Serum hepatitis B virus ribonucleic acid and its influencing factors in chronic hepatitis B


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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

Abstract

Background. Hepatitis B virus (HBV) remains one of the most serious and prevalent health problems in the world.

Objectives. To determine the serum hepatitis B virus (HBV) RNA levels in patients with chronic hepatitis B (CHB) with low HBV DNA levels and analyze the influencing factors.

Materials and methods. Seventy-two CHB patients with low HBV DNA levels were enrolled and divided into 2 groups according to hepatitis B e antigen (HBeAg) status; their age, sex, the incidence of HBV RNA level < lower limit of detection (LLD), and serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), quantitative determination of HBsAg (qHBsAg), HBV DNA, and HBV RNA levels were compared. The factors influencing serum HBV RNA levels < LLD and the correlation between serum HBV RNA levels, and serum ALT, AST, qHBsAg and HBV DNA levels were analyzed.

Results. In HBeAg-positive patients, serum AST, qHBsAg and HBV RNA levels were higher, and serum HBV DNA levels and incidence of HBV RNA < LLD were lower than those in HBeAg-negative patients (p < 0.05). Multivariate linear regression analysis revealed that HBeAg is a factor that significantly influences serum HBV RNA levels in patients with CHB (p < 0.05). Multivariate logistic regression analysis indicated that HBeAg and qHBsAg are factors that influence serum HBV RNA levels < LLD in patients with CHB. In HBeAg-positive patients, serum HBV RNA levels were positively correlated with qHBsAg and HBeAg.

Conclusions. The serum HBV RNA levels in CHB patients with low HBV DNA levels varied according to HBeAg status. The HBeAg is a factor that significantly influences serum HBV RNA levels in patients with CHB, while HBeAg and qHBsAg are factors that significantly influence serum HBV RNA levels < LLD in patients with CHB.

Key words: chronic hepatitis B, DNA, hepatitis B virus, pre-gene RNA, serum markers
Background

Hepatitis B virus (HBV) infection remains one of the most serious and prevalent health problems in the world. Every year, 0.5–1.2 million patients die from liver decompensation, liver cirrhosis, liver failure, hepatocellular carcinoma, and other HBV-related diseases. The underlying cause of persistent HBV infection is the constant presence of covalently closed circular DNA (cccDNA). Further exacerbating the issue, existing serum markers and virological replication molecular assays are unable to accurately detect the presence of cccDNA in hepatocytes. Studies have demonstrated that despite the low concentration of HBV DNA in the serum of chronic hepatitis B (CHB) patients, cccDNA still actively replicates in vivo. This is evident through the observation of enhanced inflammation and fibrosis reported in liver histology. The monitoring of cccDNA in liver tissue requires an invasive liver biopsy, which is difficult to continuously implement in clinical practice. Additionally, the uneven distribution of cccDNA in liver tissue and the presence of relaxed circular double-stranded DNA (rcDNA) also increase the difficulty of detecting HBV.

In 2017, The European Association for the Study of the Liver Clinical Practice Guidelines defined serum HBV RNA as a novel marker and pointed out that pre-gene RNA (pgRNA), a major component of HBV RNA, is released into the blood as a virus-like particle. The pgRNA is a direct transcript of cccDNA that accurately reflects the presence of cccDNA in hepatocytes. The ability to detect serum HBV RNA will provide more direct evidence of the status of viral replication and its therapeutic efficacy in patients with CHB.

Objectives

This study explores the presence and influencing factors of serum HBV RNA at low replication levels in patients with CHB and aims to provide new ideas for the clinical diagnosis and treatment of CHB.

Materials and methods

Study criteria

Our research was a retrospective case-control study. Inclusion criteria were as follows: patients who satisfy the relevant diagnostic criteria in the Guidelines for Prevention and Control of Chronic Hepatitis B (2015 Edition) (hereinafter referred to as the “Prevention and Control Guidelines”). These criteria include: confirmed HBV infection for at least 6 months, and serum HBV DNA < 1.0 × 10^4 U/mL. Exclusion criteria were as follows: the diagnosis of other Hepadnaviridae infections, alcoholic liver disease, nonalcoholic fatty liver disease, drug-induced liver disease, liver injury due to metabolic and autoimmune liver disease or other causes, HIV-infected patients, and patients with primary biliary cirrhosis.

Ethics approval

This study was performed according to the Declaration of Helsinki and approved by the Clinical Research Ethics Committee of Hunan Provincial People's Hospital (Ethical Application Ref: 2018S77), and each patient provided written informed consent before participation in the study. The research was conducted in compliance with institutional review board regulations.

Subjects

Patients with CHB who were definitively diagnosed in our hospital from May 2018 to July 2018 and who met the study criteria were selected. All patients had low levels of serum HBV DNA and were treated according to the Prevention and Control Guidelines after admission.

Methodology

Sample collection and processing

Five milliliters of fasting venous blood was collected in the morning and added to an anticoagulation tube. After centrifugation at 4000 rpm for 10 min (centrifugation radius: 10 cm), the supernatant was collected, dispensed into 1.5-mL enzyme-free Eppendorf (EP) tubes, and stored at −80°C for further analysis.

Serum HBV DNA detection

The SLAN Real-Time PCR Detection System 96 fluorescence quantitative detector (Shanghai Hongshi Medical Technology Co., Ltd., Shanghai, China) was used to detect serum HBV DNA. The reagent was purchased from Hunan Sansure Biotech Inc. (Changsha, China) and the approved by the National Medical Products Administration (NMPA) quantitative polymerase chain reaction (qPCR) method was applied. Certified laboratory staff performed the assay in accordance with the instructions. The lower limit of detection (LLD) of the kit was 1.0 × 10^2 U/mL.

Detection of HBV serological markers (HBV-M)

A fully automatic chemiluminescence immunoassay analyzer, Caris200 (Beijing Wantai BioPharm Co., Ltd., Beijing, China), was used to detect HBV serological markers
Detection of liver function indicators

The Beckman 5800 fully automatic biochemical analyzer and the reagent purchased from Shanghai Kehua Bio-Engineering co., Ltd (Shanghai, China) were used to detect liver function. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were used as observation indicators; both were detected using the dual-reagent method.

Serum HBV RNA extraction and purification

For HBV RNA detection, the HBV pre-genomic RNA (pgRNA) from all samples was extracted and purified using a nucleic acid extraction (DNA/RNA) or purification kit (magnetic beads method) from Sansure Biotech Inc. (Registration certificate for medical device No. 20173401141).

The detailed protocol was as follows: 200 μL of each serum sample, 600 μL of solution 1 and 100 μL of solution 2 were added into EP tube and heated up to 60°C for 10 min. The samples were then placed at room temperature for 10 min. All tubes were placed on a magnetic rack for 3 min, after which the supernatant was discarded. Next, 600 μL of solution 3 and 200 μL of solution 4 were added to each tube. The tubes were then briefly vortexed and centrifuged. Then, they were placed on the magnetic rack for 3 min, after which the supernatant was carefully discarded. Next, 50 μL of elution solution was added to each tube. The tubes were then vortexed and centrifuged briefly for 3 min. Next, they were placed on a magnetic rack at room temperature for 10 min. The solution was then transferred to new EP tubes and stored at 4°C for further use. Before HBV RNA detection, the purified nucleic acid was treated with DNase. Briefly, 16 μL of each purified nucleic acid solution and 4 μL of DNase solution were added to each PCR tube. The tubes were sealed and placed at 37°C for 30 min and then at 75°C for 10 min. DNase-treated solutions were used for further HBV RNA detection.

Serum HBV RNA detection

The serum HBV RNA detection was processed using Hepatitis B Viral pregenomic miRNA Quantitative Fluorescence Diagnostic Kit (PCR-Fluorescence Probing) from Sansure Biotech Inc. The kit’s LOD was reported at 50 copies/mL.

The detailed protocol was as follows: 30 μL of HBV RNA master mix was added to each PCR tube. Then, 20 μL of purified and DNase-treated nucleic acid sample solution, purified HBV RNA quantitative reference RNA (A-B-C-D), as well as negative and positive RNA controls, were added to the PCR tubes. All PCR tubes were carefully sealed and placed into the Real-Time PCR Detection Systems (including Slan 96P (Shanghai Hongshi Medical Technology Co., Ltd., China) and ABI 7500 (Applied Biosystem, USA). The PCR program was run as follows: 95°C for 1 min, 60°C for 30 min and 95°C for 1 min, and followed as 45 thermocycles as 95°C for 15 s and 60°C for 30 s with fluorescence detection. The results were analyzed according to the kit user manual.

Observation indicators

To compare patient age, sex, the incidence of HBV RNA below the LLD, as well as serum ALT, AST, qHBsAg, HBV DNA, and HBV RNA levels, all patients were divided into HBeAg-positive and HBeAg-negative patients according to their HBeAg status. The serum HBV RNA levels in patients with CHB and factors which influence serum HBV RNA levels that were below the LLD were analyzed. The correlation of serum HBV RNA levels with serum ALT, AST, qHBsAg, and serum HBV DNA levels was also analyzed.

Statistical methods

Data were processed using the SPSS v.19.0 statistical software (IBM Corp., Armonk, USA). Statistical graphs were plotted using GraphPad Prism 6.0 (GraphPad Software, San Diego, USA). Logarithmic transformation (log) was adopted for all laboratory data used for serum detection of patients with CHB. Quantitative data that satisfied normal distribution were expressed by x ± s, and the paired t-test was used for comparison between both groups. Pearson correlation analysis was applied; non-normally distributed measurement data were expressed using M (P25, P75) after logarithmic transformation. The Mann–Whitney U test was used for intergroup comparison, and Spearman’s rank correlation analysis was applied; enumeration data were expressed as percentages. The χ2 test was used for intergroup comparison; linear regression analysis and binary logistic regression analysis were used to analyze the influencing factors of HBV RNA. A p-value <0.05 was used to indicate statistical significance.

Results

Seventy-two cases of CHB patients with low levels of HBV DNA were included in this study. The average age of the patients was 49.49 ±13.539 years. Among them, 46 were men and 26 were women. Among the 72 patients, 12 were HBeAg-positive and 60 were HBeAg-negative. There was no statistically significant difference in terms
of sex or serum ALT levels between HBeAg-positive and HBeAg-negative patients ($p > 0.05$). HBeAg-positive patients were younger than HBeAg-negative patients; their serum AST, qHBsAg and HBV RNA levels were higher than those in HBeAg-negative patients, while their serum HBV DNA levels and incidence of HBV RNA below the LLD were lower than those in HBeAg-negative patients. These differences were statistically significant ($p < 0.05$, Table 1).

To perform the univariate linear regression analysis, age (assigned value: continuous variable), sex (men = 1, women = 2), ALT (assigned value: continuous variable), AST (assigned value: continuous variable), qHBsAg (assigned value: continuous variable), HBeAg (assigned value: HBeAg-positive = 0, HBeAg-positive = 1), and HBV DNA (assigned value: continuous variable) were taken as independent variables. Further, serum HBV RNA (assigned value: continuous variable) was taken as the dependent variable. The results demonstrated that age, AST, qHBsAg, and HBeAg are factors that significantly influence serum HBV RNA levels in patients with CHB ($p < 0.05$). Independent variables with $p < 0.20$ in the univariate analysis were included in the multivariate linear regression analysis. The results demonstrated that HBeAg is a factor that significantly influences serum HBV RNA levels in patients with CHB ($p < 0.05$, Table 2).

To perform multivariate logistic regression analysis of the factors which influence serum HBV RNA levels to be below the LLD, age (assigned value: continuous variable), sex (men = 1, women = 2), ALT (assigned value: continuous variable), AST (assigned value: continuous variable), HBeAg (assigned value: continuous variable), HBeAg (assigned value: HBeAg-positive = 0, HBeAg-positive = 1), and HBV DNA (assigned value: continuous variable) were taken as independent variables. Whether serum HBV RNA was below the LLD (assigned value: yes = 1, no = 0) was taken as the dependent variable. The results demonstrated that HBeAg (odds ratio (OR) = 3.85, 95% confidence interval (95% CI) [1.068, 13.880], $p < 0.039$) and qHBsAg (OR = 1.682, 95% CI [1.055, 2.681], $p < 0.029$) are factors which significantly influence serum HBV RNA levels to be below the LLD in patients with CHB.

Serum HBV RNA in patients with CHB was positively correlated with qHBsAg ($r = 0.322$, $p = 0.006$) (Fig. 1A) and HBeAg ($r = 0.235$, $p = 0.047$) (Fig. 1B) but was not correlated with ALT ($r = 0.038$, $p = 0.752$), AST ($r = 0.189$, $p = 0.557$) or HBV DNA ($r = 0.058$, $p = 0.629$). In HBeAg-positive patients, serum HBV RNA levels were positively correlated with qHBsAg ($r = 0.848$, $p < 0.001$) (Fig. 1C) and HBeAg ($r = 0.725$, $p = 0.008$) (Fig. 1D) but were not correlated with ALT ($r = 0.051$, $p = 0.876$), AST

### Table 1. Comparison of clinical data between HBeAg-positive and HBeAg-negative patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>HBeAg-positive</th>
<th>HBeAg-negative</th>
<th>Test statistic</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>12</td>
<td>60</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Age [years]</td>
<td>40.4 ±10.5</td>
<td>51.3 ±13.4</td>
<td>–2.649‡</td>
<td>0.01</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>9/3</td>
<td>37/23</td>
<td>0.301‡</td>
<td>0.58</td>
</tr>
<tr>
<td>ALT (M (P25, P75)) [U/L]</td>
<td>1.74 (1.44, 1.95)</td>
<td>1.39 (1.19, 1.70)</td>
<td>210§</td>
<td>0.23</td>
</tr>
<tr>
<td>AST (M (P25, P75)) [U/L]</td>
<td>1.63 (1.52, 1.94)</td>
<td>1.40 (1.27, 1.64)</td>
<td>191§</td>
<td>0.011</td>
</tr>
<tr>
<td>qHBsAg (M (P25, P75)) [U/mL]</td>
<td>3.45 (1.46, 3.77)</td>
<td>2.74 (1.34, 3.23)</td>
<td>216§</td>
<td>0.03</td>
</tr>
<tr>
<td>HBV DNA (M (P25, P75)) [U/mL]</td>
<td>2.06 (2.00, 2.88)</td>
<td>2.93 (2.68, 3.19)</td>
<td>174§</td>
<td>0.005</td>
</tr>
<tr>
<td>HBV RNA (M (P25, P75)) [U/mL]</td>
<td>2.39 (1.70, 4.69)</td>
<td>1.70 (1.70, 1.74)</td>
<td>212§</td>
<td>0.007</td>
</tr>
<tr>
<td>HBV RNA below the LLD, n (%)</td>
<td>5 (42.0)</td>
<td>44 (73.0)</td>
<td>4.613‡</td>
<td>0.032</td>
</tr>
</tbody>
</table>

Data are expressed as means ± standard deviation (SD). † t-value; § Z-value; HBV – hepatitis B virus; HBeAg – hepatitis B e antigen; LLD – lower limit of detection; ALT – alanine aminotransferase; AST – aspartate aminotransferase; qHBsAg – quantitative determination of HBsAg.

### Table 2. Univariate and multivariate linear regression analysis of factors influencing serum HBV RNA levels in CHB patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>B</th>
<th>Univariate analysis</th>
<th>p-value</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SD</td>
<td>t-value</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>−0.024</td>
<td>0.009</td>
<td>−2.770</td>
<td>0.007</td>
</tr>
<tr>
<td>Sex</td>
<td>−0.256</td>
<td>0.254</td>
<td>−1.007</td>
<td>0.317</td>
</tr>
<tr>
<td>ALT</td>
<td>0.292</td>
<td>0.300</td>
<td>0.976</td>
<td>0.332</td>
</tr>
<tr>
<td>AST</td>
<td>0.651</td>
<td>0.388</td>
<td>1.678</td>
<td>0.098</td>
</tr>
<tr>
<td>qHBsAg</td>
<td>0.273</td>
<td>0.092</td>
<td>2.956</td>
<td>0.004</td>
</tr>
<tr>
<td>HBeAg</td>
<td>1.164</td>
<td>0.299</td>
<td>3.897</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HBV DNA</td>
<td>0.05</td>
<td>0.224</td>
<td>0.222</td>
<td>0.825</td>
</tr>
</tbody>
</table>

HBV – hepatitis B virus; CHB – chronic hepatitis B; SD – standard deviation; HBeAg – hepatitis B e antigen; ALT – alanine aminotransferase; AST – aspartate aminotransferase; qHBsAg – quantitative determination of HBsAg.
In HBeAg-negative patients, serum HBV RNA was not significantly correlated with qHBsAg (r = 0.151, p = 0.251), HBeAg (r = 0.083, p = 0.529), ALT (r = −0.020, p = 0.878), AST (r = −0.080, p = 0.542), or HBV DNA (r = 0.145, p = 0.268).

Discussion

The current treatment of patients with CHB remains challenging, with difficulties in drug discontinuation and easy rebound among the issues. Existing serological markers do not accurately reflect the existence of cccDNA in hepatocytes. In 1996, when studying whether HBV can infect human peripheral blood mononuclear cells, Kock et al. detected HBV RNA in the serum of patients with CHB. In recent years, both national and international studies have conducted extensive research on continuously improving and optimizing HBV detection methods, and further exploring the clinical implications of serum HBV RNA.

The Prevention and Control Guidelines suggest that the ideal treatment-endpoint for HBeAg-positive and HBeAg-negative patients with CHB is the clinical observation of sustained HBsAg clearance, with or without seroconversion after drug withdrawal. However, HBsAg cannot accurately reflect the replication of HBV in hepatocytes. Furthermore, the HBeAg-negative conversion rate is not an ideal marker of CHB pathogenesis during the actual treatment. This study compared serum HBV RNA levels in HBeAg-positive and HBeAg-negative patients with CHB with low levels of HBV DNA replication and found significant differences in serum HBV RNA levels between both groups. The results of this study demonstrated that HBeAg-positive patients were younger than HBeAg-negative patients; this was similar to the results obtained by Yang et al. In that study, the authors suggested that the incidence of negative HBeAg correlated with age. Further analysis of potential influencing factors demonstrated that HBeAg is a factor that significantly influences serum HBV RNA levels in patients with CHB. Further, we observed that serum HBV RNA was positively correlated with HBeAg in patients with CHB, and serum HBV RNA levels were positively correlated with HBeAg in HBeAg-positive patients. These data indicate that serum HBV RNA can accurately reflect the replication status of the virus.

Fig. 1. Correlation of HBV RNA with serum levels of qHBsAg and HBeAg. The LLD for the HBV RNA PCR is 50 U/mL (1.70 log₁₀ U/mL).
in patients with CHB. In a previous study, Huang et al.\textsuperscript{14} found that serum HBV RNA levels may be used as a serological marker for predicting the seroconversion of HBeAg during treatment with nucleosides and nucleotides.\textsuperscript{15} 

While, to a certain extent, HBV DNA can reflect the replication activity of cccDNA in vivo, the low detection rate of HBV RNA is consistent with this phenomenon.\textsuperscript{5} However, serum HBV DNA below the lower limit of detection only indicates that the reverse transcription process of the virus is inhibited and cannot reflect the transcriptional status of cccDNA. After the reverse transcription process is inhibited, cccDNA still produces progeny virus in the form of HBV RNA virus-like particles.\textsuperscript{5} Therefore, following drug withdrawal on the premise of existing virological response, the rates of disease rebound, and recurrence are higher.\textsuperscript{5,12,16,17} The results of this study demonstrate that there was no correlation between serum HBV DNA and HBV RNA in patients with CHB, and that serum HBV DNA was not correlated with HBV RNA in patients with different HBeAg status. These data indicate that even when HBV DNA is at a low level or below the lower limit of detection, cccDNA maintains its replication activity which results in HBV RNA being detected at a relatively high level. Thus, serum HBV RNA can reflect the presence of cccDNA more accurately than serum HBV DNA, thereby providing more powerful evidence for the efficacy of antiviral treatment in patients with CHB.\textsuperscript{5,18,19} 

The incidence of serum HBV RNA below the lower limit of detection is higher in HBeAg-negative patients than in HBeAg-positive patients. This may be due to the higher cccDNA transcriptional activity in HBeAg-positive patients compared to HBeAg-negative patients, which is consistent with findings from other studies.\textsuperscript{20} Multivariate logistic regression analysis revealed that qHBsAg and HBeAg are factors that influence serum HBV RNA below the lower limit of detection in patients with CHB. In this study, the total detection rate of serum HBV RNA in patients with CHB was 32% (23/72), which was similar to the detection rate in the study by Huang et al.\textsuperscript{12} However, the detection rate in the study conducted by Li et al.\textsuperscript{21} was 85.3%. Among other factors, this difference may be due to sample selection, demographic characteristics and variable methodology. 

The results of this study demonstrate that serum HBV RNA is positively correlated with HBsAg in HBeAg-positive patients; however, there is no significant correlation between the two in HBeAg-negative patients. This may be because HBsAg can be synthesized, not only from cccDNA but also from the integrated HBV gene fragments. Studies have shown that in HBV-infected patients, hepatocytes carrying integrated HBV DNA fragments account for approximately 1% of total hepatocytes and that HBV DNA is transcribed and translated into HBsAg after integration. This may be an important factor that contributes to the failure to completely clear HBeAg in clinical practice.\textsuperscript{22,23} Therefore, HBsAg has certain shortcomings in terms of judging whether patients have reached the level of clinical cure. In contrast, serum HBV RNA is a direct transcript of cccDNA, and can more directly reflect the replication activity of cccDNA in vivo compared with HBsAg. Several studies have suggested that HBV RNA should be included in the clinical diagnosis, disease progression monitoring and the rational withdrawal of antiviral drugs.\textsuperscript{24–26} However, its use as an established serum marker in clinical practice still requires more accurate, sensitive, and standardized detection methods, as well as more sophisticated prospective studies.

Limitations

The main limitation of our study was the size of the study sample.

Conclusions

There is a marked difference in serum HBV RNA levels in CHB patients with low HBV DNA levels and different HBeAg statuses. The HBeAg is a factor that influences serum HBV RNA levels in patients with CHB, while HBeAg and qHBsAg are factors that influence serum HBV RNA levels to be below the lower limit of detection in patients with CHB. Concurrently, serum HBV RNA levels in patients with CHB are correlated with other serological markers. This suggests that serum HBV RNA levels in patients with CHB can reflect virus activity to a certain extent. We, thus, believe that HBV RNA can be utilized as a novel serum marker for the detection of HBV infection.

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