STUDY ON CHANGES IN SERUM HBV RNA LEVELS IN PATIENTS WITH CHRONIC HEPATITIS B TREATED WITH TENOFOVIR DISOPROXIL FUMARATE

Nguyen Dinh Ung[1], Le Van Nam[2], Nguyen Hong Thang[2]*
Nguyen Trong Chinh[2], Ho Huu Tho[1]

Abstract

Objectives: To determine the changes in serum HBV RNA levels in treatment-naïve chronic hepatitis B (CHB) patients who were treated with Tenofovir Disoproxil Fumarate (TDF). ***Methods:*** 77 treatment-naïve CHB patients were treated with long-term TDF monotherapy at the Department of Infectious Diseases, Military Hospital 103, Vietnam Military Medical University from 2017 to 2020. Samples were collected at several time points: At the baseline, after 3, 6, 9, and 12 months of TDF treatment. Serum HBV DNA and HBV RNA levels were quantified by the Real Time RT-PCR method. Statistical analyses were performed with Medcalc 20.019. ***Results:*** Serum HBV RNA levels tended to decrease during the TDF treatment in a biphasic pattern. In the first phase, from baseline to 3 months of treatment, HBV RNA levels decreased rapidly (the median slope of the decrease was 0.38 log copies/mL/month). In the second phase, from 3 - 12 months of treatment, serum HBV RNA levels decreased more slowly than in the first phase (the median slope of the decrease was 0.09 log copies/mL/month; $p < 0.05$). Serum HBV RNA levels decreased more slowly than serum HBV DNA levels in the first phase, but there was no significant difference in the second phase ($p > 0.05$). ***Conclusion:*** Serum HBV RNA levels decreased in a biphasic pattern with a different slope during TDF treatment. Serum HBV RNA levels decreased more slowly than HBV DNA and may complement this marker in the assessment of treatment outcomes and prognosis for chronic hepatitis B.

Keywords: Chronic Hepatitis B (CHB); HBV pgRNA.

[1]Vietnam Military Medical University
[2]Military Hospital 103, Vietnam Military Medical University
***Corresponding author: Nguyen Dinh Ung (dr.ungd4.vmmu@gmail.com)**
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INTRODUCTION

Hepatitis B virus (HBV) can cause asymptomatic infections and dangerous complications, such as decompensated liver disease, cirrhosis, and hepatocellular carcinoma [1]. In 2019, it was estimated that about 4.1% of the global population (284 - 351 million people) is chronically infected with HBV, and approximately 555,000 (487,000 - 630,000) deaths were due to diseases related to HBV [2]. Vietnam is one of the countries with a high prevalence of HBV, with about 6.6% of the population with chronic HBV infection [2]. Globally, about 42% of cirrhotic patients have chronic HBV infection. In Vietnam, the rate was about 35% [3]. The above data proved that chronic HBV infection had been a global health problem and a huge burden on the health of our country.

However, the current treatment for CHB virus still has many problems. To date, antiviral regimens have not been able to completely cure CHB, and when the drug was stopped, HBV rapidly reactivated in the peripheral blood of the patient [4]. Unfortunately, tests based on current HBV markers (HBV DNA, HBeAg, and HBsAg) are not effective in predicting important treatment outcomes, such as virological response or HBeAg seroconversion [5]. Serum HBV DNA levels were rapidly decreased during the new-generation Nucleos(t)ide analogues (NAs) treatment, such as Tenofovir or Entecavir. HBsAg was often redundantly synthesized from both cccDNA (covalently closed circular DNA) and the integrated gene of the patient genome. The above problems reduce the role of HBV DNA and HBsAg in predicting treatment response and the prognosis of complications [6].

HBV pregenomic RNA (HBV pgRNA) was transcribed directly from cccDNA in infected hepatocytes, serving as a template for the reverse transcriptase to synthesize the DNA minus strand of HBV. Several recent studies have reported that HBV pgRNA is released into the peripheral blood of CHB patients and may be a potential biomarker for early prognosis of HBeAg seroconversion better than serum HBV DNA and HBsAg levels [5]. In addition, the presence of HBV pgRNA in peripheral blood has been shown to be associated with viral reactivation after NAs discontinuation [7]. So far, there has been no consensus on changes in serum HBV

RNA levels during the NAs treatment. Therefore, we conducted this study to: *Evaluate the changes in serum HBV RNA levels during the tenofovir treatment as a basis for further studies on the role of this promising biomarker in the clinical application as well as in the management of HBV infection in the community.*

MATERIALS AND METHODS

1. Subjects

77 treatment-naïve CHB patients were treated at the Department of Infectious Diseases, Military Hospital 103, Military Medical University from 2017 to 2020.

* *Inclusion criteria:* Based on the Guidelines for the diagnosis and treatment of CHB of the Ministry of Health in 2019 [8], including: Patients who were diagnosed with CHB; Patients who were treatment-naïve of NAs; Patients who received monotherapy with TDF; Patients who consented to participate in the 12-month follow-up study.

* *Exclusion criteria:* Pregnant and lactating women, children under 15 years of age; Patients who had severe liver-related complications, such as cirrhosis or liver cancer; Patients with co-infection with human immunodeficiency (HIV) or hepatitis C virus (HCV); Patients who received therapy with any NAs; Patients who did not comply with treatment.

2. Methods

* *Study design:* A prospective cohort study.

Patients were followed up within 12 months with several times of collecting peripheral blood samples: At the baseline, after 3, 6, 9, and 12 months of TDF treatment.

* *Standard laboratory assessments:*

Serum HBV DNA levels were quantified using the Realtime Sacycler 96/HBV Real-TM Quant Dx. The limit of quantification of this assay was 30 copies/mL, a conversion factor of 1.7 copies per IU and a linear range from 30 copies/mL - 10^8 copies/mL. The presence of serum HBeAg was measured using an immunoassay on the COBAS E411 (Roche Diagnostics, Mann-heim, Germany; Elecsys® HBeAg; Analytical sensitivity was 0.3 IU/mL).

* *Quantification of serum HBV RNA levels:*

The levels of HBV RNA were detected by a quantitative one-step

real-time polymerase chain reaction in the Rotor-Gen Q system (QIAGEN, Germany) with a TaqMan® probe method. The primers and probe used to detect HBV RNA were as follows: forward primer: 5'- GCCAAAATTCGCAGTCCC-3'; reverse primer: GCCACTCATCAGTCAGCAGGATG; probe: FAM-CGCTGGATGTGTCTGCGGCGT-BHQ1. The real-time PCR mixture (40 µL) contained 8µL of 5x HTOne Ultra RT-qPCR Probe master mix (HT Biotec, Vietnam), 2µL forward primer (10µM), 2µL reverse primer (10µM), 0.8µL probe (10µM), 15.2µL DEPC water, and 12µL template. The thermal cycling program included 10 min at 50^{o}C for reverse transcription, 15 min at 95^{o}C for initial denaturation, and 45 cycles of 15s at 94^{o}C for denaturation, 30s at 63^{o}C for annealing, and 30s at 72^{o}C for extension. A positive control and a non-template control were included in each PCR assay tested. Primers and probes for quantification of HBV pgRNA were designed for the conserved region of the S gene. The reverse primer for quantification of HBV pgRNA contains a 10-nucleotide sequence at the 3' end that is specific for the HBV genome and further contains a 5' tail of unrelated sequence (underlined). The limit of quantification of this assay was 100 copies/mL.

** Antiviral Drug:*

All patients in this study received Tenofovir Disoproxil Fumarate (Savi Tenofovir 300, SAVIPHARM J.S.C), with a dose of 300 mg daily, continuously for 12 months. After that, patients continued to take the drug as prescribed by the clinician.

** Statistical analysis:*

Results of HBV DNA quantification that were negative or below the limit of detection were converted to 30 copies/mL for statistical analysis, while results of HBV RNA quantification that were negative or below the limit of detection were converted to 100 copies/mL. Statistical analyses were performed using MedCalc version 20.019 (MedCalc Software Ltd, Ostend, Belgium).

3. Ethics

This study was approved by the Ethics Committee of Vietnam Military Medical University (No. 780/QĐ-HVQY, March 28 2018).

RESULTS AND DISCUSSION

* *Characteristics of participants:*

Table 1. Characteristics of participants' age.

Gender	n	Median (IQR)**	p*
Female	10	46.50 (34.00 - 55.00)	0.08
Male	67	34.00 (29.00 - 47.00)	
Total	77	36.00 (30.75 - 49.25)	

* *Mann-Whitney test; ** Median (Interquartiles)*

As table 1 shows, the median age of participants in the study was 36 years old, which was the main working age in society. The ages between the male and female groups did not show a statistically significant difference (p = 0.08 > 0.05).

Table 2. Characteristics of serum HBV DNA levels during the treatment.

Time points (months)	Serum HBV DNA levels (log copies/mL) Median (IQR)			p*
	Baseline HBeAg negative group	Baseline HBeAg positive group	Total	
Baseline	n = 34 5.89 (4.34 - 6.47)	n = 43 6.99 (5.97 - 7.60)	n = 77 6.39 (5.42 - 7.44)	0.001
3	n = 27 2.63 (2.18 - 3.11)	n = 36 3.43 (2.70 - 4.28)	n = 63 3.12 (2.44 - 3.76)	0.002
6	n = 25 1.48 (1.48 - 1.49)	n = 34 1.50 (1.46 - 2.23)	n = 59 1.48 (1.48 - 2.15)	0.46
9	n = 24 1.46 (1.46 - 1.48)	n = 27 1.48 (1.46 - 2.30)	n = 51 1.46 (1.46 - 1.48)	0.25
12	n = 23 1.48 (1.46 - 1.48)	n = 27 1.48 (1.46 - 2.13)	n = 50 1.48 (1.46 - 1.93)	0.14

* *Mann-Whitney test*

As table 2 shows, serum HBV DNA levels tended to decrease during treatment, corresponding to the virological response of patients to antiviral drugs.

In addition, only at the baseline and after 3 months of treatment was there a difference in serum HBV DNA levels between the two groups with or without HBeAg at the baseline ($p < 0.05$). In contrast, after 6, 9, and 12 months of treatment, there was no statistically significant difference in serum HBV DNA levels between the two groups with or without HBeAg at the baseline ($p > 0.05$).

Table 3. Characteristics of serum HBV RNA levels during the TDF treatment.

Time points (months)	Serum HBV RNA levels (log copies/mL) Median (IQR)			p*
	Baseline HBeAg negative group	Baseline HBeAg positive group	Total	
Baseline	n = 34 4.13 (2.50 - 5.21)	n = 43 5.34 (3.59 - 6.93)	n = 77 4.97 (3.03 - 6.02)	0.003
3	n = 27 3.01 (2.00 - 3.99)	n = 36 3.30 (2.59 - 4.34)	n = 63 3.10 (2.21 - 4.07)	0.14
6	n = 25 2.77 (2.00 - 3.32)	n = 34 2.89 (2.00 - 3.89)	n = 59 2.79 (2.00 - 3.68)	0.12
9	n = 24 2.00 (2.00 - 2.80)	n = 27 3.01 (2.00 - 4.02)	n = 51 2.41 (2.00 - 3.49)	0.02
12	n = 23 2.00 (2.00 - 2.00)	n = 27 2.00 (2.00 - 3.88)	n = 50 2.00 (2.00 - 3.05)	0.02

** Mann-Whitney test*

As shown in table 3, serum HBV RNA levels gradually decreased during treatment, corresponding to the clinical response to antiviral drugs. In addition, serum HBV RNA levels were higher in the baseline HBeAg-positive group compared with the baseline HBeAg-negative group at the baseline; after 9 and 12 months of treatment, there was statistical significance ($p < 0.05$). In contrast, there was no statistically significant difference in serum HBV RNA levels between the two groups after 3 and 6 months of treatment (all $p > 0.05$).

Table 4. The change of serum HBV RNA level during the TDF treatment.

Phases	Slope of the decrease of serum HBV RNA levels (log copies/mL/months) (Median - IQR)	p
From baseline to 3rd months (1)	0.38 (0.10 - 0.63)	
From 3rd to 6th months (2)	0.06 (0.00 - 0.22)	*$p^{(1)-(2)} < 0.0001$
From 6th to 9th months (3)	0.02 (0.00 - 0.22)	*$p^{(1)-(3)} < 0.0001$ *$p^{(1)-(4)} < 0.0001$ **$p^{(2)-(3)-(4)} = 0.69$
From 9th to 12th months (4)	0.00 (0.00 - 0.15)	*$p^{(1)-(5)} < 0.0001$
From 3rd to 12th months (5)	0.09 (0.00 - 0.19)	

As table 4 shows, the slope of the decline of serum HBV RNA levels gradually decreased during treatment. Phase (1) from baseline 3rd months had the fastest rate of decrease in serum HBV RNA levels (median slope = 0.38 log copies/mL/month), faster than the remaining phases ($p < 0.0001$). Phase (2): From 3rd - 6th (0.06 log copies/ml/month); phase (3): From 6th - 9th (0.02 log copies/mL/month); phase (4): From 9th - 12th (0.00 log copies/mL/month). There was no difference in the slope of the decrease in serum HBV RNA levels ($p^{(2)-(3)-(4)} > 0.05$). When combining phases (2), (3), and (4) into a single phase, the slope of decline in serum HBV RNA levels in this phase was significantly different from that in phase (1) (0.09 vs log copies/mL/month; $p < 0.0001$).

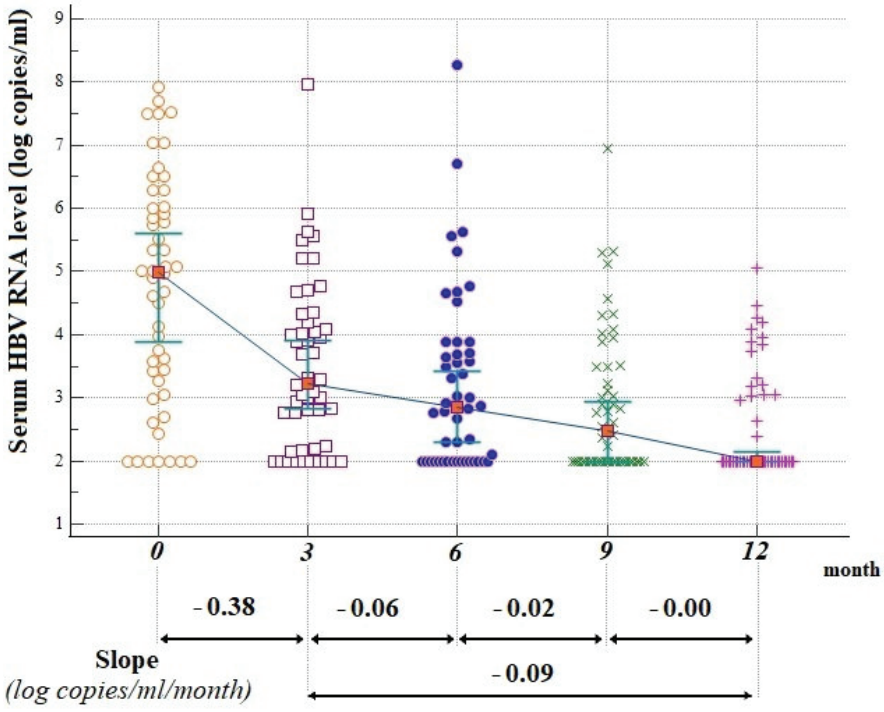

Figure 1. The model of the biphasic pattern of serum HBV RNA levels during TDF treatment.

As figure 1 shows, the change of serum HBV RNA levels over time of treatment was a reduction pattern with a biphasic form: The first phase from the baseline to after the 3rd month of treatment, and the second phase from the 3rd - 12th month of treatment. Recently, some authors have also published reports describing the biphasic decline in serum HBV RNA levels [9]. Accordingly, in 76 HBeAg-positive CHB patients who received Telbivudine and/or Adefovir, the decline in serum HBV RNA levels during NAs was biphasic: In the first phase (serum HBV DNA detectable), serum HBV RNA levels were decreased rapidly (median of the slope was 0.207 log copies/mL/month, IQR 0.402 log to 0.112 log), and in the second phase (serum HBV DNA was undetectable), serum HBV RNA levels decreased slowly (median of the slope was 0.071 log copies/mL/month; IQR: 0.105 log to 0.039 log). However, the junction between the two phases was defined as the first time when serum HBV DNA was undetectable. This difference may be due to the fact that all patients in our study were treated with TDF, which had a better antiviral effect than Telbivudine and Adefovir [10].

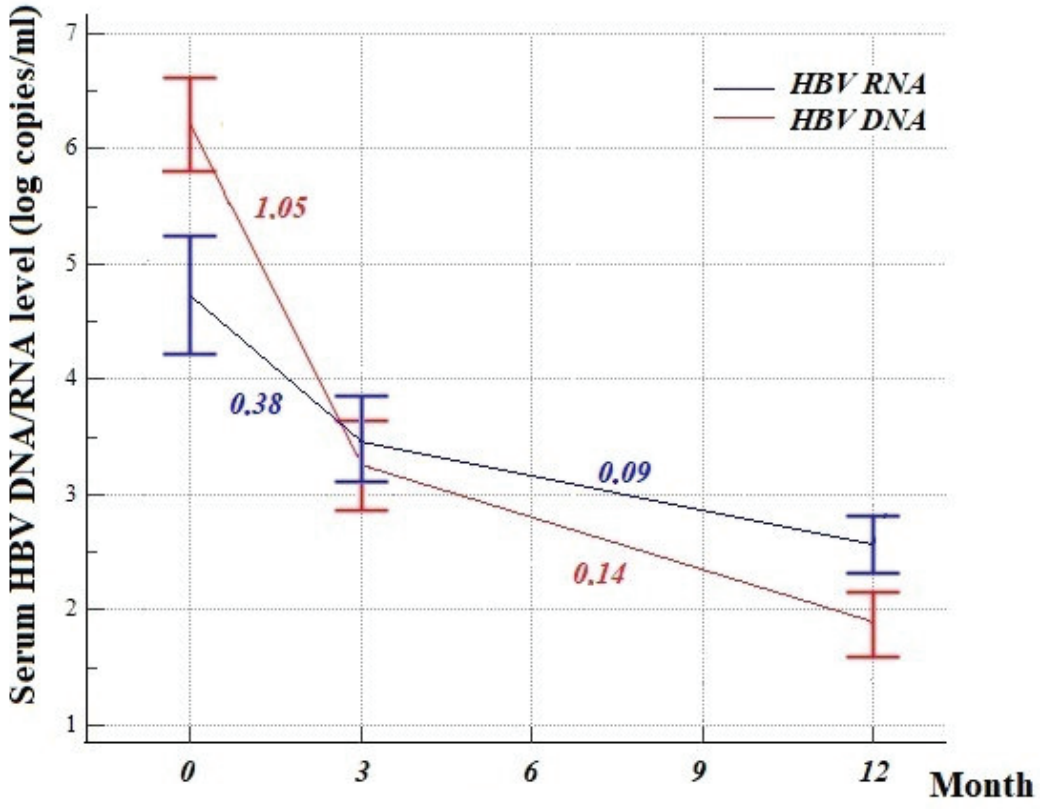

Figure 2. Comparison of the slope of decline between serum HBV DNA and HBV RNA levels during the TDF treatment.

According to figure 2, in the first phase, serum HBV DNA levels decreased significantly faster than serum HBV RNA levels ($p < 0.05$). In contrast, in the second phase, the rate of decrease in levels of these two markers was similar ($p > 0.05$). This result has also been reported in the publication of L. Jansen et al. 2016 [11]. Accordingly, serum HBV DNA and HBV RNA levels were quantified in the serum of 10 CHB patients who were treated with NAs for a period of 120 weeks. Serum HBV RNA levels decreased less than the serum HBV DNA levels, and mean HBV RNA levels were significantly higher than HBV DNA levels during all time points of the treatment. At 120 weeks of follow-up, 7 of 10 patients had HBV DNA levels below the limit of detection, while only 1 of 10 patients had low HBV RNA levels below the limit of detection (mean ± standard error of the mean, 2.0 ± 0.1 vs 3.4 ± 0.4 log10 copies/mL, respectively; $p = 0.002$).

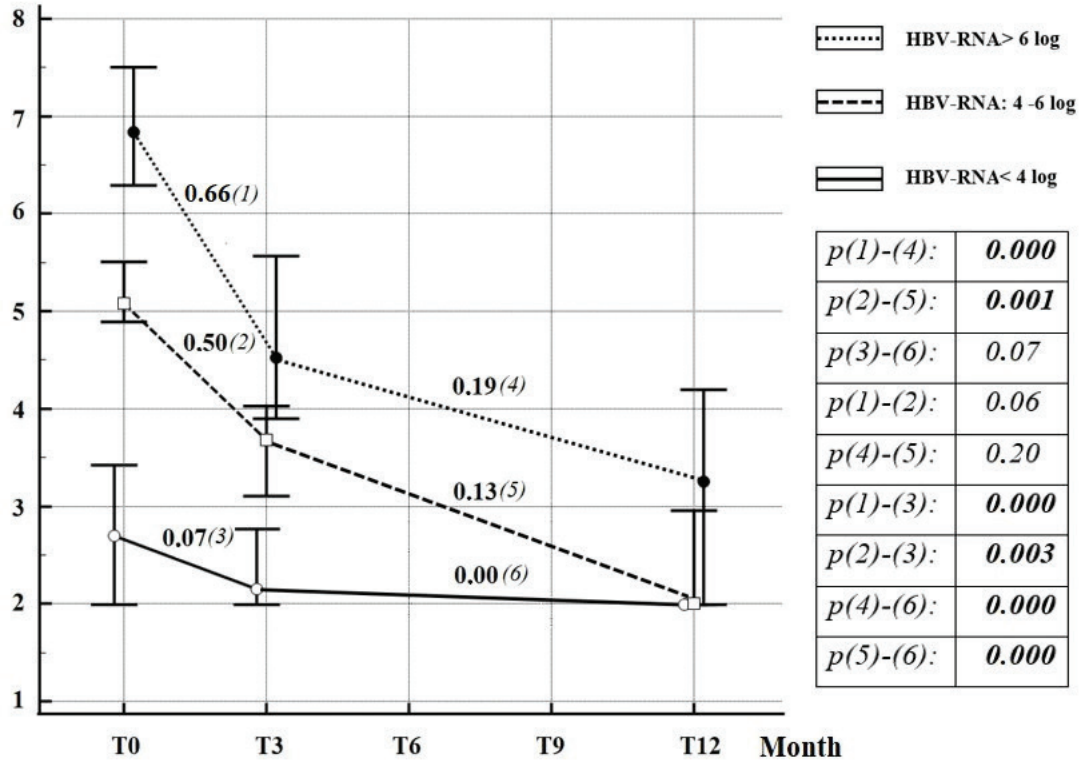

Figure 3. Model of the decline of serum HBV RNA levels, grouped by baseline serum HBV RNA levels.

Based on baseline serum HBV RNA levels, patients were divided into three groups: Group with high serum HBV RNA levels (> 6 log copies/mL); group with moderate serum HBV RNA levels (4 - 6 log copies/mL); and group with low serum HBV RNA levels (< 4 log copies/mL).

As figure 3 shows, the pattern of reduction of serum HBV RNA levels over time of treatment had a clear biphasic pattern in the baseline high serum HBV RNA level group (> 6 log copies/mL, $p^{(1)-(4)} = 0.000 < 0.05$) and the baseline medium serum HBV RNA level group (4 - 6 log copies/mL; $p^{(2)-(5)} = 0.001 < 0.05$). Meanwhile, the group of patients with low serum HBV RNA levels (< 4 log copies/mL) did not separate into distinct phases ($p^{(3)-(6)} = 0.07 > 0.05$). In addition, there was no statistically significant difference in the rate of reduction of serum HBV RNA levels in the two groups with high and medium baseline serum HBV RNA levels (all $p > 0.05$). While patients in the two groups above had a faster decrease in serum HBV

RNA levels at all phases compared with the group with low serum HBV RNA levels ($p < 0.05$).

In a retrospective study published in 2021 on 185 CHB patients who received at least two consecutive years of treatment with entecavir, I-chin Wu et al. also provided an alternative approach to the pattern of decline in serum HBV RNA levels in response to ETV treatment [12]. The author subgroups patients based on baseline serum HBV RNA levels into three groups with high, moderate, and low levels (≥ 6 log; 4 log $\leq$ & < 6 log; < 4 log). The findings of the study indicate that the group with high baseline levels of HBV pgRNA demonstrated a significant reduction within the first 3 months, followed by minimal variation thereafter. In the group with moderate levels, serum HBV pgRNA levels decreased slowly during the 12th month of treatment and stabilized thereafter. In contrast, in the group with low levels, serum HBV pgRNA levels increased after 3 months and then stabilized. Compared with the group with moderate and low HBV pgRNA levels, the group with high baseline HBV pgRNA levels had higher serum HBV pgRNA levels not only at baseline but also after the 12th and 60th month of treatment. At 3 months, the group with high and moderate HBV pgRNA levels always had higher HBV pgRNA levels than the group with low HBV pgRNA levels. At 6 months, the same was true for the group with high levels of HBV pgRNA, while there was no significant difference in serum HBV pgRNA levels between the low and medium groups at the 6th, 12th, and 60th months of treatment. Meanwhile, serum HBV DNA levels in groups divided by HBV pgRNA concentration all had a rapid decrease after 3 months, then the trend decreased gradually. There was no significant difference in HBV DNA levels in 3 groups at the 3rd, 6th, 12th, and 60th months.

The results of our study and those of I-chin Wu et al. both showed a biphasic decline of serum HBV RNA levels in the peripheral blood of patients with CHB treated by NAs as well the time of intersection between the two phases of the decline in serum HBV RNA levels was after 3 months of treatment. However, the difference here was observed in the group with low baseline HBV RNA levels below 4 log copies/mL, which not only did not decrease levels after 3 months of treatment but even recorded an increase in serum HBV RNA levels and then stabilized in the second phase. Ichin Wu has suggested that this phenomenon is due to the inhibition of antiviral drugs on reverse transcription

from the HBV pgRNA template to create HBV DNA; thus, increasing the accumulation and leading to HBV pgRNA elevation in the patient's peripheral blood. However, an increase in peripheral blood HBV pgRNA levels was not observed in the group with moderate and high HBV pgRNA levels. While the decline in serum HBV DNA levels after 3 months of treatment was also noted by the author to be very rapid, suggesting the accumulation of HBV pgRNA also increased correspondingly (due to not participating in the reverse transcriptase process, which was inhibited by the drug). Further investigation is required to clarify the differences in peripheral blood HBV pgRNA levels among patients with chronic hepatitis B who received nucleoside/nucleotide analogue therapy for three months. Specifically, the study should focus on comparing patients with moderate and high levels of HBV pgRNA with those exhibiting low levels of the virus.

CONCLUSION

The study found that treating CHB patients with TDF led to a biphasic decline in serum HBV RNA levels. The first phase of decline was more rapid than the second phase. Because serum HBV RNA levels decreased more slowly than serum HBV DNA levels, the results suggest that HBV RNA has the potential as a marker in times of undetectable HBV DNA under the antiviral inhibition of nucleos(t)ide analogues. These findings represented a significant step towards understanding the clinical application of serum HBV RNA levels in CHB patients in both general and specific contexts in Vietnam. Future studies are needed to further investigate the clinical utility of this marker.

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