Change of Cytokines in Chronic Hepatitis B Patients and HBeAg are Positively Correlated with HBV RNA, Based on Real-world Study

Qiqi Zhang1*, Hui Huang1*, Aijun Sun1*, Chunyan Liu1*, Zhidong Wang1, Feifan Shi1, Wei Duan1, Xueying Sun1, Qi Wang1, Ping Sun1, Chunwen Pu*1 and Yong Zhang2

1Department of Biobank, The Sixth Affiliated People’s Hospital of Dalian Medical University, Dalian, Liaoning, China; 2Outpatient Department of Hepatitis, The Sixth Affiliated People’s Hospital of Dalian Medical University, Dalian, Liaoning, China

Abstract

Background and Aims: The natural course of chronic hepatitis B virus (HBV) infection is widely studied; however, follow-up studies of the same patients are scanty. Here, we studied the dynamic changes of serum HBV RNA and cytokines in hepatitis B virus e antigen (HBeAg)-positive patients treated with entecavir (ETV) to explore the relationship between the serum viral nucleic acids and host immunity. Methods: Thirty-three chronic hepatitis B patients who are HBeAg-positive, with high virus load (HBV DNA >20,000 IU/mL), and received standard nucleos(t)ide analogue (NA) antiviral therapy (ETV) for more than 48 weeks were included. The serum levels of HBV nucleic acids and selected cytokines were measured at 0, 12, 24, and 48 weeks respectively. Results: Serum HBV RNA could still be detected while serum HBV DNA had fallen below the detection limit in patients treated with ETV. There was a strong positive correlation between HBV RNA and HBeAg, with a concomitant decrease in the secretion of cytokines from type 1 helper T (Th1)/type 2 helper T (Th2)/interleukin (IL)-17 producing T (Th17) cells. IL-4 and IL-10 were still be detected while serum HBV DNA had fallen below the detection limit in patients treated with ETV. There was a strong positive correlation between HBV RNA and HBeAg, with a concomitant decrease in the secretion of cytokines from type 1 helper T (Th1)/type 2 helper T (Th2)/interleukin (IL)-17 producing T (Th17) cells. IL-4 and IL-10 were the main cytokines negatively associated with serum HBV RNA. Conclusions: HBeAg can be used to reflect the load of HBV DNA indirectly, because serum HBV RNA has not been widely used in clinical practice. Meanwhile, serum IL-4 and IL-10 might be explored in combination with HBV RNA in guiding future clinical antiviral therapy.


Introduction

Globally, chronic infection with the hepatitis B virus (HBV) affects more than 250 million individuals, and can lead to the development of liver cirrhosis, liver failure, and hepatocellular carcinoma, despite the availability of effective HBV vaccines. As a highly prevalent virus, hepatitis B infection is considered to be a non-cellular disease, with its pathogenesis mediated by innate immunity and adaptive immune response. Although HBV may initially escape the innate immune defense, T cells have dual antiviral effects on HBV infection, both cytopathic and non-cytopathic. In addition, effective B cell and neutralizing antibody responses are lacking. There was evidence published showing that the virus could be cleared by activation of immunity. HBV control is correlated with the expansion of intrahepatic HBV-specific CD8+ T cells and peripheral cytotoxic T cells with the enhanced production of cytokines.

Serum HBV DNA load is the only biomarker for clinical monitoring of the number of circulating viral particles in peripheral blood. In addition, serum HBV load can be used as an independent indicator for the prediction of the patient response to antiviral treatment. However, HBV DNA was undetected in most patients treated with nucleos(t)ide analogues (NAs). Of note, there is no reliable indicator to reflect the replication of HBV in the liver. In fact, the disappearance of HBV DNA in serum only indicates an effective inhibition of the HBV reverse transcription but not the transcriptional...
had drug-induced hepatitis, alcoholic hepatitis, nonalcoholic of Helsinki. The criteria for exclusion were: (1) patients who
received combination therapy with other NAs. Moreover,
this study was carried out by following the guidelines set
out in European Association Expert Consensus published in
J Hepatol in 2017.14 and the East Asian Expert Consensus
published in Aliment Pharmacol Ther in 2020.15

The patients received ETV treatment continuously for
48 weeks, and were followed up every 12 weeks. Patients
naive to ETV treatment were defined as the baseline. The
clinical indicators include: HBV DNA load, anti-HBsAg
(anti-HBs), HBeAg, anti-HBe, anti-hepatitis B core antigen
(anti-HBc), lymphocyte count and serum alanine aminotransferase (ALT) levels. The work was approved by
the Ethics Committee of Dalian Sixth People’s Hospital (ap-
proval number: 2018-026-002).

### Table 1. Baseline characteristics of CHB patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>All</th>
</tr>
</thead>
<tbody>
<tr>
<td>Participants</td>
<td>33</td>
</tr>
<tr>
<td>Participants with HBeAg positivity</td>
<td>33</td>
</tr>
<tr>
<td>Participants with HBeAg negativity</td>
<td>0</td>
</tr>
<tr>
<td>Age in years</td>
<td>47.4±1.9</td>
</tr>
<tr>
<td>Male/Female</td>
<td>25/8</td>
</tr>
<tr>
<td>HBV DNA in log_{10}IU/mL</td>
<td>6.81±0.64</td>
</tr>
<tr>
<td>ALT in U/L</td>
<td>191.98±37.54</td>
</tr>
<tr>
<td>TBIL in μmol/L</td>
<td>37.20±10.30</td>
</tr>
</tbody>
</table>

CHB, chronic hepatitis B; HBeAg, hepatitis B virus e antigen; ALT, alanine aminotransferase; TBIL, total bilirubin.

activity of covalently closed circular DNA (cccDNA).7 Wang et al.8 revealed that HBV RNA is transcribed from the ac-
tive cccDNA in infected liver cells. Such RNA is pregenomic RNA (pgRNA) that has not been reverse-transcribed and ex-
ists in the nucleocapsid of mature virus particles. Since the transcription from cccDNA to pgRNA is one of the important
steps in HBV replication, pgRNA is regarded as a template for reverse transcription and synthesis of the HBV genome.
Therefore, serum HBV pgRNA is considered as an import-
ant new biomarker, which reflects the sustainability of HBV
infection as well as the transcriptional activity of cccDNA
in the liver. Furthermore, it is also regarded as a predictor of
the efficacy of antiviral therapy and the development of
early resistance in patients, or a biomarker for prompting
the discontinuation of NAs treatment.9,10

The serum HBV pgRNA load has been shown to be asso-
ciated with the development of the cytotoxic lymphocyte
(CTL) immunodeficiency in chronic hepatitis B (CHB) pa-
tients, as well as the induction of HBV-related immune-me-
diated inflammatory liver injury.11,12 Therefore, it is neces-
sary to understand the relationship between HBV pgRNA and
host immunity in CHB patients receiving antiviral treat-
ment. In this work, we conducted a study on the dynamic
changes of serum HBV RNA levels and representative cy-
tokines released by type 1 helper T (Th1)/type 2 helper
T (Th2)/interleukin (IL)-17 producing T (Th17) cells with
treatment with ETV. We found that serum hepatitis B virus e antigen (HBeAg) levels could be used to predict
serum HBV RNA levels in HBeAg-positive (+) CHB patients
with ETV. In addition, serum IL-4 and IL-10 were the
main cytokines negatively associated with HBV RNA. When
combined with HBV RNA, these two cytokines could be use-
ful in guiding clinical antiviral therapy.

### Methods

### Patients

We evaluated 33 HBeAg(+) individuals who had received
at least 48 weeks of ETV treatment from December 2015
to July 2019 (Table 1). In short, the criteria for inclusion
were: (1) HBsAg-positive for at least 6 months; (2) HBeAg
positive, anti-HBe (anti-HBe)-negative; (3) HBV DNA
>20,000 IU/mL; and (4) patients who were naïve to NAs
therapy before recruitment, but who received monother-
apy with ETV. The informed consent was obtained for all pa-
tients according to the ethical standards of the Declaration
of Helsinki. The criteria for exclusion were: (1) patients who
had drug-induced hepatitis, alcoholic hepatitis, nonalcoholic

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steatohapatitis, autoimmune hepatitis, liver fibrosis >F4 or
hepatocellular carcinoma, and FibroScan was used to evalu-
ate the degree of liver fibrosis (F4 >17.5);13 or (2) patients
receiving combination therapy with other NAs. Moreover,
this study was carried out by following the guidelines set
out in European Association Expert Consensus published in
J Hepatol in 2017.14 and the East Asian Expert Consensus
published in Aliment Pharmacol Ther in 2020.15

Clinical and serological parameters

The reagent kits of HBsAg, anti-HBs, HBeAg, and
anti-HBc were purchased from Abbott Laboratories in Chi-
cago, IL, USA (HBsAg: Cat# 6C36, Lot No. 24052FN01; anti-
HBc: Cat# 7C18, Lot No. 23294FN00; HBeAg: Cat# 6C32, Lot
No. 23193BE01; anti-HBe: Cat# 6C34, Lot No. 24055BE01;
anti-HBc: Cat# 6C33, Lot No. 24324BE01). Serum HBV DNA
level was quantified using the HBV PCR Fluorescence Quan-
titative Detection Kit from Tianlong Bio-technology in Su-
zhou, China (Cat# 6C34, Lot No. P1372104001), with 500
IU/mL as the lower limit of detection. ALT was quantified
using the Alanine Aminotransferase Test Kit from Ruiyuan
Bio-technology in Ningbo, China (Cat# 1040-717, Lot No.
2020100902). CELLPACK DCL from Beckman-Coulter Ex-
perimental System in Suzhou, China (Cat# 628020, Lot No.
2010141) was used to test blood cell analysis, including the
absolute value of lymphocytes, absolute values of mono-
cytes and absolute values of neutrophils.

Extraction and quantification of HBV pgRNA

The levels of HBV pgRNA in serum were detected using the
HBV-pgRNA assay kit from Hotgen in Beijing, China (Lot
No. 20191001) following the manufacturer’s protocol with
the 7500 Real-time PCR Instrument from Applied Biosys-
tems in Marsiling, Singapore. The specific primers for pgR-
NA were 5′-CACCGCCTCTGCTCTGTATCG-3′ (forward) and
5′-TAGGGGCATTTGGTGGTCTGTAAG-3′ (reverse). The lower
limit of HBV RNA was calculated to be 150 copies /mL.

Determination of cytokine levels

Serum cytokine concentration, including of interferon-gamma
(IFN-γ), tumor necrosis factor-alpha (TNF-α), IL-2, IL-4,
IL-8, IL-10, IL-12 p70 heterodimer (IL-12p70), IL-17A and
macrophage inflammatory protein-1-beta (MIP-1β), was
determined using the MILLIPLEX® MAP Human High Sensi-
tivity T Cell Magnetic Bead Panel 96-Well Plate Assay from
Merck Millipore in Darmstadt, Germany (Cat# HSTMAG-28S,
Lot No. HSTMAG-28SK-05).

### Statistical analysis

Measured concentrations of HBV nucleic acids (HBV DNA
and HBV RNA) and antigen or antibody (HBeAg or anti-HBe)
were logarithmically transformed (log10) for analysis. Non-

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parametric tests, including the Mann-Whitney test, Spearman's correlation test, and multiple linear regression analysis were performed with SPSS 23.0 (IBM Corp., Armonk, NY, USA). All tests were two-tailed, and a p-value of less than 0.05 was considered to be statistically significant.

Results

Dynamic changes of HBV RNA, HBV DNA, HBeAg and anti-HBe during ETV treatment in HBeAg(+) CHB patients

In order to understand the replication of HBV in HBeAg(+) CHB patients treated with ETV, the levels of serum HBV DNA, HBV RNA, HBeAg and anti-HBe were evaluated within 48 weeks. We found that the levels of HBV DNA, HBV RNA, ALT and HBeAg decreased (data for ALT shown in Supplementary Fig. 1). The serum concentrations of each in the ETV group (12 weeks, 24 weeks and 48 weeks) were significantly decreased, compared with the baseline group (p<0.01). At the 12th week, there were five patients with HBeAg seroconversion (SC) (5/33), and no patients achieved HBeAg seroconversion out to 48 weeks (Fig. 1).

Serum HBeAg was positively correlated with HBV RNA during ETV treatment

Serum HBV DNA, HBV RNA and HBeAg levels were found to decrease in subjects treated with ETV in this study. However, it was not clear whether the HBV nucleic acids and the HBeAg protein in the serum were interrelated. Thus, we set out to analyze the potential correlation between HBV nucleic acid and HBeAg (Fig. 2). At baseline, we found that HBV DNA was positively correlated with the level of HBV RNA (r=0.564, p<0.001) and HBeAg (r=0.464, p<0.01) respectively. However, such correlation was not observed at the 12th, 24th and 48th weeks (p>0.05). It is noteworthy that serum HBV RNA was positively correlated with HBeAg, and the correlation became stronger with the prolongation of the ETV treatment. In addition, the serum concentrations of HBsAg, anti-HBs and anti-HBc were also determined and no correlation with HBV RNA was observed (data not shown). HBeAg is not a structural component of HBV nor is it necessary for HBV DNA replication. The presence of HBeAg indicates that liver tissue is still infected with HBV continuously.
Fig. 2. Relationship between serum HBV RNA, HBV DNA and HBeAg. (A) Correlation between serum HBV DNA and HBV RNA during ETV treatment over 0–12 weeks. (B) Correlation between serum HBV DNA and HBeAg during ETV treatment over 0–12 weeks. (C) Correlation between serum HBV RNA and HBeAg during ETV treatment over 0–48 weeks. HBV, hepatitis B virus; HBeAg, hepatitis B virus e antigen; ETV, entecavir.
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The serum HBeAg was found to positively correlated to serum HBV RNA in this study but the underlying mechanism remains to be elucidated.

**Dynamic changes of cytokines in patients with HBeAg(+) CHB treated with ETV**

Chronic HBV infection leads to immune regulatory dysfunction. The balance of the ratio between Th1 and Th2 T lymphocytes is one of the main factors responsible for effective elimination of virus and virus-infected cells. Under the stimulation of HBV-specific antigens, CD4 T lymphocytes differentiate into Th1 and Th2 cells, driven by different cytokines; thus, the balance between the cytokine networks is altered. Therefore, the assessment of the relationship between HBV RNA and cytokines may provide some insight into the antiviral immune network. To this end, we first explored the number of immune cells in the peripheral blood, and found neutrophils increased significantly at 48 weeks (p<0.05) compared with that at the baseline, but there was no statistically significant difference in the number of lymphocytes and monocytes at various time points studied (Fig. 3A).

Interestingly, the levels of serum IL-4, IL-10, IL-12, IFN-γ and IL-17 were found to decrease following ETV treatment. Specifically, the serum levels of IL-4, IL-10, IL-12 and IFN-γ were significantly decreased at 24 weeks (Fig. 3B–E) and 48 weeks (Fig. 3B–D) compared with that at baseline. The se-
Table 2. Factors associated with HBV RNA during ETV treatment (final multivariate models)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>12 weeks</th>
<th>24 weeks</th>
<th>48 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Coefficient (95% CI)</td>
<td>p</td>
<td>Coefficient (95% CI)</td>
<td>p</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.102 (−0.357, 0.560)</td>
<td>0.685</td>
<td>0.053 (−0.594, 0.699)</td>
<td>0.488</td>
</tr>
<tr>
<td>IL-2</td>
<td>0.012 (−0.562, 0.586)</td>
<td>0.928</td>
<td>−0.007 (−0.055, 0.041)</td>
<td>0.719</td>
</tr>
<tr>
<td>IL-8</td>
<td>0.003 (−0.007, 0.013)</td>
<td>0.996</td>
<td>0.002 (−0.004, 0.008)</td>
<td>0.985</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>−0.021 (−0.016, 0.014)</td>
<td>0.273</td>
<td>0.011 (−0.010, 0.032)</td>
<td>0.238</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.026 (−0.096, 0.147)</td>
<td>0.623</td>
<td>0.100 (−0.043, 0.243)</td>
<td>0.219</td>
</tr>
<tr>
<td>IL-10</td>
<td>−0.037 (−0.071, 0.007)</td>
<td>0.022</td>
<td>0.000 (−0.013, 0.013)</td>
<td>0.160</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>−0.011 (−0.430, 0.195)</td>
<td>0.394</td>
<td>0.000 (−0.013, 0.013)</td>
<td>0.160</td>
</tr>
<tr>
<td>IL-17A</td>
<td>−0.006 (−0.055, 0.041)</td>
<td>0.719</td>
<td>−0.006 (−0.055, 0.041)</td>
<td>0.719</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.000 (−0.007, 0.007)</td>
<td>0.947</td>
<td>0.000 (−0.007, 0.007)</td>
<td>0.947</td>
</tr>
<tr>
<td>IL-12</td>
<td>−0.011 (−0.562, 0.586)</td>
<td>0.928</td>
<td>−0.007 (−0.055, 0.041)</td>
<td>0.719</td>
</tr>
</tbody>
</table>

HBV, hepatitis B virus; ETV, entecavir; IL, interleukin; IFN-γ, interferon-gamma; TNF-α, tumor necrosis factor-alpha; IL-12p70, IL-12 p70 heterodimer; MIP-1β, macrophage inflammatory protein-1-beta. CI, confidence interval.

Correlation and multivariate analysis of the factors related with HBV RNA

In order to clarify the relationship between serum levels of HBV RNA and cytokines, we performed correlation analysis. At baseline, serum HBV RNA and IL-12p70 were negatively correlated ($r=-0.316, p<0.05$). With ETV treatment, HBV RNA was negatively correlated with IL-10 at 24 weeks ($r=-0.055, p<0.017$), while serum IL-10 level is significantly associated with HBV RNA at 24 weeks of ETV treatment ($B=-0.602, p=0.046$) (Table 2).

Discussion

HBV RNA could reflect the status of HBV cccDNA in liver cells. Thus, HBV RNA was proposed as a serum biomarker for evaluating the dynamic changes of HBV, especially when HBV DNA could not be detected with NA treatment. In this study, we found that ETV was associated with reduced serum HBV nucleic acid when the load of HBV DNA could not be detected at 24 weeks, indicating that HBV RNA could be used to monitor the level of HBV replication during NA treatment. At the same time, not only the serum HBV nucleic acid decreased but the HBeAg protein was also significantly reduced. This might be due to the fact that pgRNA is similar in structure to HBeAg. Cornberg et al. have found that pgRNA encodes the P protein, which is an HBeAg and HBeAg premise protein. Therefore, the dynamic changes of HBV RNA were consistent with that of serum HBeAg protein level.

Clinically, HBV RNA could be utilized to evaluate the treatment of NAs. Furthermore, the relationship between HBV levels and HBeAg has been extensively explored. 

In this study, we found that the HBV RNA and HBeAg were positively correlated in untreated patients. Ji et al. found that the HBV RNA level at 12 weeks of NAs treatment could be used as an independent predictor of viral response or HBeAg seroconversion. Butler et al. found that the correlation between HBV DNA and pgRNA levels in CHB subjects was low during NA treatment, and the concentration of HBV RNA was usually higher than that in HBV DNA. In contrast, in patients not treated with NA, the concentration of serum HBV RNA was positively correlated with HBV DNA. In this study, serum HBV DNA, HBV RNA and HBeAg were only positively correlated in untreated patients at the 12th week; however, the correlation disappeared during the follow-up ETV treatment. Bömmel et al. showed that in the era of an age of NAs treatment, the decrease of HBV DNA was strongly related to HBeAg seroconversion.
HBV infection. At the 12th week of ETV treatment, IL-4 mediates liver neutrophil recruitment and inflammation and hepatocellular damage, especially the number of neutrophils increased during ETV treatment. Naumenko et al. revealed that when the liver tissue was infected by HBV, neutrophils were rapidly recruited from the peripheral circulation to the infected site, driven by inflammatory factors and chemokines, and participated in removal of the pathogen. In this study, we found that the level of chemokines (IL-8 and MIP-1b) increased at the 48th week of ETV treatment. In addition, studies by others have shown that neutrophils regulate the maturation and activation of natural killer (NK) cells. Activated neutrophils also promote the maturation of myeloid dendritic cells, the proliferation of T cells and the differentiation of Th1 and Th17, while NK cells and Th1 cells are important immune cells for virus clearance. The cytokines secreted by Th1 and Th17 cells were found to decrease in their studies, which is consistent with the findings in our study. Ye et al. also found that the balance between CD4+ Th response (Th1 and Th1 response) and the regulatory response was an important part of immune regulation. Inappropriate, excessive and non-specific Th17 and Th1 effector responses might be related to the pathogenesis of HBV-associated liver inflammation and hepatocellular damage, especially the Th17 response. IL-17-mediated liver neutrophil recruitment via the induction of IL-8 might lead to liver injury in CHB patients. Neutrophils in hepatitis patients would also trigger some structural changes and dysfunctions. Th1/Th2 cells maintain a relatively balanced state in normal circumstances, but this balance becomes distorted with HBV infection. At the 12th week of ETV treatment, IL-10 was the main cytokine found to be negatively correlated with serum HBV RNA level, compared to other cytokines. At the 24th week, IL-10 became the main factor to be negatively correlated with serum HBV RNA level, compared to other cytokines. It is generally believed that IL-4 promotes the differentiation of T lymphocytes into Th2 cells. IL-4 inhibits the function of Th1 cells and limits the Th1 type response, so that the secretion of Th1 cytokines (IL-2 and IFN-γ, etc.) is reduced, leading to the reduced immune response and weakened ability to clear viruses, culminating in viral rebound. It is also believed that the elevated serum level of IL-10 may be partly responsible for the poor clinical outcome of antiviral treatment. Because IL-10 is a powerful immune and inflammatory inhibitory factor, it not only inhibits Th1 cellular immune response but also inhibits cytokines secreted from Th1 cells. IL-10 could act on the early stage of dendritic cell maturation, inhibit the expression of costimulatory molecules, as well as reduce the expression of major histocompatibility antigen class II molecules, leading to the suppression of the maturation and differentiation of dendritic cells. As a result, CD4+ Th cells and lymphocytes become unresponsive to specific exogenous antigens, leading to immune tolerance to HBV and inhibiting its clearance.

However, the role of IL-10 in the process of antiviral treatment of hepatitis B needs remains unclear. The cytokines secreted from Th1 cells and Th17 cells are vital for the antiviral immune response in the patients we assessed. In the early stage of ETV treatment, there was a muted immune response from the Th1/Th2/Th17 cells. At the 48th week of ETV treatment, elevated chemokines stimulated neutrophils and promoted immune responses. As serum IL-4 and IL-10 were negatively associated with the serum level of HBV RNA, these two cytokines might be used in combination with HBV RNA as new indicators of antiviral drug efficacy. Future long-term prospective studies will shed light on the relationship between HBV RNA and immunity in CHB patients.

Acknowledgments

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study concept and design (CP), experimental treatment (HH, CL, QW, PS), acquisition of data (QZ, FS), interpreta-
tion of data (QZ, AS, ZW and XS), drafting of the manuscript (QZ, CP), critical revision of the manuscript for important intellectual content (CP, QZ, WD, HH), and administrative, technical, or material support, study supervision (CP, YZ).

Data sharing statement

Technical appendix, statistical code, and dataset are available from the corresponding authors. Participants gave informed consent for data sharing.

References


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