Occult hepatitis B virus infection in maintenance hemodialysis patients: prevalence and mutations in “a” determinant

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Abstract

Background Occult hepatitis B virus infection (OBI) is rare and its diagnosis is often overlooked, but there is still a risk of HBV transmission. To analysis the prevalence of OBI among maintenance hemodialysis (MHD) patients in Sichuan Provincial People's Hospital, the molecular biological characteristics of OBI and the mutation of "a" determinant were investigated.

Method: A total of 330 patients undergoing hemodialysis at Sichuan Provincial People's Hospital and its satellite dialysis units were tested for HBV markers by ELISA. HBV-DNA was detected by real-time PCR in patients with HBsAg negative/ HBcAb positive to investigate the prevalence of OBI. For plasma samples with persistent HBV DNA positive, the S gene was determined by nested PCR, and the sequence of gene mutation was compared with the standard sequence.

Result: Among 330 patients, ages range from 27 to 95 with a mean of 60.66 years old and majority of patients (57.6%) > 60 years old. Serum from 165 of 180 HBsAg negative/HBcAb positive individuals were tested for HBV DNA by real-time PCR. 7 of 165 patients had low level of HBV DNA. Of those, 5 individuals were both HBcAb and HBsAb positive, and 2 individuals were HBcAb positive alone. After 2 years follow-up, 2 individuals with HBcAb positive alone were both tested HBV DNA again, but the other patients were not. Serum from the 2 patients were used nested PCR to confirm the genotype and the mutation of S gene. We found they both were genotype B. Amino acid sequencing confirmed that one had no mutations and the other one had Q128R, T131N, M133S, F134L and D144E mutants in "a" determinant.

Conclusion: Chronic HBV infection was 7.3% (24/330) and OBI was 2.1% (7/330) in our hospital hemodialysis center. The genotype of patients with OBI were genotype B. Mutations of Q128R→T131N→M133S→F134L and D144E might be the potential sites associated with OBI.

Background

Chronic hepatitis B (CHB) infection is highly prevalent in China. The prevalence of HBV sero-epidemiology from national survey was 7.18% in the year of 2012[1, 2]. From Chinese national renal data system, the infection rate specialized in Sichuan province is about 11%. As for hemodialysis patients, the prevalence of CHB ranged from 11% to 15% in developing countries, higher than developed countries[3, 4]. HBsAg positive has long been considered the marker to diagnosis HBV infection. But in the early study, donors containing anti-HBc but lack of HBsAg led to HBV infection of recipient during blood transfusion[5, 6]. Thus, occult hepatitis B infection (OBI) which is characterized by the presence of HBV DNA in the plasma or liver of people with undetectable HBsAg was proposed[7]. Amount of evidence indicates that OBI may be potentially related to acute hepatitis, cirrhosis and hepatocellular carcinoma(HCC)[8, 9]. OBI frequently occurs in specific situations including hemodialysis, organ transplantation, chronic liver disease and blood transfusion[9, 10]. Hemodialysis (HD) patients are more possible incidence of infections as a result of the long-term blood transfusion, the potential exposure to contaminated HD machines, the immunocompromised body, as well as cross-contamination[3, 11–13]. Besides, OBI could be a source of

infection both to other patients and staff inside the hemodialysis units[13]. The prevention of CHB and OBI in maintenance hemodialysis patients remains a challenge.

The progression of the disease and the consequences of long-term infection are related to the genotypes and mutations of HBV. The most common type of HBV strains in western countries are genotype A and D, whereas those in Asia Pacific region including China are genotypes B and C, which possibly lead to different degree of disease progression [2, 14].

The main hydrophilic region (MHR) is a highly conserved region on HBsAg encoded by HBV S gene, containing antigenic determinant genes, which can induce the immune response to produce protective anti-HBs. The 124–147 aa sequence in the MHR region is relatively conserved, forming the “a” determinant, which can maintain the antigenicity of the surface antigen. The mutation or substitution at single or multiple sites can lead to changes in amino acids, which is generally believed to impair HBsAg secretion and inhibit the production of anti-HBs[9, 15].

Reports of many studies have described G145R/A, G119R, P120T, M133T/L, T140L, D144E, T131N, T126N mutants around the world [16–24]. Koyanagi analyzed a strain of RBA and used ELISA to detect HBsAg-negative HBV S gene, and found mutations such as G145A and E129N in the “a” determinant caused the appearance of glycosylation sites, which affecting the HBsAg antigenicity[25]. Present studies revealed that mutations are mainly concentrated in the “a” determinant.

This study reports on the prevalence and molecular characteristics of OBI among MHD patients in Sichuan Provincial People’s Hospital. The research focus on exploring HBV-related biomarkers from studies on genomics to further improve the efficacy of antiviral therapy.

Methods

Study design and patients

The study conducted 330 adult patients undergoing hemodialysis more than three months at Sichuan provincial people’s hospital and its satellite dialysis units from December, 2014 to December, 2015. A systematic screening of HBV sero-markers were detected by ELISA (PerkinElmer Inc., China). The markers included Hepatitis B surface antigen (HBsAg), surface antibody (HBsAb) and core antibody (HBcAb). Previous findings provided us the patients with HBsAg negative/HBcAb positive. Collected serum samples from them were anonymized and tested for HBV DNA by real-time quantitative PCR assay. After 2 years follow-up, samples with persistent HBV DNA were determined the S regions by nested PCR, and sequence of gene mutation was compared with the standard sequence.

HBV DNA testing

HBV viral load (IU/ml) was measured using real-time polymerase chain reaction (qPCR) by COBAS AmpliPrep and COBAS TaqMan HBV Test (Roche, USA) according to the standard manufacturer's instructions. The qPCR kit has a detection limit of 20 IU/ml.

Nested PCR and sequencing

PCR was carried out using PrimeSTAR Max DNA Polymerase (R045A, Takara, Japan) according to the protocol described. The mixtures were denatured at 98°C for 1 min and 30 cycles were performed in a thermal cycler. The PCR products were analyzed by electrophoresis on a 1.2% agarose gel. The sequences of the primers are 5'-GGGTCACCATATTCTTGGG-3' and 5'-CATACTTTCCAATCAATAGG-3' in the first PCR, and 5'-CTTGGAACAAGATCTACAGC-3' and 5'-CATACTTTCCAATCAATAGG-3' in the second PCR. Amplified products were sequenced by Tsingke Biological Technology from China, and amino acid sequences were aligned with standard sequences from PubMed using BioEdit software (BioEdit Sequence Alignment Editor Software, Department of Microbiology, North California State University). Three standard sequences are from the previous studies[26–28].

Statistical analysis

All data were analyzed using SPSS 19.0 software (IBM Corp, NY, USA). Proportions were used to descriptive data. Measurement data is expressed as mean $\pm$ SD. The comparison between the two groups was performed by χ^2 -test or Fisher's exact test. A *p*-value of less than 0.05 was used to determine whether relationships were statistically significant.

Results

Relative characterization of MHD patients

A total of 330 MHD-patients involved this study. Demographic characteristics of the dialysis population are shown in Figure 1 and 2. The participants were aged 27 to 95 years [median $\pm$ SD: (60.66 $\pm$ 14.32) years], with majority of patients were > 60 years old (Figure 1). The proportion of male was 55.7% (185/330), and the female is 44.3% (145/330). The etiology of chronic kidney disease (CKD) and its proportion were listed in Figure 2. Chronic renal inflammatory disease, diabetic nephropathy (DN) and hypertension are major causes of CKD.

All the MHD-patients were determined the frequencies of HBV serological markers (HBsAg, HBsAb and HBcAb) by ELISA. The results showed that the group of after immunizations via vaccination was 15.5% (51/330). 7.3% (24/330) and 21.8% (72/330) were the group non-immune and CHB infection, respectively. The significant proposition of past hepatitis B infection was 54.5% (180/330). Less common

situation was all HBsAg, HBsAb and HBcAb positive in three individuals, occurring early in HBV infection (Table 1).

Assessment of HBV DNA among the studied MHD-patients

Next, the 180 patients (54.5%) were HBsAg negative but HBcAb positive and therefore considered to enroll the further study. Of those, 165 patients have given preoperative consent and collected serum sample to the HBV DNA testing. In many cases, serum (or plasma) HBV DNA determination and analysis based on real-time PCR has been used with sufficient sensitivity to detect OBI. Hence, we diagnosed OBI by serum HBV DNA[13]. The result suggested 7 patients (7/165) had low level of HBV DNA. For description in detail, we encoded them from No.1 to No.7 and their information showed in Table 2.

Five patients (No.1, 3, 4, 5 and 7) are both HBcAb and HBsAb positive, and 2 patients (No.2 and 6) are HBcAb positive alone. We followed these 7 patients for 2 years. Unfortunately, No.3 and 4 died within two years, so they couldn't continue the following study. Only No.2 and 6 remained low level of HBV DNA. But for the other 3 patients, HBV DNA turned to negative (Table 2). This result revealed that we should paid attention to patients with HBcAb positive alone.

Nucleotide sequencing and phylogenetic analysis of the detected strains

To explore the molecular mechanism of the phenomenon we observed in this study, we amplified and identified HBV DNA by nested PCR, and sequence of gene mutation was compared with the standard sequence. Amplified products were subjected to electrophoresis and analyzed. As with electrophoresis showed in Figure 3, No.2 and 6 remained the same bands as the positive control (No.8). More than 10 HBV genotypes (A to J) and several subtypes have been implicated through the genetic divergence of the whole HBV genomic sequences [11]. We compared the two patients' sequence with standard sequence in PubMed. Findings revealed that both them were genotype B as previous studies described[2, 14].

Amino acid mutations related to vaccine escape, so we considered it to be a possible explanation of the continent HBV DNA. We compared their amino acid sequence with standard sequences (D00329, AB602818 and AB073846) using BioEdit. Results revealed No.2 did not occur any amino acid substitutions in "a" determinant. Otherwise, "a" determinant of No.6 had Q128R, T131N, M133S, F134L and D144E mutants (Table 2 and Figure 4). However, clinical implications of these mutations need further investigation.

Discussion

China is a high-prevalent area of hepatitis B infection. The incidence of HBV infection among hemodialysis patients in China was from 6.3% to 30.8%[3]. OBI, a cause of HBV infection, should be taken seriously. However, the prevalence and the mutations of OBI in Sichuan province is rarely investigated. In total, 330 patients with maintenance hemodialysis were enrolled. We detected their serum using ELISA kit, and the result showed 7.3% (24/330) patients were chronic HBV infection. Compared with other regions in China, the infection rate in our hospital was in a low level, indicating the protection was eligible. Interestingly, majority patients (165/330) were HBcAb positive but HBsAg negative, who were potentially OBI patients. when HBsAg is absent, OBI diagnosis is widely by analyzed low levels of the serum (or plasma) HBV-DNA (less than 200 IU/mL) by real-time PCR assays [7, 13, 29, 30]. Hence, the prevalence of OBI in our unit derived from HBV DNA screening. All the OBI patients were male. Man are more especially vulnerable to infect OBI than woman, which is consistent with the results of Kamal et al[31].

Prevalence of CHB and OBI were well-controlled in our unit, but several studies have shown that OBI leads to a higher risk of HBV transmission through blood exchange in maintenance hemodialysis patients[6]. Thus, regular screening of HBV DNA by sensitive PCR assays in MHD patients should be advocated.

We paid attention to the 7 persons and detected their HBV DNA again after 24months.Unfortunately, two individuals died so that we lost to follow-up. For the other patients, 3 of 5 individuals who were both HBsAb and HBcAb positive before turned to HBV DNA negative in 24 months. The other two patients with HBcAb positive alone were persistent HBV DNA positive within 24 months.

DNA extracts were amplified by highly sensitive nested PCR and then confirmed the nucleotide sequences. More than 10 HBV genotypes (A to J) and several subtypes have been implicated through the genetic divergence of the whole HBV genomic sequences [14].The two patients with HBcAb positive alone were both genotype B, which performed as previously described [2].

The HBsAg contains the major hydrophilic region (MHR) in the S gene that spans amino acid positions 100 to 160. The “a” determinant is a dominant epitope inside the MHR, which located between amino acids 124 and 147, and mutations in this region are related to the generation of vaccine escape variants and persistent infection[8, 32–35]. HBV pre-S/S gene mutations influence the immune escape of hepatitis B immunoglobulin. Mutations in the pre-S, core promoter and S regions also promote the progress of cirrhosis and HCC[14]. Actually, “a” determinant is more vital for immune response to HBV infection, and amino acid substitution in this region can generate escape mutations and result in OBI[36, 37]. Thus, sequence analysis identified mutations in “a” determinant from patients with persistent HBV DNA positive. G145R mutant occurred most commonly during OBI[6, 22, 24], which have mainly been found in genotype B [33]. But sequences alignment of two patients did not show G145R mutant.

We found mutations of Q128R, T131N, M133S, F134L and D144E from one patient. Position of T131N, F134L and D144E had been reported[19–21, 23], but Q128R and M133S have not been determined. The new amino acid changes we identified might be significantly reduced the specific anti-HBs antibodies binding.

The clinical implications and mechanism of OBI remain poorly understood. Except mutations in the “a” determinant of HBsAg, treatment-associated mutations, splicing and mutations in the pre-S region also associated with OBI[10]. For further understanding, mechanisms linked to host immune response, such as vitamin D receptor polymorphisms (VDR), cytolytic and noncytolytic T-cell responses, and apoptosis, are related to the regulation of HBV replication and protein synthesis[10]. In addition, it has been reported that memory T-cell immune response, innate immune system or cytokines such as tumor necrosis factor- α and interferon- γ are also associated with OBI [13]. In vivo, the injection of specific vtHBsAg could identify the effect of amino acid substitution. At present, K122I, G145R/I/W/E, and others, are found in the “a” determinant to damage HBsAg secretion, antibody binding and immune escape[38].

In our study, we found new positions related to OBI from reliable patients. Further studies both *in vivo* and *in vitro* should be considered in our experiment to verify the significant mutants and clinical implications.

Conclusions

The prevalence of CHB and OBI was well controlled in our unit, but we also advocate the regular screening of HBV DNA to prevent the serious complications. Mutants of Q128R and M133S were novel and their clinical implication should be further studied.

Abbreviations

OBI occult hepatitis B virus infection

MHD maintenance hemodialysis

HBV hepatitis B virus

CHB chronic hepatitis B

HBsAg Hepatitis B surface antigen

HBsAb Hepatitis B surface antibody

HBcAb Hepatitis B core antibody

PCR polymerase chain reaction

HCC hepatocellular carcinoma

HD hemodialysis

MHR main hydrophilic region

SD standard deviation

DN diabetic nephropathy

CKD chronic kidney disease

VDR vitamin D receptor

Declarations

Ethics approval and consent to participate

The study has been granted by ethics committee of Sichuan provincial people's hospital (Grant No. 2017120). Written informed consent for the study was obtained from all patients.

Consent for publication

No applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YT analysed the experimental data, and was a major contributor in writing the manuscript. XQL examined the serological assessment. DH collected information of patients. XHL analysed patients' information.

QH and GL made contribution to collected samples from patients. YZ designed and fund this work. All authors read and approved the final manuscript.

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Tables

Table 1. Hepatitis B status of our hemodialysis unit.

	Hepatitis B surface antigen (HBsAg)	Hepatitis B core antibody(anti-HBcAb)	Hepatitis B surface antibody(anti-HBsAb)	Number of patients
HBV immune via vaccination	Negative	Positive	Negative	55 (15.5%)
Chronic HBV infection	Positive	Negative	Positive	24 (7.3%)
Past Hepatitis B infection	Negative	Positive	Positive	147 (44.5%)
Past Hepatitis B infection*	Negative	Negative	Positive	33 (10%)
Non-immune	negative	Negative	Negative	72 (21.8%)
Early stage of HBV infection	Positive	Positive	Positive	3 (0.9%)

*Patients who have acquired natural immunity due to prior exposure to hepatitis B

Table 2. Characteristics and information of OBI patients.

Code	Sex	Age-ranges	HBsAg	HBsAb	HBcAb	HBV-DNA (IU/ml)	HBV-DNA (2 years follow-up)	Mutations in “a” determinant
1	Male	60-69	Negative	Positive	Positive	<20	Negative	Unknown
2	Male	70-69	Negative	Negative	Positive	<20	<20	No mutation
3	Male	60-69	Negative	Positive	Positive	<20	Died	Unknown
4	Male	80-89	Negative	Positive	Positive	<20	Died	Unknown
5	Male	60-69	Negative	Positive	Positive	<20	Negative	Unknown
6	Male	60-69	Negative	Negative	Positive	<20	<20	Q128R, T131N, M133S, F134L, D144E
7	Male	80-89	Negative	Positive	Positive	<20	Negative	Unknown

Figures

Figure 1

Distribution of patients age.

Figure 2

The etiology of CKD and its proportion in MHD patients.

A

B

Figure 3

Nested PCR of follow-up patients. (A) The first PCR. (B) The second PCR. No.8 was positive control. The arrow showed positive result.

A

B

Figure 4

The mutations in “a” determinant of No.2 and No.6 compared with standard sequences. (A) No.2 patient had no mutations in “a” determinant. (B) No.6 patient had Q128R, T131N, M133S, F134L and D144E mutants.