Detectability of Hepatitis B Virus in Peripheral Blood Mononuclear Cells Among Naive Chronic Hepatitis B Patients With Negative Viremia

A Multicenter Study

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Background and Study Aim: Studies analyzed the extrahepatic reservoir of hepatitis B virus (HBV), especially in those with chronic HBV who are hepatitis B surface antigen positive but have a negative peripheral viremia, are still scarce. Therefore, we aimed to investigate the presence of HBV-DNA in peripheral blood mononuclear cells (PBMCs) and to evaluate different factors affecting this.

Patients and Methods: A total of 1650 naive chronic hepatitis B patients were recruited. Among these patients, 320 (19.4%) (75% were male [n = 240]; mean ± SD age, 38.4 ± 12.8 years) have a persistently negative serum real-time polymerase chain reaction (PCR) for HBV-DNA without previous treatment experience. For all patients, hepatic function tests and fibrosis assay by Fibroscan and hepatitis C virus coinfection, as well as HBV-DNA-PCR in both serum and PBMCs were analyzed.

Results: More than half of them (n = 170, 53.1%) exhibited positive HBV-DNA in PBMCs. The mean logarithm 10 of quantitative HBV-DNA by PCR in PBMCs was (5.1 ± 0.3 IU/mL). Hepatitis C virus coinfection was found in 30 patients (17.6%). Most of them (320 patients) had insignificant fibrosis scores (less than F2). The multivariate logistic regression analysis for prediction of presence of detectable HBV-positive viremia in PBMCs yielded the following risk factors (odds ratio [OR]): the presence of hepatitis C virus coinfection (OR = 1.7) and a logarithm 10 of quantitative hepatitis B surface antigen more than 3 (OR = 1.1).

Conclusions: A considerable number of patients with negative plasma HBV-DNA are still harboring subtle form of virus within remote extrahepatic compartments. Thus, dual testing for both plasma and PBMCs is mandatory especially in epidemiologic studies.

Key Words: hepatitis B virus, peripheral blood mononuclear cells, polymerase chain reaction

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Hepatitis B virus (HBV) infection is an important health problem with challenging management.[1] Recent guidelines did not recommend the "to-treat" option and assigned "to-follow" for those with low or negative viremia.[2–4] This was attributed to the absence of actual viral cure through the current available antiviral drugs that suppress rather than eradicate the virus.[5] In a small percentage of HBV patients, HBV-DNA may be detected in the liver, plasma, or peripheral blood mononuclear cells (PBMCs), despite hepatitis B surface antigen (HBsAg) seroconversion or seroclearance (HBsAg cleared or reduced to levels below the detection limit). In these cases, DNA is detected using ultrasensitive molecular detection techniques; thus, occult HBV infection is diagnosed.[6–8]

Complete eradication was hindered by the viral persistence in the nuclei of infected hepatocytes, either integrated into the host genome or as a covalently closed circular DNA (cccDNA) episomal form.[9] The detection of HBV viremia in extrahepatic compartments, such as HBV, infected PBMCs; using a highly sensitive polymerase chain reaction (PCR) has been used to assess this integration.[10]

Most of guidelines rely on the sequential absence of viral DNA in the serum to assign the evidence of "no-need-to-treat" option. However, this evidence may be criticized with the emergence of detectable HBV-DNA in the extrahepatic compartments, for example, PBMCs,[11] that may be a hidden source for flare or relapse.

There are scarce data with regard to the detection of HBV viremia in PBMCs especially in patients with HBV-DNA negativity in the serum without prior viral suppressive drugs. Therefore, we aimed to evaluate the prevalence and predictors of the occurrence of HBV in plasma and PBMCs of patients with undetectable HBV viremia. Generally, the detection of HBsAg is a reliable method for the diagnosis of HBV infection.[6] Thus, this study was conducted to determine the prevalence of HBV-DNA in the serum and PBMCs of naive chronic HBV–infected individuals (with HBsAg-positive and HBsAg-negative plasma DNA PCR).

MATERIAL AND METHODS

Part of this work (descriptive data) was previously published by the same authors.[12] Thus, some degree of similarity may be detected in methodology section. However, in this article, we try to

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All patients agreed to participate in this study. The study protocol was approved by the ethics committee of Al Azhar University of Medical Sciences (number: IRB.227/2017).

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evaluate the presence of HBV in peripheral blood mononuclear cells among special group of patients (naive [without previous HBV treatment] chronic hepatitis B (CHB) patients with negative viremia). Thus, we increase the total number of studied patients. (To link the published article: https://www.sciencedirect.com/science/article/pii/S1687850721002934).

Patient Selection

Between January 2018 and December 2018, a cross-sectional study was designed to include all consecutive patients with proven CHB (as defined by HBsAg positivity for more than 6 months) who attended to the viral hepatitis outpatient clinic in 2 major tertiary referral units in Upper Egypt: Assiut University Hospital, Assiut, Egypt, and South Valley University Hospital, Qena, Egypt. From these cases, naive patients (without previous treatment) with persistently negative hepatitis B viremia (as confirmed by 3 consecutive testing for plasma HBV-DNA-PCR negativity [<20 mIU/mL] 3 months in between) were selected.

Sample Collection and Processing

Approximately 5 mL of peripheral blood was drawn from each patient into sterile ethylenediaminetetraacetic acid–containing anticoagulant tubes, and then, plasma was separated and frozen at −20°C until use. Peripheral blood mononuclear cells were isolated by Ficoll density gradient centrifugation using electronic centrifuge (Tianjin Haoyang Biological Technology Co, Ltd, Tianjin, China) at 1000*g* for 30 minutes at 20°C. After washing with phosphate-buffered saline (pH, 7.3–0.1) in triplicate, the cells were stored at −20°C.

Serological Tests

Serological markers of HBV (HBsAg, anti-HBs, HBeAg, and anti-HBc) were measured by using third-generation enzyme-linked immunosorbent assay (ELISA) testing (Prechek Bio, Inc, Taiwan) according to the manufacturer's instructions. Quantitative HBsAg (qHBsAg) was tested for 4 readings using (Roche Cobas e 411 analyzers with Elecsys) HBsAg II Quant reagent kits (Roche Diagnostics, Indianapolis, Ind).

DNA Extraction

Hepatitis B virus DNA was extracted from a 200-μL plasma aliquot and 3 to 5×10^6 of PBMCs, using Favor prep Blood Genomic DNA Extraction Mini Kit (Artus GmbH, Hamburg, Germany), according to the manufacturer's instructions. Murine cytomegalovirus (MCMV; Fast Track Diagnostics, Luxembourg) was used as internal control. Moreover, sterile micro centrifuge tubes, containing only the reaction mixtures, were processed simultaneously for both plasma and PBMC samples as the negative control.

Quantitative Real-Time Polymerase Chain Reaction of HBV DNA

Hepatitis B virus DNA in the plasma and PBMC samples were measured by using automated system QIAamp DSP Virus Spin Kit (QIA agility; QIAGEN, Germany), according to the manufacturer's protocol. Amplification of HBV-DNA was carried out by the real-time PCR kit (QIAGEN GmbH, Hilden, Germany), using the Rotor-Gene QRT-PCR apparatus.

Detection of Hepatitis C Virus Coinfection

Quantitative detection of hepatitis C virus (HCV) viral load in the plasma and PBMCs samples was performed by Artus HCV RG RT-PCR Kit (cat no. 4518265, QIAGEN1; QIAGEN) using the ABI 7500 Fast Real-Time PCR Thermal Cycler.

Assessment of the Liver

Hepatic stiffness score was measured by Fibroscan (EchoSens, Paris, France) in kilopascals according to the manufacturer's instructions (score less than 7.4 kPa equal to F0–F2, 9.5–12.4 kPa equal to F3, and 14.5 kPa or greater equal to F4 on METAVIR pathologic scoring system).[13–15] Basic laboratory function tests (eg, serum alanine aminotransferase [ALT] and aspartate aminotransferase) were done as routine.

Statistical Analysis

Frequencies, percentages, and means were used, as appropriate, for descriptive analysis. Univariate and multivariate logistic regression analyses were performed to assess the significant predictors of HBV persistence between groups. All statistical analyses were conducted by SPSS software for Windows, release 18 (SPSS, Inc, Chicago, Ill). A *P* value less than 0.05 was considered significant.

RESULTS

Basic Characteristics

A total of 1650 naive CHB patients were recruited; among these patients, 320 (19.4%; 75% were male [n = 240]; mean ± SD age, 38.4 ± 12.8 years) have a persistently negative plasma real-time PCR for HBV-DNA without previous treatment experience. All of them had negative HBeAg, positive anti-HBe, and positive anti-HBcr (anti-HB core antigen). Most of them exhibited nonsignificant fibrosis score (F0/F1; 96.8%, n = 310) and 28% (n = 90) were coinfection with HCV. The basic demographic and laboratory parameters were listed in Table 1.

TABLE 1. Basic Characteristics of the Studied Group

Characteristics	Total Patients (N = 320)
Age, mean ± SD, y	38.44 ± 12.72
Sex, male:female, n (%)	240 (75%):80 (25%)
Fibrosis score by Fibroscan, n (%)	
F0	250 (78.1%)
F1	60 (18.8%)
F2	10 (3.1%)
More than F2	0
Anti-HCV, positive (HCV Ab positive):negative (HCV Ab negative), n (%)	90 (28.1%):230 (71.9%)
Real-time HBV-DNA-PCR from PBMCs, positive:negative, n (%)	170 (53.1%):150 (46.9%)
PBMCs HBV-DNA, mean ± SD, Log_{10}IU/mL	2.71 ± 2.56
Plasma HCV-RNA, mean ± SD, Log_{10}IU/mL	5.63 ± 0.62
Serum ALT level (0–40 mg/dL), mean ± SD	30.4 ± 12.2
Serum bilirubin level (0–1.0 mg/dL), mean ± SD	0.925 ± 0.13
Serum albumin level (3.5–5.0 mg/dL), mean ± SD	4.5 ± 0.3
Serum creatinine level (0.7–1.5 mg/dL), mean ± SD	1.0 ± 0.2
Total qHBsAg level, mean ± SD, Log_{10}IU/mL	2.6 ± 0.8

anti-HCV, hepatitis C virus antibody.

TABLE 2. Univariate Analysis of Factors Associated With HBV-Positive Viremia in PBMCs

Characteristics	HBV Viremia From PBMCs		*P*
	Positive (n = 170)	Negative (n = 150)	
Age, mean ± SD, y	37.41 ± 12.733	39.60 ± 12.63	0.373*
Sex, male:female, n (%)	140 (82.4%):30 (17.6%)	100 (66.7%):50 (33.3%)	0.001†
Fibrosis score by Fibroscan, n (%)			
F0	130 (76.5%)	120 (80%)	0.010†
F1	30 (17.6%)	30 (20%)	
F2	10 (5.9%)	0	
More than F2	0	0	
Anti-HCV, positive:negative, n (%)	30 (17.6%):140 (82.4%)	60 (40%):90 (60%)	0.001†
PBMCs HBV viremia, mean ± SD, Log_{10}IU/mL	5.1 ± 0.3	—	0.001*
Plasma HCV-RNA-PCR, mean ± SD, Log_{10}IU/mL	0.38 ± 1.5	1.06 ± 2.13	0.001*
Serum ALT level, mean ± SD, mg/dL	31.7±14.9	28.9±8.0	0.001*
qHBsAg level, mean ± SD, Log_{10}IU/mL	3.12 ±0.57	2.12±0.72	0.163*

*Student *t* test.
†χ^2 test.
anti-HCV, hepatitis C virus antibody.

Prevalence and Characteristics of HBV-Positive Viremia in PBMCs

Of the 320 patients with negative plasma HBV, more than half (n = 170, 53.1%) exhibited positive HBV-DNA PCR in PBMCs (negative <20 IU/mL). The mean ± SD logarithm 10 (Log_{10}) of quantitative HBV-DNA by PCR in PBMCs was 5.1 ± 0.3 IU/mL. In those with positive HBV-DNA in PBMCs, HCV coinfection was found in 3 patients (17.6%). Most of them had insignificant fibrosis scores (less than F2). The detailed comparison between the positive and negative PBMCs viremia was shown in Table 2.

Predictors of HBV-Positive Viremia in PBMCs Among the Studied Group

The multivariate logistic regression analysis for the prediction of presence of detectable HBV-positive viremia in PBMCs yielded the following risk factors (odds ratio [OR]): the presence of HCV-RNA (OR = 1.7) and a Log_{10} of qHBsAg more than 3 (OR = 1.1), as shown in Table 3.

DISCUSSION

Chronic hepatitis B infection carries the risk of hepatocellular carcinoma even without cirrhosis as noted in several epidemiologic studies.[16,17] This may be explained by the persistence of HBV within the nuclei of infected liver cells, either integrated into the host genome or as a cccDNA episomal form.[9] The HBV integration phenomena represent the main difficulty in HBV eradication, because it needs a liver biopsy to precisely prove this form, which is unplausible to apparently healthy subjects.[18–20]

Testing the virus genome in extrahepatic compartments such as the PBMCs may help bypass this invasiveness.[21,22] In addition, until now, there was no universal methodology for the presence of cccDNA in patients' sera.[20,21,23–25] Studying HBV replication in PBMCs yielded conflicting results. Some denied its role in harboring the cccDNA form and support its role with only the linear and circular relaxed HBV genome,[26] while others accept that estimating HBV viremia within the PBMCs, represented as an easily accessible mirrors to assess the cccDNA forms.[26–28] This related to their detection of the cccDNA form in a variable proportion of patients with chronic and occult HBV infection.[27–31]

Occult HBV infection can remain dormant without detectable clinical manifestations for many years or can progress up to hepatocellular carcinoma development.[32,33] Several factors make the patients vulnerable for development of occult status, for example, immune suppression of the host, associated HCV infection, the window phase after acute HBV infection, genetic mutations in the "a" immune determinant of the S gene.[32,34,35] Therefore, we, herein, tried to focus on detection of hidden HBV in naive patients with persistently negative plasma HBV viremia, by detection of PBMCs harboring HBV, which may have a significant role in persistence, transmission, and drug resistance.[36] Interestingly, researchers found that HBV genotype profiles isolated from plasma and PBMCs of the same patients commonly were not identical. This finding provide evidence that favors the occurrence of immune escape mutation with subsequent formation of vaccine and therapy escape strains, which was critically important.[28]

In our study, plasma DNA was undetectable in all samples, but in PBMC counterparts, it was detected in more than half of those patients (53.1%). This is a relatively high number that may reflect the importance of extrahepatic reservoirs, such as PBMCs, in maintaining HBV chronicity, which represents a hidden and occult source of HBV chronic infection and the subsequent complications.[37,38] This observation may raise the alarm of the insufficiency of testing HBV viremia in only sera. With our results, Xu et al[39] conclude that PBMC is the main extrahepatic site privileged for HBV

TABLE 3. Logistic Regression Analysis of Significant Predictors for the Presence of HBV Viremia in PBMCs

Variables	*B*	SE	Wald	*df*	Sig *P*	OR CI
Presence of HCV positivity	7.446	1.777	17.568	1	0.000	1.7
Mean qHBsAg level	16.260	2.819	33.278	1	0.000	1.1
Constant	−51.667	8.926	33.503	1	0.000	0.000

anti-HCV, hepatitis C virus antibody.

replication and has a great role in HBV intrauterine transmission; interestingly, no association was detected between PBMC transfer and positive serum HBV-DNA or HBsAg in their neonates. Patients who had HBV in PBMCs exhibited insignificant liver pathologic changes (their fibrosis score was less than F2), and all had normal serum ALT. This is a logic finding as most of cases were apparently healthy subjects without any liver insufficiency.

The form of "cccDNA" is considered as an active transcription form used as a template for HBV replication.[40] Failure of its detection in the serum or PBMCs might be related to its integration within HBV-DNA cellular genome.[41] The cccDNA form represents a very small fraction compared with total DNA; for this reason and because of lack of a sensitive laboratory method for its detection, it was suspected that failure of its detection in the serum or PBMCs early during therapy did not exclude the persistence of low viral replication in these sites.[41] Reactivation of occult HBV infection has been reported after DAAS (direct acting antivirals) therapy in patients with HBV-HCV coinfection, so accurate diagnosis of occult HBV is a must.[42]

Hepatitis C virus coinfection was found in approximately 17% of cases and was independently associated with HBV viremia in PBMCs (OR = 1.7). The interaction between HCV and HBV coinfection also was a risk factor for harboring HBV-DNA in PBMCs. With our results, Sagnelli et al[38] reported that approximately half of HCV coinfected patients had detectable HBV-DNA in PBMCs only, with negative results in plasma. In contrary, Wagner et al[43] found that HCV coinfection does not associated with a different distribution of HBV-DNA and cccDNA in serum and/or PBMCs of HBV-positive patients. In HCV/HBV coinfection, usually one virus overdominates the other, but which one is downregulating the other is still a perplexing phenomenon.[41]

We found also that the presence of qHBsAg (quantitative HBsAg) with a mean more than 3 (Log_{10}IU/mL; OR = 1.1) were also significantly associated with HBV viremia in PBMCs. Occult HBV, as defined simply by the presence of HBV-DNA in the liver, with or without detectable HBV-DNA in the plasma with the absence of HBsAg,[44] has been reported to have HBV viremia within the PBMCs.[45–47] Hence, the HBsAg is a traditional marker of the disease, the quantification of HBsAg by qHBsAg assays may indirectly reflect the number of infected hepatocytes, and we reported its association with HBV persistence.[48–50] Thus, we recommend its incorporation within the diagnostic workup and also follow-up setting.

Our study had some limitations. First, it was a cross-sectional one and lacked the follow-up. However, we recommend a longitudinal follow-up study to test the kinetics of HBV viremia and disease progression in those patients. Second, we did not test the infected PBMCs with the specific immunohistochemical stains such as antibodies against CD3, CD4, CD8, CD38, and Tim-3, which may help in further in-depth identification of their immunologic characteristics. In addition, we did not test the HBV genotype. Genotype testing was not ordered in the Egyptian protocol for treating HBV and was not considered as an essential for enrolling patients. In conclusion, the HBV may infect extra hepatic reservoirs such as PBMCs that may harbor HBV-DNA even in those with persistently negative peripheral viremia and assigned as a healthy subject. Thus, we recommend to build-up a diagnostic algorithm that test HBV-DNA in both PBMCs and sera especially in those with HCV coinfection and high levels of qHBsAg.

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