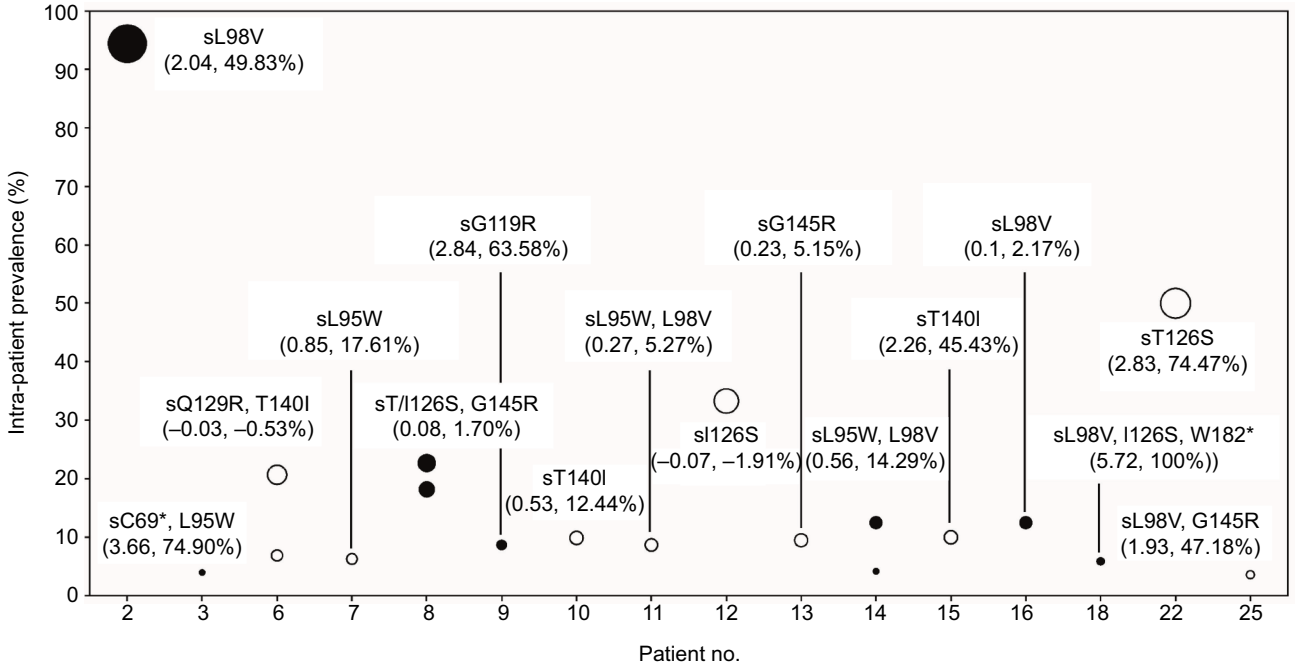

Figure 4 Intra-patient prevalence of HBs mutations was associated with lower HBsAg synthesis/secretion in this study.
Notes: Intra-patient prevalence was expressed as percent of clones with the specific mutation. At least 1 related mutation was detected in 16 (61.5%) patients. Of these, 14 (87.5%) patients' prevalence of related HBs mutation was <35% (mean: 10.3%, range: 3.6%–33.3%). The relative dimension of black (Sn <0.58, n=7) and white (Sn ≥0.58, n=9) dots represents the intra-patient prevalence of related HBs mutations. The numbers indicate the ΔHBsAg and the relative percentage of HBsAg decrease. *Indicates the stop codon.
Abbreviations: HBs, hepatitis B surface; HBsAg, hepatitis B surface antigen; Sn, normalized Shannon entropy.

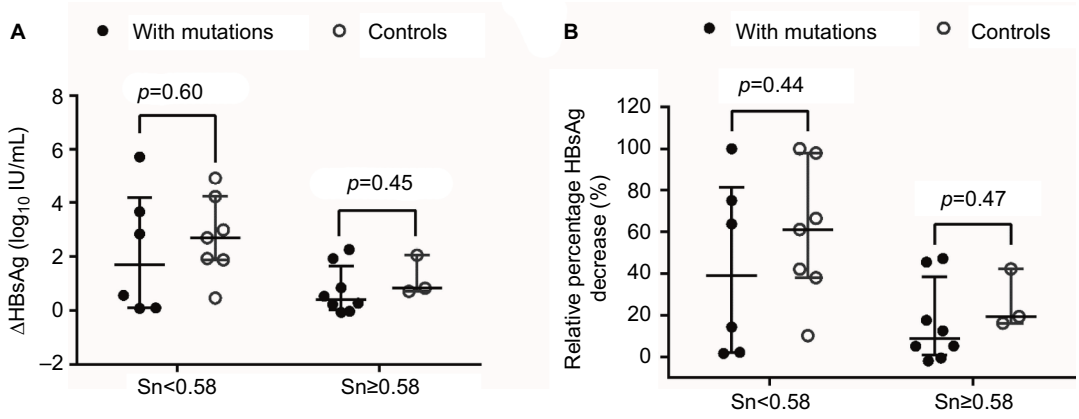

Figure 5 Effect of HBs-specific mutations on the ΔHBsAg (**A**) and the relative percentage of HBsAg decrease (**B**) both in the Sn <0.58 and ≥0.58 groups.
Abbreviations: HBs, hepatitis B surface; HBsAg, hepatitis B surface antigen; Sn, normalized Shannon entropy.

ferent 2 groups, with an intra-patient prevalence of ≥50% (94.4% and 50%, respectively). Although the 2 mutations were characterized by a high HBsAg decline (2.04 $\log_{10}$ IU/mL and 2.83 $\log_{10}$ IU/mL), the Sn did not change if left out these 2 mutations. It seems unlikely that HBsAg-related mutation is the main source of the differences in baseline Sn.

Discussion

In this study, we evaluated the relationship between baseline SHBs diversity and HBsAg decline in HBeAg-positive patients treated with Peg-IFN-α. Our results show that the presence of higher baseline SHBs diversity was associated with lower HBsAg reduction in response to Peg-IFN-α therapy. Moreover, the differences in the "a" determinant upstream flanking region and the first loop of the "a" determinant (nucleotides 341–359, 371–389, and 381–399) were the main source for the higher baseline SHBs diversity.

Charuworn et al examined baseline interpatient hepatitis B viral diversity using mean genetic distance in tenofovir-treated patients who lost HBsAg and compared it with that of control patients with high HBsAg levels.[14] Their results suggested that patients with HBsAg loss have less interpatient viral diversity within the *HBs gene*. Although we used Sn in the present study, we also obtained the similar result that the patients with lower baseline SHBs diversity has more HBsAg decline compared with those with higher baseline SHBs diversity in Peg-IFN-α therapy. Both the mean genetic distances and Sn are ways to evaluate viral diversity. In previous studies, we evaluated the HBV diversity using both methods and found that the results were consistent. This demonstrates that the bias from the evaluation method is minimal and can be ignored. In addition, similar results have also been demonstrated in patients infected with HCV in Peg-IFN-α therapy.[21,22] These results suggest that the high viral diversity of pretreatment was associated with poor treatment outcomes, although studies did differ in pathogenic agents (HBV and HCV), medications (NA and Peg-IFN-α), and clinical end points (degree of HBsAg decline and virological response).

However, several studies reported the distinct results that increased viral diversity within the HBV X/pre-core/core regions contributes to HBeAg seroconversion in genotypes B and C patients during Peg-IFN or NA therapy.[18,28] This discrepancy might be because of several differences between those studies and ours. First, in the aforementioned studies, the X/pre-core/core regions were analyzed, whereas the SHBs region was evaluated in our study. There is no doubt that the patterns of HBV genetic variability are significantly different between different regions, particularly those that locate in structural-coding areas with rich immune epitopes.[32,33] Indeed, Charuworn et al confirmed that HBV X and pre-core/core regions generally displayed higher viral diversity than other regions.[14,32] Second, only 10 clones per sample were used to evaluate viral diversity, whereas this study used 25–30 clones per sample. The number of clones is too small to well reflect the complexity of the intra-patient virus population and can, therefore, cause bias when calculating viral diversity.

Our data revealed that the "a" determinant upstream flanking region and the first loop of the "a" determinant (nucleotides 341–359, 371–389, and 381–399) were the main sources for higher baseline SHBs diversity. These regions included critical epitopes for humoral and cellular immunity.[34] A possible explanation was that more sequence variations within these regions perturb host immune control, resulting in the survival of more viral variants. Finally, HBsAg levels declined more slowly in these patients.[35] Further studies are needed to confirm this hypothesis.

There were certain limitations to this study. First, the HBsAg decline was chosen as the study outcome but not hard end points, including HBsAg loss or seroconversion. However, HBsAg loss or seroconversion, whether spontaneous or treatment-induced, is still a rare event in CHB patients. From this point of view, it seems more reasonable to choose HBsAg decline as the end point in the current study. In addition, considering the individual differences in baseline HBsAg levels, we included the ΔHBsAg and the relative percentage HBsAg decrease in the HBsAg decline as study end points. Second, the sample size for the study was small, and all participants were HBeAg-positive. Thus, the generalizability of the study results is constrained to HBeAg-positive patients for those receiving Peg-IFN-α therapy.

Conclusion

This study showed that the higher baseline SHBs diversity was associated with lower HBsAg reduction in response to 48-week Peg-IFN-α therapy. Moreover, more sequence variations within the "a" determinant upstream flanking region and the first loop of the "a" determinant were the main sources for the higher of baseline SHBs diversity. The evaluation of SHBs diversity at baseline could be used to guide patient screening and inform both patients and clinicians of the likelihood of HBsAg reduction in the setting of Peg-IFN-α therapy. Whether this observation extends to an HBeAg-negative population and other treatment strategies remains to be determined in further studies. However, our findings suggest that consideration should be given to the SHBs diversity at the time of therapy initiation, especially when we seek to achieve functional HBV cure (HBsAg loss) in the setting of CHB therapy.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (81171561 and 81772171), the National Science and Technology Major Project of China (2012ZX10002007001, 2017ZX10202203-007 and 2017ZX10202203-008), and the Chongqing Postgraduate Research Innovation Project (Grant Number: CYB16095).

Disclosure

The authors report no conflicts of interest in this work.

References

1. WHO [webpage on the Internet]. Hepatitis B. Fact sheet, updated July 2017. [cited, 2017]. Available from: http://wwwwhoint/mediacentre/factsheets/fs204/en/. Accessed November 17, 2017.
2. Terrault NA, Bzowej NH, Chang KM, Hwang JP, Jonas MM, Murad MH; American Association for the Study of Liver Diseases. AASLD guidelines for treatment of chronic hepatitis B. *Hepatology.* 2016;63(1):261–283.
3. Sarin SK, Kumar M, Lau GK, et al. Asian-pacific clinical practice guidelines on the management of hepatitis B: a 2015 update. *Hepatol Int.* 2016;10(1):1–98.
4. European Association for the Study of the Liver. Electronic address eee, European Association for the Study of the L. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. *J Hepatol.* 2017;67(2):370–398.
5. Chevaliez S, Hezode C, Bahrami S, Grare M, Pawlotsky JM. Long-term hepatitis B surface antigen (HBsAg) kinetics during nucleoside/nucleotide analogue therapy: finite treatment duration unlikely. *J Hepatol.* 2013;58(4):676–683.
6. Marcellin P, Buti M, Krastev Z, et al. Kinetics of hepatitis B surface antigen loss in patients with HBeAg-positive chronic hepatitis B treated with tenofovir disoproxil fumarate. *J Hepatol.* 2014;61(6):1228–1237.
7. Martinot-Peignoux M, Asselah T, Marcellin P. HBsAg quantification to optimize treatment monitoring in chronic hepatitis B patients. *Liver Int.* 2015;35(Suppl 1):82–90.
8. Zoutendijk R, Hansen BE, van Vuuren AJ, Boucher CA, Janssen HL. Serum HBsAg decline during long-term potent nucleos(t)ide analogue therapy for chronic hepatitis B and prediction of HBsAg loss. *J Infect Dis.* 2011;204(3):415–418.
9. Yu C, Gong X, Yang Q, et al. The serum IL-23 level predicts the response to pegylated interferon therapy in patients with chronic hepatitis B. *Liver Int.* 2015;35(5):1549–1556.
10. Zoulim F, Carosi G, Greenbloom S, et al. Quantification of HBsAg in nucleos(t)ide-naive patients treated for chronic hepatitis B with entecavir with or without tenofovir in the BE-LOW study. *J Hepatol.* 2015;62(1):56–63.
11. Hosaka T, Suzuki F, Kobayashi M, et al. Clearance of hepatitis B surface antigen during long-term nucleot(s)ide analog treatment in chronic hepatitis B: results from a nine-year longitudinal study. *J Gastroenterol.* 2013;48(8):930–941.
12. Chan HL, Chan CK, Hui AJ, et al. Effects of tenofovir disoproxil fumarate in hepatitis B e antigen-positive patients with normal levels of alanine aminotransferase and high levels of hepatitis B virus DNA. *Gastroenterology.* 2014;146(5):1240–1248.
13. Tian Q, Jia J. Hepatitis B virus genotypes: epidemiological and clinical relevance in Asia. *Hepatol Int.* 2016;10(6):854–860.
14. Charuworn P, Hengen PN, Aguilar Schall R, et al. Baseline inter-patient hepatitis B viral diversity differentiates HBsAg outcomes in patients treated with tenofovir disoproxil fumarate. *J Hepatol.* 2015;62(5):1033–1039.
15. Dong H, Zhou B, Kang H, et al. Small surface antigen variants of HBV associated with responses to telbivudine treatment in chronic hepatitis B patients. *Antivir Ther.* 2017;22(1):43–51.
16. Xiang KH, Michailidis E, Ding H, et al. Effects of amino acid substitutions in hepatitis B virus surface protein on virion secretion, antigenicity, HBsAg and viral DNA. *J Hepatol.* 2017;66(2):288–296.
17. Gencay M, Hubner K, Gohl P, et al. Ultra-deep sequencing reveals high prevalence and broad structural diversity of hepatitis B surface antigen mutations in a global population. *PLoS One.* 2017;12(5):e0172101.
18. Bhardwaj N, Ragonnet-Cronin M, Murrell B, et al. Intrapatient viral diversity and treatment outcome in patients with genotype 3a hepatitis C virus infection on sofosbuvir-containing regimens. *J Viral Hepat.* 2018;25(4):344–353.
19. de Queiroz AT, Maracaja-Coutinho V, Jardim AC, Rahal P, de Carvalho-Mello IM, Matioli SR. Relation of pretreatment sequence diversity in NS5A region of HCV genotype 1 with immune response between pegylated-INF/ribavirin therapy outcomes. *J Viral Hepat.* 2011;18(2):142–148.
20. Fan X, Mao Q, Zhou D, et al. High diversity of hepatitis C viral quasispecies is associated with early virological response in patients undergoing antiviral therapy. *Hepatology.* 2009;50(6):1765–1772.
21. Cannon NA, Donlin MJ, Fan X, Aurora R, Tavis JE; Virahep C Study Group. Hepatitis C virus diversity and evolution in the full open-reading frame during antiviral therapy. *PLoS One.* 2008;3(5):e2123.

22. Donlin MJ, Cannon NA, Yao E, et al. Pretreatment sequence diversity differences in the full-length hepatitis C virus open reading frame correlate with early response to therapy. *J Virol.* 2007;81(15):8211–8224.
23. Wang N, Li Y, Han Y, Xie J, Li T. HIV sequence diversity during the early phase of infection is associated with HIV DNA reductions during antiretroviral therapy. *J Med Virol.* 2017;89(6):982–988.
24. Palumbo PJ, Wilson EA, Piwowar-Manning E, et al. Association of HIV diversity and virologic outcomes in early antiretroviral treatment: HPTN 052. *PLoS One.* 2017;12(5):e0177281.
25. Epaulard O, Signori-Schmuck A, Larrat S, et al. Ultradeep sequencing of B and non-B HIV-1 subtypes: viral diversity and drug resistance mutations before and after one month of antiretroviral therapy in naive patients. *J Clin Virol.* 2017;95:13–19.
26. King AJ, Sonsma JA, Vriend HJ, et al; Medical Microbiological Laboratories and Municipal Health Services. Genetic diversity in the major Capsid L1 protein of HPV-16 and HPV-18 in the Netherlands. *PLoS One.* 2016;11(4):e0152782.
27. Vinuesa V, Bracho MA, Albert E, et al. The impact of virus population diversity on the dynamics of cytomegalovirus DNAemia in allogeneic stem cell transplant recipients. *J Gen Virol.* 2017;98(10):2530–2542.
28. Wu S, Imazeki F, Kurbanov F, et al. Evolution of hepatitis B genotype C viral quasi-species during hepatitis B e antigen seroconversion. *J Hepatol.* 2011;54(1):19–25.
29. Li H, Song XF, Hu TT, Ren H, Hu P. A strong conservative tendency in HBV transcriptase (RT): a majority of natural RT mutations derived from the S gene. *Liver Int.* 2016;36(7):963–970.
30. Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol.* 2016;33(7):1870–1874.
31. Zhu HL, Li X, Li J, Zhang ZH. Genetic variation of occult hepatitis B virus infection. *World J Gastroenterol.* 2016;22(13):3531–3546.
32. van de Klundert MA, Cremer J, Kootstra NA, Boot HJ, Zaaijer HL. Comparison of the hepatitis B virus core, surface and polymerase gene substitution rates in chronically infected patients. *J Viral Hepat.* 2012;19(2):e34–e40.
33. Vita R, Overton JA, Greenbaum JA, et al. The immune epitope database (IEDB) 3.0. *Nucleic Acids Res.* 2015;43(Database issue):D405–D412.
34. Desmond CP, Bartholomeusz A, Gaudieri S, Revill PA, Lewin SR. A systematic review of T-cell epitopes in hepatitis B virus: identification, genotypic variation and relevance to antiviral therapeutics. *Antivir Ther.* 2008;13(2):161–175.
35. Salpini R, Colagrossi L, Bellocchi MC, et al. Hepatitis B surface antigen genetic elements critical for immune escape correlate with hepatitis B virus reactivation upon immunosuppression. *Hepatology.* 2015;61(3):823–833.

Supplementary materials

Table S1 Summary of known mutations associated with lower HBsAg synthesis/secretion in vivo and in vitro

Mutation	Source		Intracellular HBsAg	Extracellular HBsAg
E2G	CHB	HepG2	↓	↓
C69*	CHB	HepG2	↓	↓
W74*	CHB	Huh7	↓	↓
L95W	CHB	HepG2	↓	↓
L98V	CHB	HepG2	↓	↓
G119R	OBI	Huh7/mice	↑	↓
T125A	CHB	Huh7	–	↓
I/T126S	OBI	Huh7/mice	↑	↓
Q129R	OBI	Huh7/mice	↑	↓
S136P	OBI	Huh7/mice	↑	↓
T140I	OBI	Huh7/mice	↑	↓
K141E	OBI	Huh7/mice	↑	↓
D144A/G	OBI	Huh7/mice	↑	↓
G145R/A	OBI	Huh7/HepG2/mice	↓	↓
W182*	CHB	HepG2	↓	↓
M103I+G145A	OBI	Huh7/HepG2	↓	↓
R122K+G145A	OBI	Huh7/HepG2	↓	↓
M103I+R122K+G145A	OBI	Huh7/HepG2	↓	↓

Notes: *Stop codon mutation. ↑, increase; ↓, decrease.
Abbreviations: CHB, chronic hepatitis B; HBsAg, hepatitis B surface antigen; OBI, occult hepatitis B infection.

Table S2 Intra-patient prevalence of mutations associated with lower HBsAg synthesis/secretion in this study

Group	Patient	C69*	L95W	L98V	G119R	T/I126S	Q129R	T140I	G145R	W182*
1	2	0	0	94.4	0	0	0	0	0	0
1	3	4	4	0	0	0	0	0	0	0
1	8	0	0	0	0	18.2	0	0	22.7	0
1	9	0	0	0	8.7	0	0	0	0	0
1	14	0	12.5	4.2	0	0	0	0	0	0
1	16	0	0	12.5	0	0	0	0	0	0
1	18	0	0	5.9	0	5.9	0	0	0	5.9
2	6	0	0	0	0	0	20.7	6.9	0	0
2	7	0	6.3	0	0	0	0	0	0	0
2	10	0	0	0	0	0	0	9.9	0	0
2	11	0	8.7	8.7	0	0	0	0	0	0
2	12	0	0	0	0	33.3	0	0	0	0
2	13	0	0	0	0	0	0	0	9.5	0
2	15	0	0	0	0	0	0	10	0	0
2	22	0	0	0	0	50	0	0	0	0
2	25	0	0	3.6	0	0	0	0	3.6	0

Notes: Data are presented as percentage (%). Group 1: Sn <0.58; Group 2: Sn ≥0.58. *Indicates the stop codon.
Abbreviations: HBsAg, hepatitis B surface antigen; Sn, normalized Shannon entropy.