



Detection of Occult Hepatitis B Virus Among Iranian HCV-Infected Patients with Hemophilia Treated with Direct-Acting Antiviral Agents

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Received 2018 August 14; Revised 2018 November 16; Accepted 2018 November 19.

Abstract

Background: One of the challenges to treat hepatitis C virus (HCV) infection is the activation of hepatitis B virus (HBV) that occurs during treatment of HCV infection with direct-acting antivirals (DAAs) in some patients. The detection of serum or liver HBV DNA in the absence of serum HBsAg (HBV surface antigen) is described as occult HBV infection (OBI).

Objectives: The current study aimed at determining the prevalence of OBI in Iranian DAA-naïve HCV-infected patients with hemophilia.

Methods: The current study was conducted on 100 patients with hemophilia receiving DAAs. The sera obtained from these patients were tested for the presence of HBsAg. Then, the presence of the HBV DNA was detected in peripheral blood mononuclear cell (PBMC) and also plasma samples using nested polymerase chain reaction (PCR).

Results: Among the 100 study subjects, 81 (81%) were male and 19 (19%) female. The mean age of the patients was 37 ± 10.50 years. All patients had previously received HBV vaccine. In the current study, HBV DNA was observed in 1% of plasma and in 3% of PBMC samples. In addition, none of the patients who had positive result for HBV detection test previously had markers of HBV infection (anti-HBc (HB core antigen) antibodies, anti-HBs antigens, and positive result of DNA PCR) and all had negative results for HCV RNA after treatment.

Conclusions: Generally, the prevalence of OBI was low, but however, HBsAg negativity was not sufficient to completely exclude the presence of HBV DNA. Thus, the serological markers of HBV infection should be confirmed by molecular tests for the presence of possible occult infection.

Keywords: Hepatitis B Virus, Hepatitis C Virus, Direct-Acting Antivirals, Occult HBV

1. Background

Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are the most common causes of chronic liver diseases in the world (1). More than 75% of individuals exposed to HCV develop chronic infection (2). In addition, more than 71 million individuals are infected with HCV in the world (3, 4). On the other hand, more than 248 million individuals with chronic HBV infection were identified by their HBV surface antigen (HBsAg) seroprevalence worldwide (5). The presence of liver or serum HBV DNA in the absence of serum HBsAg is defined as occult HBV infection (OBI) (6, 7). In addition, nearly 20% of cases with OBI do not have markers of previous HBV infection. Various reasons

are suggested to explain this situation, such as a mutation in the S gene and suppressor role of HCV on HBsAg. HBV activation could be associated with severe hepatitis and liver failure (6-13). There are conflicting data whether HBV replication may be inhibited by coinfection with other agents, especially HCV or HIV (14-20). Although HBV replication is commonly inhibited in the presence of HCV coinfection, a total HBV activation rate of 14.5% is reported following interferon (IFN)-induced HCV eradication (21). Thus, the individuals with active HBV and HCV infections likely show a high HCV viral load, and low or undetectable HBV DNA levels. After HCV suppression, their HBV could become re-activate, or more aggressive with high HBV DNA levels and low HCV RNA detection (22). HBV reactivation was reported

in patients with HCV infection treated with direct-acting antivirals (DAAs) (9, 23). The early direct-acting antiviral agents known as IFN-free treatment were approved by the Food and Drug Administration (FDA) as the treatment of chronic HCV infection in 2013, which greatly eradicated HCV from infected livers (9, 24). Also, DAAs were related to HBV activation both in HBsAg-positive patients, and HBsAg-negative subjects with OBI (6, 25). Currently, there are four classes of DAAs: NS3/NS4A serine protease inhibitors, NS5A inhibitors, NS5B polymerase inhibitors, and cyclophilin inhibitors (26). The major goal of antiviral therapy is to eradicate HCV from the serum and achieve a sustained virological response (SVR), defined as the lack of HCV in the serum 12-24 weeks after completion of therapy (26, 27). Recently, some studies assessed the risk of HBV reactivation in patients infected with HCV receiving interferon (IFN)-free DAAs. However, the incidence and the clinical aspects of HBV reactivation after IFN-free DAAs for HCV are not completely determined (28).

2. Objectives

The current study aimed at analyzing the total risk of occult HBV in Iranian DAA-naïve HCV-infected patients with hemophilia.

3. Methods

3.1. Study Population

From April 2015 to September 2017, one hundred patients with hemophilia receiving IFN-free DAA regimen to treat HCV infection were enrolled in the Comprehensive Hemophilia Care Center in Iran. Before treatment onset, plasma HCV RNA level, and HCV genotype and subtype were collected from patients' medical records. Patients were followed up during treatment and every four weeks after completion of the treatment for 12 weeks, and their clinical parameters and serum HCV RNA levels were assessed by Cobas Taqman HCV (Roche Molecular Systems). In the current descriptive, cross sectional study, demographic and medical characteristics of patients (age, gender, history of blood transfusion, intravenous drug abuse, history of surgical procedures, medical history (HBV infection or other diseases), family history of hepatitis, and history of hepatitis B vaccination) and laboratory tests data (e.g., aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels, HbsAg, triglyceride, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and fasting blood glucose (FBS)) were collected (Table 1) and transferred into a checklist.

The protocol of the study was approved by Ethics Committee of the Iranian Comprehensive Hemophilia Care Center. All participants signed written informed consent, and the study objectives were explained to all of them.

Table 1. Demographic Characteristics and Laboratory Tests Data of the Study Participants^a

Variable	Patients with Hemophilia, N = 100
Age, y	37 ± 10.50
Gender	
Male	81 (81)
Female	19 (19)
Total bil, mg/dL	1.53 ± 1.08
Direct bil, mg/dL	0.32 ± 0.17
AST, U/L	30.94 ± 19.91
ALT, U/L	28.77 ± 22.45
PT, sec	12.82 ± 8.98
INR	1.24 ± 0.72
Hb, mg/dL	14.07 ± 2.88
WBCs, × 10 ³ /μL	7.1 ± 3.26
RBCs, × 10 ⁶ /μL	4.78 ± 0.84
FBS, mg/dL	99.83 ± 23.32
Urea, mg/dL	31.55 ± 9.59
Creatinine, mg/dL	0.99 ± 0.29
Uric acid, mg/dL	5.78 ± 1.93
Cholesterol, mg/dL	152.70 ± 44.26
TG, mg/dL	110.38 ± 43.01
LDL Chol, mg/dL	75.18 ± 25.99
HDL Chol, mg/dL	40.50 ± 11.12
HIV	Negative
HBsAg	Negative
HBcAb	Negative (93%), Positive (7%)
HBsAb, mIU	73.21 ± 45.21

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; Bil, bilirubin; FBS, fasting blood sugar; HB, hemoglobin; HBcAb, hepatitis B core antibody; HBsAb, hepatitis B surface antibody; HBsAg, hepatitis B surface antigen; HDL, high density lipoprotein; LDL, low density lipoprotein; PT, prothrombin time; RBC, red blood cell; TG, triglyceride; WBC, white blood cell.

^a Values are expressed as No. (%) or mean ± SD.

3.2. Serological Tests Using ELISA

A 5 - 6-mL blood sample obtained from each patient was drained in a sterile tube containing ethylene diamine tetra-acetic acid (EDTA) 12 weeks after DAAs therapy. Sera were separated by centrifugation and tested for the presence of HBsAg, HBcAb, and HBsAb using the enzyme-linked

immunoassay (ELISA) commercial kits (Dia. Pro, Italy) according to the manufacturer's protocol.

3.3. Preparation of PBMCs and Plasma

At first, a 5 - 6-mL peripheral blood sample obtained from each patient was drained into a sterile EDTA-containing Vacutainer tube. After separation of plasma by centrifugation, the peripheral blood mononuclear cells (PBMCs) of the samples were separated using the standard method of Ficoll density gradient centrifugation (LympholyteHTM; Cedarlane and Hornby; Canada). The PBMC pellets were washed with phosphate buffered saline (PBS; pH 7.4), and stored at -80°C for HBVDNA extraction. The studies showed that the long term storage of human PBMCs can be performed by cryopreservation with 10% dimethylsulfoxide (DMSO) as freezing medium containing intracellular cryoprotectant plus 90% heat inactivated fetal calf serum (29, 30).

3.4. Molecular Assays Using Nested PCR

All DNAs of PBMC and plasma samples were extracted using NucleoSpin[®] blood kit (Machery-Nagel, Germany) and NucleoSpin[®] Dx virus kit (Machery-Nagel, Germany) according to the manufacturer's protocol, respectively. DNAs were eluted and stored at -20°C for HBV DNA detection. The primers were designed by NCBI-Primer BLAST database. Then, SnapGene software was employed to investigate the validity of sequences (Table 2). To confirm OBI, the nested polymerase chain reaction (PCR) was performed in a $30\text{-}\mu\text{L}$ reaction volume containing $5\ \mu\text{L}$ of DNA sample for the first and $1\ \mu\text{L}$ for the second round of PCR, $10\ \text{pmol}$ of each primer, and PCR-Master mix (amplicon, Korea). In the first and the second round of PCR, DNA was amplified for five cycles at 94°C for one minute, 55°C for one minute, and 72°C for 90 seconds, followed by 35 cycles of denaturation at 90°C . For positive and negative controls, the plasma and PBMC samples of 10 patients with HBV infection and 10 healthy blood donors were used, respectively. In addition, pretreatment plasma samples were checked for the presence of HBV DNA in HBV-positive samples.

3.5. Data Analysis

The data obtained from demographic information of patients were expressed as the mean \pm standard deviation (SD) with SPSS or Excel software. The frequency or percentage was used for descriptive analysis.

4. Results

4.1. Study Population

The blood samples of 100 Iranian patients with hemophilia were collected 12 weeks after receiving DAA therapy. The patients with HCV infection were treated with DAA regimen including daclatasvir/sofosbuvir and ledipasvir/sofosbuvir. The DAA regimens including daclatasvir/sofosbuvir fixed dose ($n = 18.18\%$) and ledipasvir/sofosbuvir fixed dose ($n = 82.82\%$) were administered to patients (with or without ribavirin). All of patients were HBsAg-seronegative and HbCAb test result was positive in seven (7%) patients. The mean serum level of HBSAb was 73.21 ± 45.21 . None of them were infected with HIV. Table 1 shows the demographic characteristics and laboratory tests data of the study participants. The mean age of the patients was 37 ± 10.50 years, ranged 23 to 64, of which 81 (81%) were male and 19 (19%) female.

4.2. Detection of OBI in the Study Participants

Of the 100 patients achieving SVR (undetectable HCV RNA 12 weeks after treatment), HBV DNA was found in one (1%) plasma and three (3%) PBMC samples. All these patients were male ($n = 3$, mean age = 32 years). One patient had a positive PCR test both for plasma and PBMC samples. All of the HBV-positive patients were seronegative for HbCAb and HbsAg markers. DAA regimens of HBV-positive samples were ledipasvir/sofosbuvir and pretreatment HBV PCR results were negative. In addition, the activities of serum ALT and AST were collected from patients' medical records. For all patients, mean serum levels of ALT and AST were 28.77 ± 22.45 and $30.94 \pm 19.91\ \text{mg/mL}$, respectively. The current study findings showed that AST activity was within the normal range for both PBMC and plasma positive samples. In two patients, ALT levels were normal, but its level remarkably increased in the PBMC-positive sample ($53\ \text{U/L}$) compared with the normal range (Table 3).

5. Discussion

The current study aimed at determining the prevalence of OBI in DAA-naïve HCV-infected patients with hemophilia. Different concerns caused HBV activation after successful clearance of HCV through the use of DAA therapy in patients with HCV infection (31). HCV treatment and the alteration of immune synergy in coinfection could be considered as a reason for the risk of HBV activation (13). The current study demonstrated that in 100 HBsAg-negative patients, HBV DNA was detected in 1% of the plasma and 3% of PBMC samples. Indeed, 3% of patients were at the risk of HBV activation after DAA therapy.

Table 2. Oligonucleotide Primers to Detect Occult HBV DNA Through Nested PCR Amplification

Gene	Primer Sequence		Position on Chr, bp	Polarity
HBV genomic regions (surface region)	Forward inner primer	5'-AGGTATGTGCCCCTTTCCT-3'	383	S
	Reverse inner primer	5'-GGGTTAAATGTATACCCA-3'		A
	Forward outer primer	5'-CCTGCTGGTCCAGTTC-3'	948	S
	Reverse outer primer	5'-CCACAATCTTGACATACTTCCA-3'		A

Table 3. Characteristics of Patients with Occult HBV Infection

Variables	Patient ID		
	H23	H71	H83
Gender	Male	Male	Male
Age, y	32	31	35
ALT level, mg/dL			
Baseline	105	84	44
Post treatment	53	18	11
AST level, mg/dL			
Baseline	75	41	36
Post treatment	32	24	29
HBV PCR			
Baseline	Negative	Negative	Negative
Post treatment	Positive (PBMC)	Positive (PBMC and plasma)	Positive (PBMC)
HBcAb			
Baseline	Negative	Negative	Negative
Post treatment	Negative	Negative	Negative
HBsAb			
Baseline	< 100	> 100	> 100
Post treatment	< 4	> 100	> 100

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBcAb, hepatitis B core antibody; HBsAb, hepatitis B surface antibody; HBsAg, hepatitis B surface antigen.

The examinations were performed on Iranian DAA-naïve HCV-infected patients with hemophilia evaluated for OBI through the analysis of both plasma samples and PBMCs. Results of the current study demonstrated no additional risk of OBI after cessation of DAA therapy for HCV infection. To date, OBI is reported in patients with HIV, chronic HCV infection, hepatocellular carcinoma (HCC), advanced cryptogenic liver fibrosis, intravenous drug users, and the patients requiring permanent blood transfusion and hemodialysis (32).

A previous study showed that the prevalence of OBI was 0.5% among about 200 patients undergoing hemodialysis in Tehran, Iran. This result was confirmed by positive results of PCR testing for both the serum and PBMCs (32). In some studies, the prevalence of HCV/HBV coinfection was reported 15.6% to 48.9% (33). So far no other publi-

cation showed the frequency of OBI in HCV-infected patients treated with DAAs without any history of HBV infections. However, several studies pointed out that HBV reactivation related to DAA treatment occurred in 2.1% - 57.1% of the HBsAg-positive patients and frequently during DAA treatment (30). In general, both HCV and HBV have developed mechanisms to escape the host immune responses to develop chronic infection (33). Some studies represented that HBV infection can be inhibited by HCV infection with a suppressing effect of HCV core proteins on HBV replication (34). Indeed, actively replicated HCV could induce a host immune system suitable to control HBV replication. Therefore, treatment of HCV infection with drugs could change immune response against HBV replication resulting in HBV activation (9, 35). For instance, Londono et al., showed that HBV reactivation occurred as an increase

in HBV DNA levels ≥ 1 log in patients with chronic hepatitis C (CHC) receiving an IFN-free regimen (36). Wang et al., demonstrated that HBV reactivation had a prevalence of about 3.1% in 327 HBsAg-positive patients receiving DAAs oral regimen for HCV infections (37). Similarly, Calvaruso et al., reported eight HBsAg-positive cases (7.7%) in 104 patients treated with DAAs (38). In China, Huang et al., showed that 50% of HBsAg-positive and 1.6% of anti-HBc-positive patients presented HBV reactivation during treatment with DAAs without any clinical features (34). A cohort study by Tyson et al., indicated that HBV transmission was predominant in the United States adults, since the prevalence of HBV coinfection in HCV-positive American veterans was low (39). Some reports from Egypt and Turkey represented that the prevalence of HBV coinfection in patients with HCV infection was 0.7% and 2.6%, respectively (40, 41). In contrast to other studies, Ozaras et al. indicated that treatment with DAAs led to hepatitis caused by HBV reactivation in OBI-positive patients with CHC (42). Similarly, Kawagishi et al., showed that five (5.7%) of 87 patients with previous HBV infection presented HBV-reactivation and/or detectable HBV-DNA during IFN-free DAA treatment (43).

5.1. Conclusions

In conclusion, results of the current study showed that the overall prevalence of OBI was 3% among 100 patients with hemophilia receiving DAAs, confirmed by positive results of nested PCR testing for both the plasma samples and PBMCs. As observed in the current study, the prevalence of OBI in such patients was likely low due to regular vaccination for HBV or other unknown agents. However, further studies with more exact laboratory techniques and larger sample sizes are required to improve OBI screening, especially in blood transfusion centers.

Footnotes

Conflict of Interests: The authors declared no conflict of interests.

Ethical Considerations: The study protocol was approved by the Ethics Committee of the Iranian Comprehensive Hemophilia Care Center. All participants signed the written informed consent form and accordingly, the study objectives were explained to them.

Funding/Support: Iranian Comprehensive Hemophilia Care Center (ICHCC).

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