International Journal of Infectious Diseases 52 (2016) 77–82

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

Quantitative intrahepatic HBV cccDNA correlates with histological liver inflammation in chronic hepatitis B virus infection

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ARTICLE INFO

Article history:
Received 31 May 2016
Accepted 19 September 2016

Corresponding Editor: Eskild Petersen, Aarhus, Denmark.

Keywords:
Hepatitis B virus
Covalently closed circular DNA
Rolling circle amplification
Histological liver inflammation

SUMMARY

Background: The aim of this study was to determine the role of baseline hepatitis B virus (HBV) forming covalently closed circular DNA (HBV cccDNA) in liver inflammation in patients infected with HBV with serum alanine aminotransferase (ALT) levels under two times the upper limit of normal (2 × ULN).
Methods: After liver biopsy and serum virological and biochemical marker screening, patients diagnosed with chronic HBV infection with serum ALT levels under 2 × ULN and histological liver inflammation of less than grade G2 were prospectively recruited into this study. Recruitment took place between March 2009 and November 2010 at the Center of Infectious Disease, Sichuan University. Patient virological and biochemical markers, as well as markers of liver inflammation, were monitored.
Results: A total of 102 patients were recruited and 68 met the inclusion criteria; the median follow-up was 4.1 years (range 3.9–5.2 years). During follow-up, 41 patients (60.3%) exhibited signs of inflammation. Baseline HBV cccDNA >1 copy/cell (odds ratio 9.43, $p = 0.049$) and liver inflammation grade ≥G1 (odds ratio 5.77, $p = 0.046$) were both independent predictors of liver inflammation.
Conclusions: A higher baseline intrahepatic HBV cccDNA level may increase the risk of liver inflammation. Further investigations will be required to validate HBV cccDNA as an intrahepatic virological marker of patients who require extended outpatient management.

1. Introduction

According to the World Health Organization (WHO), infection with hepatitis B virus (HBV) continues to be a major health problem worldwide. In 2015, the WHO reported that two billion people had been infected with HBV and that 240 million were chronic carriers of HBV surface antigen (HBsAg). Chronic HBV infection is associated with the progression of liver diseases and an increased risk of cirrhosis, liver failure, and hepatocellular carcinoma.[1]

Seeger and Mason recently summarized the molecular biology of HBV infection.[2] When HBV enters the cytoplasm of host hepatocytes, its partially double-stranded, relaxed-circular DNA genome (rcDNA) is released and transferred into the nucleus, forming covalently closed circular DNA (HBV cccDNA), which is the original template for viral replication. One of its transcription products, the pregenomic RNA (pgRNA), serves as a template for reverse transcriptional synthesis of progeny viral DNA.[3] pgRNA is packaged, along with viral proteins also transcribed and translated from cccDNA, eventually developing into mature HBV particles. The formation of HBV cccDNA is essential to the life cycle of HBV. HBV cccDNA persists in hepatocyte nuclei and plays an important role in the maintenance and relapse of HBV infection.[4,5] Therefore, intrahepatic HBV cccDNA is believed to be an important marker of disease progression and is frequently measured to evaluate the efficacy of anti-HBV therapy and to estimate treatment endpoints.[6–8]

In clinical practice, it was found that most patients who underwent liver biopsy had normal or slightly elevated serum alanine aminotransferase (ALT) levels and mild histological inflammation, and most were not considered to be suitable for antiviral treatment, according to the Chinese guidelines for the treatment of hepatitis B.[9] However, liver inflammation may be aggravated once these patients enter the immune clearance phase. If outpatient management is insufficient, these patients may develop severe liver function abnormalities without the

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http://dx.doi.org/10.1016/j.ijid.2016.09.022

appropriate therapy. Therefore, predicting which patients will be prone to inflammation is particularly important.

Intracellular HBV cccDNA levels were recently reported to be positively correlated with serum ALT levels and the histological grade of liver inflammation in some cross-sectional studies.[10,11] Nevertheless, the capacity of the intracellular HBV cccDNA level to predict the histological grade of liver inflammation remains controversial.[12,13] The objective of this prospective study was to determine the role of baseline intracellular HBV cccDNA in predicting liver inflammation in patients infected with HBV and with serum ALT levels under two times the upper limit of normal ($2 \times$ ULN).

2. Patients and methods

2.1. Study design and patient eligibility

Consecutive patients with chronic HBV infection who underwent liver biopsy and serum virological and biochemical marker screening, and who were eligible, were recruited into this prospective study at the Center of Infectious Disease of the hospital between March 2009 and November 2010. All recruited patients were examined by clinicians. The inclusion criteria were as follows: (1) age between 18 and 65 years; (2) HBsAg was detected in the serum for at least 6 months; (3) serum ALT levels were below $2 \times$ ULN; (4) the patient consented to liver biopsy and assessment of serum virological and biochemical markers; and (5) written informed consent was obtained for treatment and inclusion in the database. The exclusion criteria were (1) patient had ever received antiviral therapy against HBV; (2) infection with hepatitis A, hepatitis C, hepatitis D, hepatitis E, or human immunodeficiency virus; (3) diagnosis of both compensated and decompensated liver cirrhosis, or hepatocellular carcinoma; (4) jaundice caused by obstructive or hemolytic diseases; (5) prolonged prothrombin time induced by blood system diseases; (6) Wilson's disease, alcoholic liver disease, or autoimmune hepatitis; and (7) comorbidities, or an uncontrolled metabolic or psychiatric condition. The study protocol was developed in accordance with the ethical guidelines of the Declaration of Helsinki and was approved by the independent ethics committee of the hospital.

2.2. Clinical data, laboratory data, and follow-up

The clinical and laboratory characteristics of all patients were recorded prospectively in a database. These included (1) biochemical tests reflecting hepatocytic damage, including serum ALT, aspartate aminotransferase (AST), albumin, total bilirubin, cholinesterase, and creatinine, all assayed by colorimetric methods (Modular EVO, Roche, Switzerland); (2) international normalized ratio (INR) for prothrombin time, assessed according to the manufacturer's instructions (Sysmex CA-7000 Systems, Sysmex, Japan); (3) HBV markers, such as HBV antigens and antibodies, detected using commercially available enzyme immunoassays (Alisei Quality System, RADIM, Italy); (4) HBV DNA, quantified using a fluorescent quantifying PCR with a lower limit of detection of 10^3 copies/ml. All of these tests are certified by the American College of Pathologists. The clinical and laboratory databases and compliance of all patients were monitored every 3 months until November 19, 2014.

2.3. Amplification of intrahepatic HBV cccDNA

The amplification of intrahepatic HBV cccDNA was performed according to previously reported methods.[14] Briefly, total DNA was extracted from 10-μm sections of about 80-μm formalin-fixed and paraffin-embedded liver biopsy tissue using a QIAamp FFPE DNA Mini Kit (Qiagen, Germany) in accordance with the manufacturer's instructions. HBV rcDNA and replicative double-stranded DNA (dsDNA) and single-stranded DNA (ssDNA) were digested using the plasmid-safe ATP-dependent enzyme (PSAD) (Epicentre Technologies Corp., Chicago, IL, USA) at 37 °C for 30 min and terminated at 70 °C for 30 min. The reaction mixture contained 6.8 μl extracted total DNA, 4 U PSAD, 2 mmol/l ATP, and 2 μl 10× reaction buffer with water to a final volume of 10 μl.

Four pairs of primers were designed for the first round of rolling cycle HBV cccDNA amplification (RCA). Ten microliters of PSAD-digested DNA was mixed with primers at a concentration of 10 μmol/l with 1 μl of 10× reaction buffer. The mixture was denatured at 95 °C for 3 min and then cooled to room temperature stepwise from 50 °C for 15 s, 30 °C for 15 s, and 20 °C for 10 min. The solution was then added to 8.5 μl of reaction mixture containing primers at a concentration of 10 μmol/l each, 0.2 μl 100× bovine serum albumin, 2.5 mmol/l of dNTP, 1 μl of the Phi29 DNA polymerase (New England Biolabs, Ipswich, MA, USA), and 1 μl of reaction buffer. The reaction was carried out at 30 °C for 18 h and terminated at 65 °C for 10 min.

HBV cccDNA was further specifically amplified and quantified using TaqMan real-time PCR with a pair of cccDNA-selective primers and a probe targeting the gap region between the two viral genome direct repeat regions (DR1 and DR2). Briefly, 25 μl of reaction solution was prepared containing 3 μl of RCA-amplified DNA template, 2.5 mmol/l dNTP, 25 mmol/l $MgCl_2$, 2.5 μl of 10× Taq polymerase PCR buffer, 0.2 μmol/l of selective primers and probe, 0.1 U of Taq polymerase, and 11.3 μl of ddH_2O. The reaction was carried out by real-time PCR (LightCycler 480, Roche, San Francisco, CA, USA) at 94 °C for 3 min, followed by 40 cycles of 94 °C for 15 s and 58 °C for 45 s.

2.4. Quantitation of intrahepatic HBV cccDNA

Ten-fold serial dilutions (10^2–10^9 copies/ml) of the plasmid pcDNA3.1 (+)-HBV-WT-C containing the entire wild-type HBV genotype C genome were used to establish standard curves to quantify HBV cccDNA. The plasmid was constructed and stored at −20 °C in the Center of Infectious Disease of the hospital. To adjust for cell numbers, a set of primers and a probe for human β-actin were also included in the real-time PCR process. The primers and probes used for HBV cccDNA and β-actin are listed in Table 1. Each sample was run in duplicate on the same plate. Liver biopsy tissues from HBV-uninfected patients were used as negative controls. The amount of HBV cccDNA was expressed as the number of copies per cell, with the estimation of 6.667 pg of DNA/cell; the methods have been reported previously in the literature.[15,16]

2.5. Statistical methods

All quantitative data were expressed as the mean ± standard deviation (SD) and were compared using the *t*-test. The Chi-square test or Fisher's exact test was performed to determine differences in qualitative data. Pearson's correlation was used to analyze the correlations between continuous variables, including log HBsAg, log HBV DNA, and log cccDNA. Spearman's test was used to assess the correlation of risk factors with inflammation scores. Logistic regression analysis was used to assess the influence of independent prognostic factors on the risk of liver inflammation. A *p*-value of <0.05 was considered to indicate statistical significance. Data were analyzed using the statistical software Intercooled Stata version 8.2 for Windows (StataCorp LP, College Station, TX, USA).