

Study on the Prevalence of Occult Hepatitis B Virus Infection in Patients Undergoing Hemodialysis

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Abstract- Occult hepatitis B (OBI) is a major challenging clinical entity characterized by the absence of hepatitis B surface antigen (HBsAg). The persistence of OBI may progress to fibrosis, cirrhosis, and hepatocellular carcinoma. This study was aimed to investigate the prevalence of OBI among HD patients. In the present cross-sectional study, 89 sera samples of hemodialysis individuals were tested for HBsAg and HBe-IgG by Enzyme-linked Immunosorbent Assay (ELISA). In addition, the HBV DNA has tested in sera and peripheral blood mononuclear cell (PBMC) samples by nested-PCR. Out of 89 patients, 51 (57.3%) were males, and 38 (42.7%) females. The ages ranged from 24 to 90 years (with a mean of 57.5 ± 1.37 years). All the sera samples had normal levels of Aspartate Aminotransferase (AST) and Alanine Transaminase (ALT) but had high levels of Creatinine (Cr) (6.9 ± 2.17) and Blood Urea Nitrogen (BUN) (61.83 ± 2.03). 2/89 (2.2%) sera samples were positive for both HBsAg and HBe-IgG test; in addition, HBV DNA was detected in both sera and their PBMC samples. The sera of 15/89 (16.85%) were only positive for the HBe-IgG test, including 10/51 (19.6%) males and 5/38 (13.2%) females ($P=0.5$). The high 16.85% prevalence OBI has been found among HD patients. To manage OBI infection, screening of HBV DNA should be implemented for HD patients by sensitive molecular means such as nested-PCR and real-time PCR.

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Introduction

Hepatitis B virus (HBV) infection is globally a great issue for public health. Approximately two billion of the world population have been infected HBV with about 1 million death annually (1). HBV has been associated with acute, fulminant hepatitis and chronic infection. Persistence of HBV infection may result in cirrhosis and hepatocellular carcinoma (HCC) (2). Occult hepatitis B infection (OBI) is a new clinical form of hepatitis B and reported in many regions of the world. OBI presents in two forms, seropositive OBI and seronegative OBI. Seropositive OBI is defined, undetectable HBsAg in serum but positive HBeIgG and detectable HBV DNA in serum or PBMC and liver. Seronegative OBI is termed as undetectable HBsAg, HBeIgG, and HBV DNA in serum, but HBV DNA is detectable in peripheral blood mononuclear cells (PBMC) and liver (3). The mutations

in the preS1, preS2, and S regions of the HBsAg gene lead to the reduction of HBV surface protein expression, which in turn resulting in undetectable HBsAg by commercial enzyme-linked immunosorbent assay (4). In addition, several factors such as mutations, integration of HBV DNA into the host genome, cellular epigenetic changes, co-infection with hepatitis C virus (HCV), or human immunodeficiency virus (HIV) and immunosuppression may lead to the development of OBI (5,6). It has been estimated that about 20% of the occult carriers are seronegative, while up to 70% of patients are seropositive for one or more HBV markers (7,8). At present, the standard method for OBI diagnosis is done by the application of highly sensitive molecular means such as nested-PCR and real-time PCR (9). The recent description is the presence of anti-HBe alone is a predictive signal of potential OBI (10); however, the standard OBI definition is the detection of HBV DNA in

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the liver biopsy (11). The integration of HBV DNA into the host genome subsequently results in escaping HBV from the immune system (12). The transmission of OBI via blood transfusion has been reported (13). Since, most of HD patients receiving multiple blood transfusion, therefore, they are at risk of acquiring OBI. The prevalence of OBI among the hemodialysis patients has been reported (14-16). Therefore, this study was aimed to determine the prevalence of OBI among HD patients by nested PCR.

Materials and Methods

Population study and sample collection

In this cross-sectional study, paired serum and PBMC samples were collected from 89 patients HD patients who referred to the dialysis unit of Golestan Hospital in Ahvaz during June to August 2018. Data such as age, sex, history of blood transfusion, AST, ALT, Cr, and BUN were evaluated in this study.

Serological assay

All sera samples were tested for the presence of Hbc-IgG and HBsAg using the ELISA test kit (Diapro, Milano, Italy) according to the manufacturer's instructions.

Preparation of PBMCs

PBMCs were separated from 5 ml ethylenediaminetetraacetic acid (EDTA) using the Ficoll density gradient (Bahar Afshan CO., Tehran, Iran), according to the manufacturer's instruction. Briefly, phosphate-buffered saline (PBS) without calcium and magnesium was mixed equally (1:1) with a blood sample, and then the Ficoll was layered over the diluted blood. After centrifugation at $400\times g$ for 30 minutes at $20^{\circ}C$, PBMCs were isolated and washed three times with PBS. Isolated PBMCs pellets were centrifuged and resuspended in 200 μ l of PBS, and then the cells were stored at $-20^{\circ}C$ until performing DNA extraction.

DNA extraction

DNA was extracted from PBMC and sera samples using High Pure Nucleic Acid Kit (Roche Applied Science, Germany) according to the manufacturer's instructions. The extracted DNA was stored at $-20^{\circ}C$ till used.

HBV-DNA detection by nested-PCR

Nested-PCR was carried with the outer primers, forward: 5'-GAGTCTAGACTCGTGGTG GACTTC-3',

and reverse: 5'-AAATKGCCTAGTAACTGAGCCA-3'; and inner primers, forward: 5'-CGTGGTGGACTTCTCTCAATTTTC-3', and reverse: 5'-GCCARGAGAAACG GRCTGAGGCC-3' (17). For the first round, the reaction mixture containing, 1.5 mM MgCl₂, PCR buffer 10X (Roche, Germany), 200 mM dNTP, ten pmol of primers and, Taq DNA polymerase 1U was subjected to Thermocycler (Peqlab, Germany) and programmed for thermal condition $94^{\circ}C$ for 5 min following 30 cycles of $94^{\circ}C$ for 45 sec, $56^{\circ}C$ for 30 sec and $72^{\circ}C$ for 30 sec and a final extension at $72^{\circ}C$ for 5 min. The second round was carried out using inner primers with the same conditions as the first round. The 417 bp PCR product was electrophoresed on a 2% agarose gel, stained with a DNA-safe stain (CinnaGen, Iran), and visualized by UV-trans illuminator.

Sequencing of isolated HBV DNA

The results of the two PCR products were sequenced (Applied Biosystems, Bioneer, South Korea). The partial sequences of the HBV genome were sent to Gene Bank to obtain the accession number.

Phylogenetic tree analysis

The phylogenetic tree was constructed using the Neighbor-joining method. The sequences of two isolated partials were compared with different HBV genotypes retrieved from GeneBanks. The Neighbor-joining with distances by Kimura two-parameter model. 1,000 replications of bootstrap were used.

Ethical compliance

All of the HD patients participating in this study were orally informed about the aim of this study. Informed consent was obtained from all patients. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration. The project was approved by the ethics committee of Ahvaz Jundishapur University of Medical Sciences under the ethical code of IR.AJUMS.REC.1394.190.

Statistical analysis

Chi-square and t-tests were used by SPSS 16 Package software for statistical analysis (SPSS Inc., Chicago, IL, USA). Descriptive statistics are reported as mean \pm standard deviation and number (percent) as appropriate. *P* less than 5% were considered to be statistically significant.

Results

Among 89 HD patients, 51 (57.3%) were males and 38 (42.7%) females. These patients ranged from 24 to 90 years in age, with a mean of 57.5 ± 1.37 years. All HD patients had received multiple blood transfusions. All the sera samples had normal levels of AST and ALT but had high levels of Cr (6.9 ± 2.17) and Bun (61.83 ± 2.03). 2/89 (2.2%) sera samples were positive for both HBsAg and HBe-IgG tests; in addition, HBV DNA was detected in both sera and their PBMC samples. The sera of 15/89 (16.85%) were only positive HBe-IgG test but negative for HBsAg and HBV DNA, indicating the presence of OBI among the dialysis patients. Among the 15 patients with OBI, 10/51 (19.6%) were males, and 5/38 (13.2%) were females ($P=0.5$). Both positive HBsAg and HBV DNA samples belong to female patients. The frequency of HBV DNA among genders was not significant ($P=0.1$).

Results of sequencing

The sequences of two positive HBV DNA samples (MK886714 and MK886715) were deposited in GenBank. Based on the analysis of sequencing by NCBI and the Hepatitis B Virus database (<https://hbvdb.ibcp.fr/HBVdb/HBVdbGenotype>), both HBV DNA (MK886714 and MK886715) were genotype D1. Both the isolated HBV shows 100% nucleotide identity with HBV genotype D1 (AB674418.1).

Phylogenetic tree analysis

The analysis of the phylogenetic tree also exhibits both the isolated HBV (MK886714 and MK886715) are cluster with HBV genotype D1 (AB674418.1) (Figure 1).

Determination of HBV subtypes

The results of partial amino acid alignment of "a" determinant region of both isolated HBV (MK886714 and MK886715) are consensus with the amino acid of the reference HBV genotype D1 (AB674418.1). Both isolated HBV comprises of the amino acid at position 122R+127P+140T, 145G, 159G+160K, and found HBV subtype ayw2 (18).

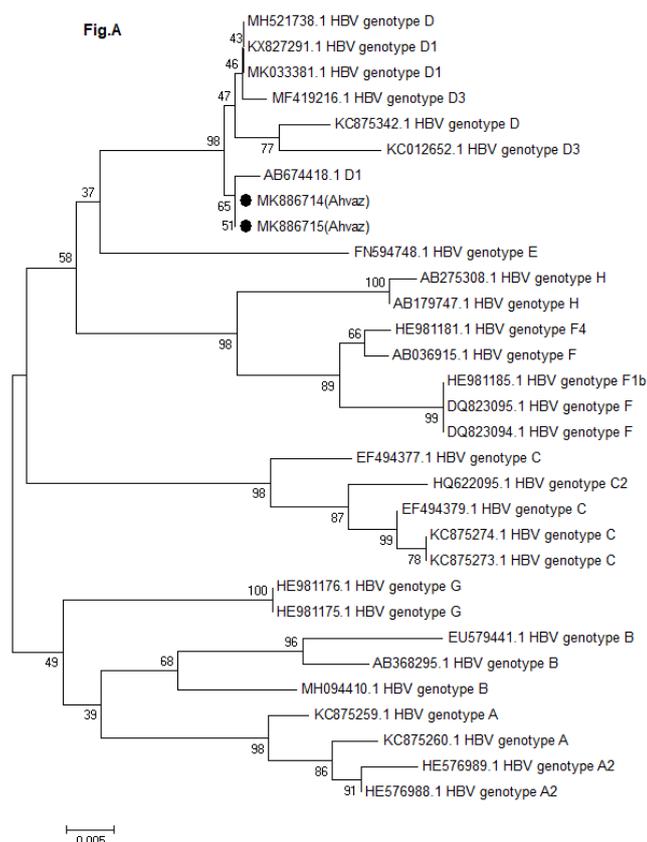


Figure 1. A Phylogenetic tree with the Neighbor-Joining method was constructed with two partial sequences of isolated HBV DNA. The two isolated HBV were compared with different HBV genotypes (A-H) retrieved from GenBank with their relevant accession numbers and isolated from different regions of the world. The two isolated HBV DNA from Ahvaz city, Iran, are marked with a black circle and are cluster with HBV DNA (AB674418.1) genotype D1 isolated from Turkey. Numbers on the branches exhibit bootstrap values acquired after 1,000 replications of bootstrap sampling. Scale bars: 0.005

Discussion

Recently, a new description of OBI is defined as the presence of anti-HBc alone is a predictive signal of potential OBI (10); however, the standard OBI definition is based on the detection of HBV DNA in the liver biopsy (12). As the majority of HD patients receiving multiple blood transfusions, thus, they are the high-risk group to contract OBI (19). In the present study, all HD patients had received multiple blood transfusion history.

The rate of OBI varies from 0% to 58% among HD patients in the various geographic regions (20). In the present study, 16.86% of HD patients were OBI. Elgohry et al. have reported a high prevalence of 26.9% OBI among the HD in Egypt (15). El Makarem *et al.*, have described low detection of 4.1% OBI among the HD patient in Egypt (20).

In our study, all the HD patients including OBI cases, had normal liver enzymes activity. It has been hypothesized that reduction of aminotransferases in HD patients could be caused by high lactate serum levels, which, rapidly consume Nicotinamide Adenine Dinucleotide Phosphate (NADPH) and result in low levels of aminotransferases; the presence of uremic factors that would inhibit the activity of these enzymes (21,22).

Different rates of OBI have been reported among HD patients. Ramezani *et al.*, have reported a low prevalence of 1% OBI among the HD patients in Tehran, Iran (23). In another study, Aghakhani *et al.*, (Tehran, Iran), have detected 3.1% OBI among 289 HD patients (24). Kalantari et al. have not detected OBI in HD patients in Isfahan city, Iran (25). The prevalence of HBV infection in the Iranian general population is about 2.2% (intermediate) (26). Minuk *et al.*, have detected OBI in 3.8% HD patients in North America (27). Similarly, Yakaryilmaz *et al.*, have reported, the rate of OBI was 2.7% among HD patients in Turkey (28).

In the present study, the phylogenic tree reveals that both isolated HBV (MK886714 and MK886715) are cluster with HBV genotype D1 (AB674418.1) isolated from Turkey.

The results of partial amino acid alignment of "a" determinant region of both isolated HBV (MK886714 and MK886715) are consensus with the amino acid of the reference HBV genotype D1 (AB674418.1) isolated from Turkey. Both isolated HBV (MK886714 and MK886715) comprise of the amino acid at position 122R+127P+140T, 145G, 159G+160K, and found HBV subtype ayw2 (18). Genotype D is dominant in Iran

(29,30). HBV genotype D is the common genotype among the neighboring Iranian countries, including Pakistan and Iraq (31-33).

Taken together, our findings and previous studies suggest that OBI should be regarded as a possible source of HBV infection among HD patients. Thus all the HD patients should be screened for HBV markers such as anti-HBc Antibody periodically

The seronegative status in OBI infection may act as a source of infection. The detection of HBV DNA in the liver biopsy is the gold standard methods for diagnosis of seronegative OBI although is invasive and has several drawbacks, such as sampling error, cost, and risk of complications (34,35).

In conclusion, since majority of HD patients receiving multiple blood transfusion, thus, they are high risk group to acquire OBI. Our results show high prevalence of 16.86% OBI have been detected in HD patients. To prevent and manage OBI infection, all the HD patients should be screened for HBV markers such as anti-HBc Antibody periodically Besides, HBV vaccination is very effective for HD patient and should be implemented for HD patients prior to first process of dialysis which can prevent the spreading of HBV infection.

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Occult hepatitis B virus infection in patients undergoing hemodialysis

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