Research Article
Viral Hepatitis

EASL | JOURNAL OF HEPATOLOGY

Relationship between serum HBV-RNA levels and intrahepatic viral as well as histologic activity markers in entecavir-treated patients

Jing Wang[1,†], Yiqi Yu[1,†], Guojun Li[2,†], Chuan Shen[3,†], Zhefeng Meng[4,†], Jianming Zheng[1,†], Yanhong Jia[3], Shaolong Chen[1], Xiao Zhang[5], Mengqi Zhu[1], Jiangjiang Zheng[6], Zhangzhang Song[2], Jing Wu[1], Lingyun Shao[1], Peiyu Qian[4], Xiaona Mao[7], Xuanyi Wang[8,9], Yuxian Huang[1], Caiyan Zhao[3], Jiming Zhang[1], Chao Qiu[1,4,8,9,*], Wenhong Zhang[1,*]

[1]*Department of Infectious Diseases, Huashan Hospital, Fudan University, Shanghai, China;* [2]*Department of Hepatology, The Second Hospital of Yinzhou of Ningbo, Ningbo, China;* [3]*Department of Infectious Diseases, The Third Hospital of Hebei Medical University, Shijiazhuang, China;* [4]*Minhang Hospital, Fudan University, Shanghai, China;* [5]*Continuing Education Office, Healthy School of Huangpu District, Shanghai, China;* [6]*Department of Pathology, The Second Hospital of Yinzhou of Ningbo, Ningbo, China;* [7]*Department of Clinical Laboratory, The Second Hospital of Yinzhou of Ningbo, Ningbo, China;* [8]*Institutes of Biomedical Sciences, Fudan University, Shanghai, China;* [9]*Key Laboratory of Medical Molecular Virology, Fudan University, Shanghai, China*

Background & Aims: In diagnostics, serum hepatitis B virus (HBV)-RNA levels are valuable when the HBV-DNA load in circulation is effectively suppressed by nucleos(t)ide analogue (NUC) therapy. This study aimed to determine the intrahepatic viral replication activity reflected in serum HBV-RNA and whether HBV-RNA contributes to liver histological changes in patients treated with NUC.

Methods: A cross-sectional set of serum and liver biopsy samples was obtained from patients treated with entecavir, who had undetectable levels of serum HBV-DNA. The correlations between serum HBV-RNA concentration and levels of peripheral and intrahepatic viral replicative forms, as well as histological scores, were analyzed. Quasispecies of serum HBV-RNA and intrahepatic viral replicative forms were examined by deep sequencing. HBV-RNA-positive hepatocytes were visualized by *in situ* hybridization.

Results: Serum HBV-RNA was detected in 35 of 47 patients (74.47%, 2.33–4.80 $\log_{10}$ copies/ml). These levels correlated not only with the intrahepatic HBV-RNA level and the ratio of intrahepatic HBV-RNA to covalently closed circular DNA (cccDNA), but also with the histological scores for grading and staging. Regarding quasispecies, serum HBV-RNA was dynamic and more genetically homogenous to simultaneously sampled intrahepatic HBV-RNA than to the cccDNA pool. *In situ* histology revealed that HBV-RNA–positive hepatocytes were clustered in foci, sporadically distributed across the lobules, and co-localized with hepatitis B surface antigen.

Conclusion: Serum HBV-RNA levels reflect intrahepatic viral transcriptional activity and are associated with liver histopathology in patients receiving NUC therapy. Our study sheds light on the nature of HBV-RNA in the pathogenesis of chronic HBV infection and has implications for the management of chronic hepatitis B during NUC therapy.

Lay summary: Serum HBV-RNA levels are indicative of the intrahepatic transcriptional activity of covalently closed circular DNA and are associated with liver histological changes in patients with chronic B hepatitis who are receiving nucleos(t)ide analogue therapy.

Introduction

Hepatitis B virus (HBV) infection is associated with significant morbidity and mortality. At present, 240 million people are estimated to have chronic hepatitis B (CHB) infection worldwide.[1,2] Considering its prevalence, monitoring viral replication activity is a vital part of the clinical management of CHB infection. Thus far, serum HBV-DNA load is the only licensed laboratory indicator that directly indicates the quantity of circulating viral particles, thereby reflecting the activity of viral production in the liver.[3,4] Patients with higher levels of serum HBV-DNA are more susceptible to advanced liver disease than those with lower levels of HBV-DNA.[5,6] Fortunately, with the availability of nucleos(t)ide analogues (NUCs), serum HBV-DNA levels can be potently suppressed to levels below the detection limits of sensitive PCR assays, within months of treatment initiation. Such virologic suppression leads to biochemical normalization, histological improvement, and prevention of complications, such as cirrhosis and hepatocellular carcinoma.[7,8]

As polymerase inhibitors, NUCs can only curb HBV replication at reverse transcription; viral RNA transcription and protein translation machineries remain intact.[9] Intrahepatic viral nucleic acids, such as total HBV-DNA and covalently closed circular DNA (cccDNA), persist even after prolonged NUC treatment.[10] Therefore, NUCs are not capable of eradicating the virus from the liver reservoir. Owing to the low levels of intrahepatic viral products and residual ongoing viral replication, patients treated with NUCs continue to have higher mortality

Keywords: Chronic hepatitis B; Viral RNA; Nucleos(t)ide analogues; Viral replication; Histopathology.
Received 23 February 2017; received in revised form 17 August 2017; accepted 18 August 2017

* Corresponding authors. Address: Department of Infectious Diseases, Huashan Hospital, Fudan University, 12 Wulumuqi Road, Jingan District, Shanghai 200040, China.
E-mail addresses: qiuchao@fudan.edu.cn (C. Qiu), zhangwenhong@fudan.edu.cn (W. Zhang).
† Co-first authors.

Please cite this article in press as: Wang J et al. Relationship between serum HBV-RNA levels and intrahepatic viral as well as histologic activity markers in entecavir-treated patients. J Hepatol (2017), https://doi.org/10.1016/j.jhep.2017.08.021

than the general population, with some of these patients progressing to end-stage liver disease.[7,11–14]

Apart from HBV-DNA and hepatitis B surface antigen (HBsAg), HBV-RNA containing virus-like particles represent an additional serum medium for the detection of viral replicative intermediates produced in the liver.[15–17] HBV-RNA is valuable in diagnostics because it carries genetic information and its quantity assessment is not influenced by immune complexes of antibodies and viral antigens. In the era of NUC therapy, the detection of serum HBV-RNA levels instead of HBV-DNA levels is more useful for predicting treatment response,[17–19] guiding discontinuation of therapy,[16] and monitoring the emergence of viral mutations.[20,21] However, whether the level of serum HBV-RNA correlates with the levels of intrahepatic HBV-RNA or other viral replicative intermediates remains unknown.

The cytopathic effects or immunopathology caused by HBV-RNA accumulation could be the driving force of liver disease progression in patients treated with NUC. The 5′-ε region of HBV pre-genomic RNA (pgRNA) induces the production of interferons and inflammatory cytokines in hepatocytes, which may lead to histological changes in patients receiving NUC therapy.[22] In addition, the immune responses against viral proteins translated from HBV-RNA, albeit at a low level, are a coherent impetus for pathogenesis of CHB.

In this study, we aimed to determine whether serum HBV-RNA levels are correlated with the levels of intrahepatic viral replicative forms and the severity of histological necroinflammation and fibrosis in patients treated with NUC.

Materials and methods

Patients and samples

A cross-sectional set of 47 patients with CHB infection who had received entecavir monotherapy for >1 year were included in this study (Table 1). A total of 25 patients were HB envelope antigen (HBeAg) positive and 22 patients were HBeAg negative. The study protocol was approved by the Ethical Committee of Huashan Hospital and was carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants. Serum samples were obtained from all patients at biopsy, during treatment and after >1 year of treatment. In addition, we included consecutive serum samples from seven patients taken before the initiation of entecavir therapy and 4–12 weeks, 24 weeks, and 48 weeks after treatment, as well as serum samples from a separate set of four patients collected from 52 to 104 weeks after treatment. None of the patients showed indications of hepatocellular carcinoma or hepatitis C virus infection. Needle biopsy samples were obtained from all patients. An adequate amount (>15 mm in length) of each biopsy sample was formalin-fixed, paraffin-embedded, and used for histopathology evaluation, immunohistochemical staining, and *in situ* hybridizing for HBV-RNA in tissue sections. Surplus needle biopsy samples from 31 patients were immersed in RNAlater (Qiagen#76106), an RNA stabilization reagent, to prevent RNA degradation and were subjected to quantifications of intrahepatic viral replicative forms. Samples were stored at −80 °C until examination.

Demographical and clinical information including age, sex, and clinical laboratory parameters of CHB were obtained from patients' medical records. According to the guidelines for the prevention and treatment of CHB in China,[23] using METAVIR criteria, histological sections were evaluated by two experienced hepatopathologists. Histological scoring was performed and averaged for the final scores.

Extraction of viral nucleic acids

Initial amount of 500 μl of serum was used because of the low abundance of HBV-RNA after entecavir therapy. According to the manufacturers' instructions, serum viral RNA was isolated using the QIAamp Viral RNA Mini Kit (Qiagen#52906) and then treated with DNase (Promega#M6101), followed by reverse transcription using PrimeScript™ RT Master Mix Kit (Takara #RR036). To compensate for the effects of inhibition and controls the preparation and amplification processes, 60 μl serum with a known titer of HCV-RNA was added to each sample.

Approximately 10 mg of lysate from the liver biopsy was equally divided (by volume) for DNA and RNA extractions. The QIAamp DNA Mini Kit (Qiagen#51306) and RNeasy Mini Kit

Table 1. Clinical characteristics of the patients receiving entecavir therapy.

	(A) HBeAg-positive (n = 25, 14†)	(B) HBeAg-negative (n = 22, 17†)
Age, years‡	40 (23–50)	42 (27–72)
Duration of treatment, years‡	3 (1–10)	3 (1–7)
Gender, M/F	18/7	18/4
Serum HBV RNA, $\log_{10}$ copies/ml‡	3.45 (2.33–4.80)*	2.72 (2.33–3.73)*
ALT, U/L‡	23 (10–79)	25 (12–52)
Serum HBsAg, $\log_{10}$ IU/ml‡	3.06 (0.35–4.15)	2.61 (0.35–3.82)
Serum HBeAg, $\log_{10}$ S/CO‡	0.86 (0.01–2.70)	-
METAVIR, grading		
Grading score <2	13	9
2≤ Grading score ≤3	12	13
METAVIR, staging		
Staging score <2	12	7
2≤ Staging score ≤3	9	12
Staging score >3	4	3
Intrahepatic HBV DNA, copies/10^3 cell‡	79.68 (0.260–6,339.22)	65.25 (3.17–822.67)
Intrahepatic HBV cccDNA, copies/10^3 cell‡	0.34 (0.06–17.85)	0.20 (0.01–1.24)**
Intrahepatic HBV RNA, $\log_{10}$ relative fold‡	3.13 (1.14–4.75)	2.46 (0–4.10)

ALT, alanine aminotransferase; HBeAg, hepatitis B envelope antigen; HBV, hepatitis B virus.
* The level was below the lower detection limit in six patients.
** The level was below the lower detection limit in one patient.
† The number of serum samples and liver biopsy specimens.
‡ Median (range). Expression of serum HBV-RNA was based on detectable data.

Please cite this article in press as: Wang J et al. Relationship between serum HBV-RNA levels and intrahepatic viral as well as histologic activity markers in entecavir-treated patients. J Hepatol (2017), https://doi.org/10.1016/j.jhep.2017.08.021