P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• BASIC RESEARCH •

HBV cccDNA in patients' sera as an indicator for HBV reactivation and an early signal of liver damage

Ying Chen, Johnny Sze, Ming-Liang He

Ying Chen, Johnny Sze, Ming-Liang He, The Institute of Molecular Biology, The University of Hong Kong, Hong Kong, China
Supported by CRCG grant from the University of Hong Kong, CERG grant from University Grant Council of Hong Kong, and Research Fund from Science and Technology Commission of Shanghai, China
Correspondence to: Dr. Ming-Liang He, The Institute of Molecular Biology, The University of Hong Kong, Hong Kong, China. mlhe@hkucc.hku.hk
Telephone: +852-2299-0758 **Fax:** +852-2817-1006
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Abstract

AIM: To evaluate the covalently closed circle DNA (cccDNA) level of hepatitis B virus (HBV) in patients' liver and sera.

METHODS: HBV DNA was isolated from patients' liver biopsies and sera. A sensitive real-time PCR method, which is capable of differentiation of HBV viral genomic DNA and cccDNA, was used to quantify the total HBV cccDNA. The total HBV viral DNA was quantitated by real-time PCR using a HBV diagnostic kit (PG Biotech, LTD, Shenzhen, China) described previously.

RESULTS: For the first time, we measured the level of HBV DNA and cccDNA isolated from ten HBV patients' liver biopsies and sera. In the liver biopsies, cccDNA was detected from all the biopsy samples. The copy number of cccDNA ranged from from 0.03 to 173.1 per cell, the copy number of total HBV DNA ranged from 0.08 to 3 717 per cell. The ratio of total HBV DNA to cccDNA ranged from 1 to 3 406. In the sera, cccDNA was only detected from six samples whereas HBV viral DNA was detected from all ten samples. The ratio of cccDNA to total HBV DNA ranged from 0 to 1.77%. To further investigate the reason why cccDNA could only be detected in some patients' sera, we performed longitudinal studies. The cccDNA was detected from the patients' sera with HBV reactivation but not from the patients' sera without HBV reactivation. The level of cccDNA in the sera was correlated with ALT and viral load in the HBV reactivation patients.

CONCLUSION: HBV cccDNA is actively transcribed and replicated in some patients' hepatocytes, which is reflected by a high ratio of HBV total DNA vs cccDNA. Detection of cccDNA in the liver biopsy will provide an end-point for the anti-HBV therapy. The occurrence of cccDNA in the sera is an early signal of liver damage, which may be another important clinical parameter.

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INTRODUCTION

Chronic hepatitis B Virus (HBV) infection is one of the most common diseases leading to a high morbidity and mortality due to the development of liver failure, liver cirrhosis (LC) and hepatocellular carcinomas (HCC)[3-28]. There are over 300 million people suffering from HBV infection worldwide, and more than 10% of Chinese are HBV carriers[3,5,14]. Infection by hepatitis B virus causes complicated biochemical, immunological and histological changes in host[7-9,11,16,18,23,24,27]. Viral kinetic studies have shown that the viral load goes through a complex series of stages during anti-HBV chemotherapy[21,22,29-31]. The HBV reactivation often occurs after cessation of anti-HBV treatment[22,24,32], which is reflected by the increases of hepatitis B e antigen (HBeAg) and DNA levels in serum. Clinically, this may manifest as hepatitis, hepatic failure, and even death. Despite its clinical importance, there are few data on the incidence and risk factors of hepatitis due to HBV reactivation after chemotherapy. In particular, the relationship of various HBV virological parameters and HBV reactivation is unclear.

HBV covalently closed circular (ccc) DNA is a critical intracellular replicative intermediate, which acts as the template for transcription of viral RNAs serving either as viral pregenome RNAs, or as mRNAs coding for the multifunctional polymerase, core, X and envelope (S) proteins[25,26]. All the HBV proteins play crucial roles in HBV gene transcription, replication, viral packaging and recycling. Due to lack of proofreading functions of polymerase, HBV goes through a fast mutagenesis and creates drug resistant strains[13,17,19,24,26,29,32], which contributes to the viral cccDNA pool. Because cccDNA of HBV is resource of new HBV viruses and resistance to drug treatment, it is believed that cccDNA is the major reason for HBV reactivation after stopping the anti-HBV therapy[21,24,25]. Monitoring of HBV viral load, antigenes, mutations and cccDNA levels will therefore provide a direct indication of HBV activity in the body[12,13,15,17-21, 28-32].

We previously developed a sensitive method to quantify HBV cccDNA, which has been successfully used to determine the amount of HBV cccDNA isolated from liver biopsy[1]. Here we report that HBV cccDNA can be detected both in liver biopsy and in patients' sera. The sera cccDNA level is correlated with ALT and viral load in HBV reactivation patients, but cannot be detected in patients' sera without HBV reactivation. Our results indicate that the occurrence of cccDNA is an early signal of liver damage, which may be another important clinical parameter.

MATERIALS AND METHODS

cccDNA standard[1, 2]

A plasmid containing Chinese HBV genome (pHBV-adr) was a gift from Professor Yuan Wang. The supercoid plasmid (cccDNA) was isolated by CsCl purification. The cccDNA concentration was determined by measurement of OD260 and verified by agarose gel electrophoresis. The copy number was determined by its molecular weight.

HBV viral DNA preparation[1]

HBV viral DNA was extracted from either 200 ul of patients' sera or weighed liver biopsies using a QIAamp DNA blood or

tissue mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

Quantification of HBV cccDNA using real-time PCR

HBV cccDNA quantification was performed as described previously[1,2], with minor modifications. Briefly, a pair of primers (forward primer: 5'-ACTCTTGGACTCBCAGCAATG-3', reverse primer: 5'-CTTTATACGGGTCAATGTCCA-3'), which can specifically amplify a DNA fragment from HBV cccDNA but not viral genomic DNA by PCR, were used for real-time PCR. In a typical real-time PCR reaction, 250 nM of the probe (5'-FAM-CTTTTTCACCTCTGCCTAATCAT CTCWTGTTCA-TAMRA-3') and 900 nM of the two PCR primers were used. For total HBV quantification, PCR amplification was performed with an HBV DNA diagnostic kit (PG Biotech. Ltd, China) using ABI 7900HT sequence detection system. The PCR program consisted of an initial denaturing step at 95 ℃ for 10 min, followed by 40 amplification cycles at 95 ℃ for 15 sec and at 61.5 ℃ for 1 min.

Patient samples

The patients were treated with chemotherapy at Queen Mary Hospital, Hong Kong, from January 2000 to May 2002. In accordance with the standard protocols, all the patients who received chemotherapy were screened for HBsAg, HBsAb, human immunodeficiency virus antibody (HIV Ab), and HBV DNA by PCR and hepatitis C antibody (anti-HCV), with commercially-available enzyme immunoassays (Abbott Laboratories, Chicago, IL, USA). HBcAb was tested by RIA (Corab; Abbott). For all HBsAg positive patients, further serological testing for hepatitis B e antigen (HBeAg), hepatitis B e antibody (HBeAb) and serum HBV DNA was performed by PCR, and chemotherapy was administered with lamivudine. All HbsAg-positive recipients were tested at 2-week intervals for liver function (including serum alanine aminotransferase, serum albumin and bilirubin) and serum HBV DNA during chemotherapy. Hepatitis serology (HBsAg, HBeAg, HBeAb, HBV DNA by PCR, and HCV RNA by RT-PCR) was performed on the serum collected preceding and during the events whenever there was any clinical suspicion of liver damage due to hepatitis B infection. The occurrences of hepatic events (acute hepatitis, chronic hepatitis, anicteric and icteric hepatitis, hepatic failure) were recorded. Hepatitis was defined as a more than three-fold elevation of serum aminotransferase above the upper limit of normal, on two consecutive determinations at least five days apart. HBV reactivation was defined to occur when preceded or accompanied by an elevation of serum HBV DNA to more than ten times that of the pre-exacerbation baseline, or when the serum HBV DNA turned from negative to positive, or when the HBsAg became positive and remained so for two consecutive readings five days apart.

All serum and biopsy samples were stored at -70 ℃. All patients who developed post-chemotherapy hepatitis due to HBV reactivation were treated with lamivudine 100 mg once daily.

RESULTS

HBV cccDNA existed in all patients' hepatocytes but only in a subset of patients' sera

To elucidate the cccDNA status in HBV patients, we quantified the cccDNA and total HBV DNA level in the liver biopsies. The total DNA was isolated from weighed liver biopsies and quantified with an HBV diagnostic kit by real-time PCR. The HBV cccDNA was also measured by real-time PCR. HBV cccDNA was detected in all the HBV patients' liver biopsies. The copy number of cccDNA in patients' hepatocytes ranged from 0.05 to 168 copies per cell, which is consistent with the estimates from Southern blot data[10]. The copy number of total HBV DNA in patients' hepatocytes ranged from 0.08 to over 3 000 copies per cell. The ratio of cccDNA to total HBV DNA ranged from 1 to 3 406 (Table 1), indicating that cccDNA in patients' hepatocytes had active replication and relative silent status.

To investigate whether cccDNA could be released into sera due to liver inflammation and necrosis, viral DNA was isolated from patients' sera and quantified by real-time PCR. Interestingly, cccDNA could only be detected in a subset of patients' sera, while HBV DNA was detected in all the patients' sera. The copy number of viral load ranged from 5.4×10^5 to 1.8×10^{11} per ml while the copy number of cccDNA ranged from undetectable level (less than 1 000 copies/ml[11]) to 3.1×10^7 per ml. The ratio of cccDNA to total viral DNA ranged from 0 to 1.77%. There was no correlation between the copy number of cccDNA and total viral DNA in the randomized patients (Table 1).

cccDNA is an early signal of liver damage

To investigate the medical significance of cccDNA in sera, longitudinal studies were performed. Patients with or without HBV reactivation during lamivudine treatment were chosen for this study. The patients' sera were collected every two weeks and HBV DNA was isolated for quantification of HBV cccDNA and total HBV DNA. According to our results, HBV cccDNA was not detectable by sensitive real-time PCR in patients without HBV reactivation (data not shown), but was detectable in patients with HBV reactivation (Figures

Table 1 HBV cccDNA in patients' hepatocytes and sera

Patients	HBV DNA in the Biopsies (copy/cell)			HBV DNA in the sera (copy/ml)		
	cccDNA	HBV DNA[a]	HBV/ccc[b]	cccDNA	HBV DNA[c]	ccc/HBV[d]
1	2.05	195.5	98.7	3.4×10^6	4.8×10^{10}	0.01%
2	0.03	0.17	5.8	UN	6.6×10^7	– -
3	173.1	799.5	4.6	3.1×10^5	6.0×10^{10}	0.001%
4	0.16	8.13	51.7	UN	1.9×10^7	– -
5	0.03	0.08	2.8	UN	5.4×10^5	– -
6	16.02	17.4	1.1	2.4×10^6	5.3×10^8	0.46%
7	18.03	3717	206	3.1×10^7	1.8×10^{11}	0.02%
8	0.79	13.1	16.5	UN	2.4×10^{10}	– -
9	0.05	153.3	3406	2.8×10^5	3.0×10^9	0.01%
10	2.06	16.4	8	2.7×10^6	7.5×10^8	1.77%

[a]The total HBV copies per cell in the patients' liver biopsies; [b]The ratio of total HBV DNA to cccDNA in the patients' liver biopsy; [c]The total HBV copies per ml of the patients' sera; [d]The ratio of cccDNA to total HBV DNA in the patients' sera. UN, untedectable.

1A and 1B). The cccDNA level was correlated with viral load (Figures 1A and 1B), which occurred earlier than ALT. Before the ALT value increased, both the cccDNA level and viral load rose to a high level. Once the ALT value increased, the cccDNA level dropped rapidly. These results suggest that patients whose sera contain cccDNA are at a high risk of HBV reactivation, and that HBV cccDNA develops in the sera earlier than ALT elevation.

Our results suggest that serum cccDNA level may be an important parameter for anti-HBV treatment, and that a low level of cccDNA in hepatocytes should be an end point of anti-HBV treatment.

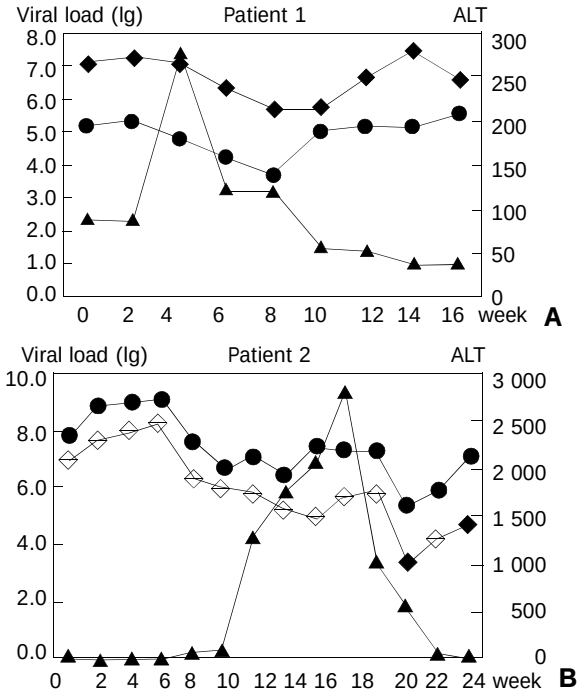

Figure 1 Kinetics of cccDNA in HBV reactivation patients. The copy numbers of total HBV and cccDNA were determined by real-time PCR. The cccDNA level was parallel to the total viral load both in the patients 1 and 2; the cccDNA level increased before ALT rose. Diamond: HBV viral load, dot: cccDNA, triangle: ALT.

DISCUSSION

Little information is available about HBV cccDNA and its activities *in vivo* due to the difficulty of differentiation of HBV cccDNA and viral genomic DNA, although a lot of work has been done on the HBV viral kinetics[21,22,29-31]. In the hepatitis B virus life cycle, cccDNA serves as templates for viral gene transcription and replication in hepatocytes[25,26]. It has been shown that cccDNA is the major reason for HBV reactivation after cessation of anti-HBV treatment[24,25]. Therefore, quantification of HBV cccDNA will provide useful information for the end-point of anti-HBV therapy.

In this paper, we first report the quantitative data on HBV cccDNA in patients' liver biopsy and sera. We previously established a quantitative real-time PCR method which can specifically differentiate HBV viral genomic DNA and cccDNA[1]. This sensitive method provided us an opportunity to investigate HBV cccDNA status in the liver biopsy and patients' sera. Our data indicated that cccDNA in the patients' liver might have two status: active and relative silent status. This was reflected by the ratio of viral total DNA to cccDNA. To our surprising, HBV cccDNA was also detected in a part of patients' sera. The release of cccDNA of hepatitis B virus to the sera might be the consequence of liver damages, such as liver inflammation, necrosis. Longitudinal studies revealed that the release of cccDNA to the sera was an early signal of liver damage, which is correlated with ALT and viral load in HBV reactivated patients. Therefore, measurement of the cccDNA level in the liver may provide an end-point of anti-HBV therapy, and detection of the cccDNA level in the sera may provide a better guidance to protect patients from HBV reactivation.

Because this is the first time to provide the quantitative data on HBV cccDNA status, and the limitation of patient samples, more clinic studies on the cccDNA kinetics are required to better understand HBV biology and provide a better guidance for the anti-HBV treatment.

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