

Detectability and clinical significance of serum hepatitis B virus ribonucleic acid

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Abstract: Serum hepatitis B virus (HBV) RNA is detected during treatment with nucleoside analogue as a consequence of interrupted reverse transcription (RT) and unaffected replicative intermediates. The presence of serum HBV RNA in chronic HBV patients is confirmed by using ribonuclease digestion. Serum HBV RNA is differentially inhibited by interferon, but not by nucleoside analogue. The inhibitory effect of interferon on HBV RNA replicative intermediates may potentiate the suppression of HBV replication. Clinical significance of serum HBV RNA includes: (I) reflect the antiviral potency of nucleoside analogue; (II) predictor of early emergence of viral mutation during lamivudine therapy; (III) independently predict initial virologic response or earlier HBV suppression during nucleoside analogue therapy; (IV) predict HBV reactivation after discontinuation of nucleoside analogue. Thus, serum HBV RNA might be useful to optimize treatment efficacy in patients with chronic HBV, including shifting of oral antivirals or conversion to immunomodulatory agent i.e., interferon. Furthermore, serum HBV RNA levels correlate better with serum quantitative HBsAg (qHBsAg) than with serum HBV DNA levels. The predictive role of serum HBV RNA in long-term treatment effects of nucleoside analogue needs further study.

Keywords: Nucleoside analogue; interferon; replicative intermediates

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Hepatitis B virus (HBV) and its replication

HBV infection remains a global health problem in spite of the availability of effective vaccines against HBV infection for more than three decades. More than 350 million people are chronic carriers of HBV worldwide (1,2). In the United States, 1.2 million persons have chronic HBV infection (3). HBV infection leads to a wide spectrum of clinical presentations, including acute or fulminant hepatitis, inactive carrier state, chronic hepatitis, cirrhosis, as well as hepatocellular carcinoma (2,4,5).

HBV is a unique DNA virus that replicates via pre-genomic RNA. The steps in HBV replication are: (I) the asymmetric DNA in virions becomes covalently closed circular DNA

(cccDNA) in the nucleus of infected hepatocytes; (II) cccDNA is transcribed to pre-genomic RNA; (III) reverse transcriptase synthesizes viral DNA minus strand; and (IV) synthesis of viral DNA plus strand to mature genomic DNA (6).

Detectability of serum hepatitis B virus (HBV) ribonucleic acid

Both interferon and nucleos(t)ide analogues have been approved for the treatment of chronic hepatitis B (CHB). Both suppress the virus, interferon additionally possesses immunomodulatory property (7). Lamivudine, as the first approved nucleoside analogue, has no effect on cccDNA,

the integrated HBV DNAs and their transcripts, the RNA replicative intermediates (8). During nucleoside analogue therapy, serum HBV RNA gradually declined but persistently detectable, suggesting the persistence of cccDNA in the hepatocytes and continuous replication of the virus. This might partially explain that to achieve sustained viral suppression, lamivudine and other nucleos(t)ide analogues usually need indefinite duration of therapy. Interferon, on the other hand, has a finite duration of therapy and a higher rate of hepatitis B surface antigen (HBsAg) clearance (9).

Our study and others demonstrated that serum HBV RNA could be detected during lamivudine or entecavir therapy (10-14). The main effect of lamivudine and entecavir are to inhibit reverse transcriptase, resulting in accumulation of HBV RNA in hepatocytes. It is still unclear how the HBV RNA in hepatocytes is released into the serum and is not degraded. Nevertheless, the presence of HBV RNA in serum of chronic HBV patients treated with nucleoside analogue was confirmed by using ribonuclease treatment (11). Ribonuclease digestion reduced the amount of HBV DNA detected by real-time reverse transcription (RT)-PCR to about 1% of the originally detected (11).

Differential inhibition on serum hepatitis B virus (HBV) ribonucleic acid

Clinical observations

Serum HBV RNA is persistently detectable during nucleoside analogue therapy, whereas it is inhibited under sequential lamivudine and interferon treatment (12). The difference between these two groups was significant in a relatively small number of patients, suggesting the substantial HBV RNA inhibitory effect of interferon. The decline in serum HBV RNA level was not simply due to discontinuation of lamivudine because serum HBV RNA was persistently detectable even after discontinuation of short-term lamivudine (12). Furthermore, the inhibition of serum HBV RNA was found in patients treated with lamivudine converting to conventional interferon and in those with conventional interferon converting to lamivudine. In the latter patients, serum HBV RNA inhibition might be due to the delayed response of interferon (12).

Simultaneous combination therapy of interferon- α and lamivudine had more profound on-treatment viral suppression and higher sustained response rates than lamivudine monotherapy (9). Sequential therapy of

lamivudine and interferon increased HBeAg seroconversion rate (15), had a higher response rate of sustained HBeAg seroconversion, ALT normalization, HBV DNA loss, and less relapse after stopping therapy (16). In addition, the initial use of lamivudine before interferon leads to a higher sustained virologic response as compared to interferon monotherapy (17). Although the underlying mechanisms of these findings remain unclear and deserve further studies, the inhibitory effect of interferon on serum HBV RNA in lamivudine-treated patients might in part explain the higher sustained response rate than those treated with lamivudine monotherapy (12).

Serum HBV RNA was detectable persistently after discontinuation of short-term lamivudine therapy. This finding suggested that although new viral particles containing HBV RNA were no longer produced after lamivudine discontinuation, the existing viral particles containing HBV RNA were not quickly degraded. Rokuhara *et al.* demonstrated that significantly more decline in serum HBV DNA than RNA during lamivudine therapy (18). Their findings confirmed ours that the lack of nucleoside analogue immediately inhibits serum viral particles containing HBV RNA. Nevertheless, further studies are needed to evaluate how long these viral particles containing HBV RNA persist in the serum. Following sequential combination therapy of lamivudine and conventional interferon, serum HBV RNA was inhibited and undetectable at the end of treatment. The shift to interferon led to the inhibition of serum HBV RNA (9).

We reported the discrepant measurement of HBV nucleic acid by the Transcription-mediated amplification and hybridization protection assay (TMA-HPA) and the Amplicor HBV Monitor test (11). Because the TMA-HPA uses RNA transcription and amplification of transcripts by T7 RNA polymerase (19), we assumed that the discrepancy was a result of persistent serum HBV RNA in patients treated with nucleoside analogue. Zhang *et al.* reported the presence of serum HBV RNA in a patient treated with lamivudine (10). They analyzed mainly the truncated HBV RNA, which was assumed to be transcribed from the integrated HBV genome. They disclosed a huge difference between truncated HBV RNA and HBV DNA. In our study, HBV DNA and HBV nucleic acid were assayed by real-time PCR and real-time RT-PCR, and less than one log difference was shown, thus the effect of truncated serum HBV RNA was minimal in our study. Furthermore, Rokuhara *et al.* also revealed the incorporation of HBV RNA into virus particles with the evidence that HBV RNA

made a single peak for the same fraction whereas both HBV DNA and HBV core-related antigen made several single peaks in sucrose gradient analyses at three different time points during lamivudine therapy (18).

It is therefore clear that interferon can inhibit serum HBV RNA during therapy with nucleoside analogue. The persistence of serum HBV RNA as a consequence of unaffected HBV RNA replicative intermediates may cause indefinite therapy with nucleoside analogue. In contrast, interferon inhibition on HBV RNA replicative intermediates lead to profound suppression of HBV replication.

In vitro and in vivo experimental data

The presence of serum HBV RNA in nucleoside analogue treated patients was confirmed by *in vitro* data in HBV-transfected HepG2.2.15 cell lines. Doong *et al.* showed the reduced HBV-specific RNAs in cell lysate after lamivudine and other nucleosides therapy (8). Our unpublished data revealed persistently detectable HBV RNA in cell culture supernatant from day 4 to day 17 of nucleoside analogue therapy when all the cells died. Lamivudine and other nucleoside analogues did not affect the integrated HBV DNAs that transcribe HBV RNA (8). Further studies are needed to evaluate the effect of long-term nucleoside analogue treatment on serum HBV RNA.

The inhibition of serum HBV RNA by interferon- α was in-line with studies on transgenic mice. Intrahepatic HBV replicative intermediates were cleared by a single injection of the interferon- α/β inducer polyinosinic-polycytidylic acid (20). The mechanisms involved in posttranscriptional steps of HBV life cycle were hypothesized as the clearance of intrahepatic HBV replicative intermediates but not the steady-state content of HBV RNA (20). Same group of researchers further showed that interferon- α/β inhibitory effect was at the level of pre-genomic RNA containing capsids through either step of preventing their assembly or accelerating their degradation (21). Interferon might directly inhibit HBV synthesis or act via cellular immune response against HBV infected hepatocytes (22). The HBV inhibition by interferon- β and interferon- γ in immortalized hepatocyte cell lines from HBV transgenic mice was in-line with the non-cytolytic inhibition pathway (23). This inhibition might act through the 2',5'-oligoadenyl synthetase/RNase L pathway (24). Interferon could induce this multi-enzymes pathway including 2',5'-oligoadenyl synthetase, endoribonuclease RNase L, and 2',5'-oligoadenyl phosphodiesterase. Among them, RNase L theoretically

inhibits all viral replication that utilizes an RNA intermediate step (22). Furthermore, activation of this ribonuclease is proposed as the major driver by which interferon inhibits viral replication (24).

Serum HBV RNA reflects antiviral potency of nucleoside analogues

We had demonstrated the dynamic change of serum HBV DNA and RNA during mono- or combination therapy (12). The peak serum HBV RNA level under entecavir treatment was significantly higher than that under lamivudine (12,13). The detectability of serum HBV RNA was also higher in entecavir treated patients as compare to those under lamivudine (12,13). Taking together, these findings suggest that serum HBV RNA level might reflect the antiviral potency of nucleoside analogue.

Entecavir is more potent than lamivudine in the inhibition of serum HBV DNA (7). Thus, entecavir more potently inhibits reverse transcriptase than lamivudine, leading to a higher level of serum HBV RNA. In contrast, entecavir or lamivudine does not directly affecting serum quantitative HBsAg (qHBsAg) as shown by the poor predictive value of serum qHBsAg levels on nucleoside analogue treatment outcomes (25) as well as the comparable serum qHBsAg levels between entecavir or lamivudine treated patients (13). These findings support the serum HBV RNA level, but not qHBsAg, might reflect the antiviral potency of nucleoside analogue.

We had also disclosed that serum HBV RNA might be a predictor of early emergence of viral mutation during lamivudine therapy (11). We assumed that the risk of viral mutant development is high in patients with large amount of serum HBV RNA. Whether the high level of serum HBV RNA originates from a large amount of cccDNA template in hepatocytes or from active transcription (or both) remains unknown.

Randomized double-blind trials revealed the mean log HBV DNA difference between lamivudine and entecavir treatment is around 0.5 to 0.8 copies/mL at treatment week 12 and 24 (26,27). Our study showed the mean log HBV RNA difference between lamivudine and entecavir therapy was 2.7 and 3.3 copies/mL at treatment week 12 and 24, respectively. Stronger suppression of HBV DNA by entecavir could not explain this difference. Instead, this difference suggested entecavir might cause higher level of serum HBV RNA. Further studies are needed to address this important issue.

Serum HBV RNA as on-treatment predictors

The most important determinant of therapeutic outcomes for CHB patients under nucleoside analogue is the intensity of on-treatment viral suppression (28). Although the correlation of baseline parameters and nucleoside analogue therapeutic outcomes has been shown, on-treatment predictors are far from clear (29-31). A roadmap approach utilizing on-treatment serum HBV DNA has been proposed (32). In addition, on-treatment serum qHBsAg level to predict nucleoside analogue treatment outcomes is unsatisfactory (25).

We disclosed that serum HBV RNA at treatment week 12 as a novel predictor of initial virologic response, independent of serum HBV DNA level at week 12, qHBsAg level at week 12 or pre-treatment serum ALT level (13). Nucleoside analogue treated CHB patients with interval from detectable to undetectable serum HBV DNA level of <16 weeks had a significantly lower week 12 serum HBV RNA level when compared to those with interval ≥ 16 weeks. Furthermore, a low week 12 serum HBV RNA level independently predicted a shorter interval to undetectable HBV DNA. Besides serum HBV DNA level, serum HBV RNA was the only independent on-treatment predictor of initial virologic response (13).

We also demonstrated that on-treatment (week 12) serum HBV DNA and RNA levels might predict HBV reactivation after discontinuation of nucleoside analogue, thus might be useful marker for safe discontinuation of nucleoside analogue treatment in CHB patients (14). The predictive value of serum HBV DNA and RNA levels at the end of treatment remains unknown (33).

The AASLD guidelines for CHB patients under lamivudine therapy recommend testing of serum HBV DNA every 3-6 months (12-24 weeks) (7). With roadmap approach, primary non-response in CHB patients treated with nucleoside analogue was detected at treatment week 12 (32). In addition, primary treatment failure is defined by alterations in serum HBV DNA levels at week 12 on monitoring for viral resistance development (34). As mentioned above, serum HBV RNA at lamivudine treatment week 12 predicted early emergence of YMDD mutation (11). We further showed that serum HBV RNA level at treatment week 12 predicted time to undetectable serum HBV DNA (13) as well as HBV reactivation after discontinuation of nucleoside analogue (14). Taking all together, monitoring at treatment week 12 is useful for patients treated with nucleoside analogue.

Serum HBV RNA levels correlated better with serum qHBsAg than with serum HBV DNA levels (13). Serum qHBsAg predicted treatment outcomes of nucleoside analogue poorly, except for HBeAg-positive patients with elevated ALT, where qHBsAg tended to decline (25). This decline was parallel with the gradual decline of serum HBV RNA during nucleoside analogue therapy (12). In contrast, serum HBV DNA usually displays more rapid decline and thus, not as well correlates with serum HBV RNA.

Conclusions

Serum HBV RNA could be detected during nucleoside analogue therapy as a consequence of interrupted RT and unaffected RNA replicative intermediates. In contrast with rapid decline under combination of nucleoside analogue and interferon, serum HBV RNA in nucleoside analogue monotherapy gradually declines (12). Thus, the inhibition of interferon on HBV RNA replicative intermediates might potentiate the suppression of HBV replication (12). We further disclosed that low on-treatment serum HBV RNA could predict earlier HBV suppression and response to nucleoside analogue therapy (13). Taken together, serum HBV RNA might be useful to optimize treatment outcomes in CHB patients, including shift to more effective oral antiviral drugs or to immunomodulatory interferon.

In daily clinical practice, complete collection of samples at several time points (pre- and on-treatment) and maintenance of good quality of easily degradable RNA samples by timely handling as well as storage in -80°C remain a daunting challenge. Furthermore, the predictive role of serum HBV RNA in long-term outcomes of nucleoside analogue treated patients was unclear. The evaluation of long-term outcomes of such patients was difficult due to the variable duration of nucleoside analogue therapy and the shift to interferon therapy in some. In addition, the predictive value of serum HBV RNA levels at the end of treatment was also unknown.

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