Research Article

The Investigation of HBV Pre-S/S Gene Mutations in Occult HBV Infected Blood Donors with anti-HBs Positive

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Introduction. The coexistence of hepatitis B virus (HBV) and hepatitis B surface antibodies (anti-HBs) in occult hepatitis B virus infection (OBI) is a contradictory phenomenon, and the underlying mechanism is not fully understood. The characteristics of pre-S/S mutations in OBI genotypes B and C (OBI_B and OBI_C) in the presence or absence of anti-HBs were analyzed extensively in this study.

Methodology. The amino acid substitutions of envelope proteins of 21 OBI strains, including 4 HBs (+) OBI_B, 6 HBs (−) OBI_B, 6 HBs (+) OBI_C, and 5 HBs (−) OBI_C samples, were analyzed and fully compared among groups of HBV genotypes and the presence of anti-HBs.

Results. The mutation rates in pre-S1, pre-S2, and S proteins of OBI_C were significantly higher than wild-type HBV (wt-HBV) genotype C strains, but only the mutation rate of S protein in OBI_B was significantly higher compared to wild-type HBV genotype B. The mutation rates in S protein of anti-HBs (−) OBI were higher than anti-HBs (+) OBI samples (4.40% vs. 2.43% in genotype B, P > 0.05; 6.81% vs. 3.47% in genotype C, P < 0.05). For these high-frequency substitutions in the pre-S/S region, the mutations sN40S and sK122R were found in 27.3% and 45.5% of anti-HBs (−) OBI strains, respectively. 7 substitutions were uniquely found in OBI_C strains and 9 substitutions were commonly detected in OBI_B and OBI_C strains.

Conclusions. These results suggested that the mutations might occur randomly and were not selected by antibody pressure.

1. Introduction

Hepatitis B virus (HBV) infection is a major threat to human health worldwide, and nearly 2.57 billion people worldwide are estimated to be infected with HBV. China is a higher intermediate prevalence area of HBV. The prevalence of HBsAg for populations aged 1~59 years is 7.18%. It is estimated that there are more than 93 million HBV-infected individuals in China, which results in a public health issue [1]. Occult hepatitis B virus infection (OBI) is a special type of hepatitis B virus (HBV) infection, which is characterized by the presence of a low viral load in the liver and/or in the blood of individuals and being negative for HBV surface antigen (HBsAg) [2]. OBI could be transmitted through blood, and the minimal HBV DNA infectious dose is below the detection limit of the current nucleic acid amplification technology assays (NAT) [3], so a residual risk of transfusion-transmitted OBI still exists in qualified blood donors even after the routine serological and nucleic acid screening, which results in a threat to the safety of blood transfusions and poses extra challenges to the prevention and control of HBV infection.

OBI can be grouped into two types: seropositive OBI [hepatitis B core antibody (anti-HBc) and/or anti-hepatitis B surface antibody (anti-HBs) positive] and seronegative OBI (anti-HBc and anti-HBs negative) [2]. The anti-HBs antibody is usually considered a marker of successful virus clearance and long-term protection. However, the coexistence of HBV and anti-HBs is quite common in OBI [4, 5]. Mu et al. reported that the prevalence of occult HBV infection was 10.9% in HBV vaccinated children in Taiwan [6]. 124 of 2919 (4.2%) HBV vaccinators at the age of 19~21 were
found to be HBsAg (−), anti-HBs (+), and anti-HBc (+), in which HBV DNA was detectable in 81 sera samples using nested PCR [7]. Pande C’s investigation results were even more surprising [8]. In 213 babies born to HBsAg (+) mothers who received recombinant HBV vaccinations at 0, 6, 10, and 14 weeks, 9/213 (4%) developed overt HBV infection, and 89/213 (42%) developed occult HBV infection at a median of 24 months of age. It is worth noting that 51% (45/89) of OBI babies received hepatitis B immunoglobulin (HBIG) and recombinant HBV vaccine, suggesting that OBI in infants with HBV-infected mothers might not be prevented by HBV vaccination.

In general, the level of anti-HBs is low in OBI patients; for example, 4 of 120 HBsAg(−) healthcare workers with low (<10 IU/L) and moderate levels (>10 to <100 IU/L) of anti-HBs were positive for HBV DNA examined by sensitive real-time PCR [9]. It seems that low and moderate levels of anti-HBs have limited neutralization capacity to prevent OBI completely, but a high level (>100 IU/L) of anti-HBs could not provide full protection either, as reported by Zheng et al. [10].

The mechanisms behind the occurrence of OBI associated with anti-HBs pressure are not fully understood. In this study, the pre-S/S mutations in OBI blood donors associated with anti-HBs elicited by vaccination or the host immune response to HBV were analyzed extensively, providing new data for the coexistence of HBV anti-HBs and even the mechanisms of OBI.

2. Methodology

2.1. Blood Sample Collection and Screening. 136425 blood samples were collected by the Shaanxi Blood Center from January to October 2015. Blood samples were individually screened for anti-HIV, anti-HCV, HBsAg, and anti-TP by enzyme immunoassays (EIA) using two different reagents for two rounds (kits were from Wantai Biological Pharmacy Enterprise Co. Ltd., Zhuhai Livzon Diagnostics Inc., Shanghai Kehua Bio-engineering Co. Ltd., Italy’s Diasorin Company and USA Bio-Rad Company). The level of alanine aminotransferase (ALT) was determined by the rate method (Beckman Coulter Au Chemistry Systems, USA).

HCV, HIV, and HBV nucleic acids were detected in 6 samples mixed-mode (6x167 µL) of the Roche COBAS S 201 Nucleic Acid Detection System (F. Hoffmann La Roche Ltd., Basel, Switzerland). The reactive pools were identified according to the single sample mode. All HBsAg (−)/HBV DNA (+) samples were confirmed and quantified by electrochemiluminescence immunoassay (ECLIA) using a reagent kit from Wantai (Wantai Biological Pharmacy Enterprise Co. Ltd.). The study protocol was approved by the Ethics Committee of the Shaanxi Blood Center.

2.2. HBV DNA Quantification and Amplification. Real-time fluorescence-based quantitative PCR was performed to measure HBV DNA levels. Sample handling procedures were strictly according to the instructions supplied by the reagent kit (Sansure Biotechnology, Hunan, China). The minimal detection limit for HBV DNA is 10 IU/mL, and the linear range of the standard curve is from 20 IU/mL to 2.0 x 10^9 IU/mL.

The Pre-S/S (1434 bp)/S (480 bp) region of the HBV genome was amplified by nested PCR according to a previous study [11]. HBV DNA was extracted from plasma using TIANamp Viral DNA/RNA kits (Sansure Biotechnology, Hunan, China). The two sets of PCR primers for the Pre-S gene were:

- P1: 5'-ACATACTCTTGGAGAAGCGG-3'; R1: 5'-CGTCGCAAACACATGCG-3'; P2: 5'-GCCTCATTTTG YGGGTCA-3'; R2: 5'-AGCAAAAACCCAAAGACC-3'.

The two sets of PCR primers for the S gene were:

- S1: 5'-CTCGTGTACAGCGGCTTTTTC-3'; R1: 5'-CATCA TCCATATAGCTGAAGCCCAA ACA-3'; S2: 5'-TTGGTC ACAAGAATCACTCACAATACC-3'; R2: 5'-GCGCTACAGA ACCACTGAAAC ATGG-3'. The products were purified and sequenced by Jinweizhi Biotechnology Co., Ltd. using ABI 3730 DNA sequencer (Applied Biosystems).

2.3. Sequence Analysis of HBV DNA. In the Kimura-2-parameter model with 1000 bootstrap replicates, phylogenetic trees were constructed using Mega-X software using the neighbor-joining method. The HBV reference sequences for multiple genotypes (A to H) were obtained from the National Center for Biotechnology Information genotyping tool. The deduced amino acid sequences of the pre-S/S regions (pre-S1, pre-S2, and S) of OBI were compared to the corresponding genotype B or C consensus sequences of HBV wild-type strains. The control HBV wild-type strains were all Chinese strains obtained from the GenBank database. Accession number of genotype B were FJ386582, FJ386600, FJ386608, FJ386610, FJ386634, FJ386654, FJ386655, FJ386658, FJ386668, FJ386669, FJ386680, FJ386681, FJ386683, FJ386684, FJ386688; accession number of genotype C were FJ386577, FJ386579, FJ386585, FJ386587, FJ386603, FJ386604, FJ386614, FJ386619, FJ386639, FJ386644, FJ386649, FJ386657, FJ386661, FJ386662, FJ386685.

2.4. Statistical Analysis. The SPSS software 18.0 (SPSS, Chicago, IL, USA) was employed for statistical analysis. Group results were compared using a t-test or the chi-square test, as appropriate. A difference with P < 0.05 (bidirectional) was statistically significant.

3. Results

3.1. Serological Characteristics of OBI in Blood Donors. Of the 136425 sera samples, 95 were verified as HBsAg (−)/HBV DNA (+), accounting for 0.0696% of the investigated blood donors. These samples can be classified into 6 different groups based on the serological index (Table 1). 10 samples were seromarker negative, indicating that those samples were from blood donors in a window period of infection or with seronegative/primary OBI. They were removed for further study due to their uncertainty. The other 85 samples
were defined as OBI and used in the following study. Of the 85 OBI samples, 34 were detected as anti-HBs positive, including 7 samples with anti-HBs concentrations of over 100 IU/L, 15 samples with anti-HBs concentrations between 10 IU/L and 100 IU/L, and 12 samples with anti-HBs concentrations lower than 10 IU/L. The other 51 samples were anti-HBs negative. All the samples had normal ALT levels (< 40 IU/L). The average age, the ratio of women to men, and the viral load in the anti-HBs (+) and anti-HBs (−) donors were 40.71 vs. 43.82; 9:25 vs. 9:42; 47.76 ± 28.91 IU/mL vs. 21.86 ± 10.43 IU/mL, respectively.

3.2. The Amplification of HBV Pre-S/S Gene and Classification of HBV Genotypes. The pre-S/S region of HBV was amplified in 11 samples, including 8 anti-HBs (+) and 3 anti-HBs (−). The S region was amplified in those samples that failed to detect the pre-S/S region, and 10 samples, including 2 anti-HBs (+) and 8 anti-HBs (−), were amplified successfully. Finally, the pre-S/S genetic regions were successfully amplified in 21 of 85 OBI samples (24.71%). HBV genotypes were classified.

Based on phylogenetic analyses of OBI S region sequences, 10 and 11 strains were classified as OBI_B and OBI_C.
respectively (Figure 1). The ratio of females to males was similar for OBI\textsubscript{B} and OBI\textsubscript{C} (2:8 vs. 2:9). The age, ALT levels, and viral load were various between genotypes B and C, but no significant differences were found between the two genotypes ($P = 0.41–0.85$).

3.3. Comparison of the Mutations in the Pre-S/S Region of HBV Strains from OBI Samples with that in Wt-HBV Strains. Deduced amino acid sequences of envelope proteins obtained from OBI samples, including 11 pre-S/S and 10 S region sequences from 10 OBI\textsubscript{B} and 11 OBI\textsubscript{C} strains, were
Table 3: The mutations in the pre-S/S region of OBI<sub>B</sub> and OBI<sub>C</sub> strains with anti-HBs positive or negative.

| Protein | Lenth | Mutation Sites | Genotype B Number of mutation Sites/frequency (mutation rate) | Genotype C Number of mutation Sites/frequency (mutation rate) | Sites common B/C Number of mutation Sites/frequency (mutation rate) |
|---------|-------|----------------|---------------------------------------------------------------|---------------------------------------------------------------|------------------------------------------------------------------|
| pre-S1  | 119   | anti-HBs+/-anti-HBs- | 0/0 (0%)                                                      | 3/11 (1.32%)                                                  | 2/4 (0.31%)                                                      |
|         |       | anti-HBs+           | 3/3 (0.84%)                                                   | 17/22 (3.70%)                                                 |                                                                  |
|         |       | pre-S2             | 2/2 (1.68%)                                                   | 6/6 (2.52%)                                                   |                                                                  |
| pre-S2  | 55    | anti-HBs+/-anti-HBs- | 1/2 (0.91%)                                                   | 2/6 (1.56%)                                                   | 0/0                                                              |
|         |       | anti-HBs+           | 6/6 (3.64%)                                                   | 11/13 (4.73%)                                                 |                                                                  |
|         |       | pre-S2             | 1/1 (1.82%)                                                   | 3/3 (2.73%)                                                   |                                                                  |
| S       | 144   | anti-HBs+/-anti-HBs- | 8/20 (1.39%)                                                  | 10/37 (2.34%)                                                 | 20/75 (2.48%)                                                    |
|         |       | anti-HBs+           | 13/14 (2.43%)                                                 | 20/30 (3.47%)                                                 |                                                                  |
|         |       | pre-S2             | 27/38 (4.40%)                                                 | 31/49 (6.81%)                                                 |                                                                  |
| pre-S/S | 318   | anti-HBs+/-anti-HBs- | 9/22 (1.03%)                                                  | 15/54 (1.93%)                                                 | 22/79 (1.60%)                                                    |
|         |       | anti-HBs+           | 22/23 (2.09%)                                                 | 48/65 (3.75%)                                                 |                                                                  |
|         |       | pre-S2             | 30/41 (3.95%)                                                 | 40/58 (5.43%)                                                 |                                                                  |

OBI, occult hepatitis B virus infection; anti-HBs, hepatitis B surface antibody.

aligned with the corresponding consensus sequences of wt-HBV strains (15 genotype B and 15 genotype C), and the results of the comparison were presented (Table 2). Compared with genotype C wt-HBV strains, the mutation rates in pre-S1, pre-S2, and S proteins of OBI<sub>C</sub> were significantly higher (P < 0.01). However, the pre-S1 and pre-S2 regions appeared well conserved between OBI<sub>B</sub> and genotype B wt-HBV strains (P = 1.000, P = 0.095), only the mutation rate in S proteins of OBI<sub>B</sub> was significantly higher (P < 0.01) than that of wt-HBV strains. When comparing the sequences of OBI<sub>C</sub> with OBI<sub>B</sub> the substitution rate in the pre-S1 region was significantly higher in OBI<sub>C</sub> than in OBI<sub>B</sub> (P < 0.05), but the substitution rates in pre-S2 and S showed no statistical significance (P = 0.527, P = 0.073).

3.4. Comparison of the Mutations in the Pre-S/S Region of HBV Strains from OBI Carriers in Groups of anti-HBs Positive and Negative. Site mutations in the pre-S/S region of HBV strains from OBI carriers in the presence or absence of anti-HBs were aligned together (Figure 2). The differences in numbers, frequency, and rates of mutations were evaluated among groups (Table 3). The mutation rate in S protein of anti-HBs (−) OBI<sub>B</sub> samples was statistically higher than that in anti-HBs (+) OBI<sub>B</sub> samples (6.81% vs. 3.47%, P < 0.05), but no statistical difference was found in anti-HB (−) OBI<sub>B</sub> and anti-HB (+) OBI<sub>B</sub> samples (4.40% vs. 2.43%, P > 0.05). The substitution rate in pre-S1 and pre-S2 proteins between groups of anti-HBs (−) and anti-HBs (+) in either OBI<sub>C</sub> or OBI<sub>B</sub> carriers appeared with no statistical significance (P > 0.05). The overall mutation rate considering Pre-S/S in OBI<sub>C</sub> strains was higher than in OBI<sub>B</sub> strains. However, for anti-HBs (−) OBI<sub>B</sub> strains, only the mutation rate in S protein presented a statistical difference (P = 0.046) between genotype B and C, whereas only the mutation rate in pre-S1 protein presented a statistical difference for anti-HBs (+) OBI<sub>B</sub> strains between genotype B and C (P = 0.01).

Furthermore, amino acid substitutions in the major hydrophilic region (MHR) and the “α” determinant were analyzed. The mutation rate of MHR in anti-HBs (−) OBI<sub>B</sub> strains was significantly higher than that in anti-HBs (+) OBI<sub>C</sub> strains (10.3% vs. 4.52%, P < 0.01), and the mutation rate of MHR in anti-HBs (−) OBI<sub>C</sub> strains was higher than that in anti-HBs (−) OBI<sub>B</sub> strains (10.3% vs. 4.52%, P < 0.01). Nevertheless, the mutation rates in the “α” determinant among different anti-HBs status and genotypes presented no statistical difference.

The high frequency of pre-S/S substitutions was classified as preferentially associated with either the anti-HBs (+) or anti-HBs (−) OBI strains considering different genotypes (Table 4). The substitution sN40S was uniquely found in 27.3% of anti-HBs (−) OBI<sub>B</sub> carriers, and sK122R was uniquely found in 45.5% of anti-HBs (−) OBI preferentially in OBI<sub>C</sub> strains. The substitutions ps1A60V, ps1G73S, ps1V90A, ps2I42T, sT118R/V/M, sD144E/N/A were uniquely found in OBI<sub>C</sub> strains in both anti-HBs (+) and anti-HBs (−) populations. The substitutions sV/T47K/A/E, sM133T/L, sF134I/L/R, sK160R/S/K, sV168A, sS174N, sL175S, and sV177A were commonly detected in OBI<sub>B</sub> and OBI<sub>C</sub> strains in both anti-HBs (+) and anti-HBs (−) populations.

4. Discussion

OBI is a worldwide health problem, and the reported prevalence significantly varies depending on locations, populations, and the sensitivity of diagnostic assays. The detection rate of OBI in blood donors was 1:1436 in our study, which was approximately two times lower than that in the data from South China reported by the National Institute of Diagnostics and Vaccine Development in Infectious Diseases of China (1:631) [12] but was higher than that in the data from Shenzhen Blood Center (1:3239) [4, 13]. Due to the similarity of the populations and diagnostic assays in
In this study, ps1A60V, ps1G73S, ps1V90A in pre-S1 and ps2I42T in pre-S2 regions were only detected in OBIC strains, which suggested that the presence of anti-HBs (+) OBI was still unclear, and the research mainly focuses on the following aspects to explore it at present: (1) Pre-S/S gene. The pre-S/S gene encodes three envelope proteins named large S (encoded by regions of pre-S1, pre-S2, and S), middle S (encoded by regions of pre-S2 and S), and small S (encoded by only the S region) protein. The pre-S (pre-S1 and pre-S2) region contains several functional sites and is crucial for viral replication. The pre-S region also plays an essential role in interacting with the immune responses because it is rich in B/C cell epitopes [19]. The major hydrophilic region (MHR) is between aa100 and aa169 in the HBV S gene, and the “α” determinant is a relatively conserved region within MHR, which serves as the most important immunodominant region in all HBV strains and is essential to the detection of HBsAg and development of HBV vaccines [20]. Mutations or insertion or deletion in the HBV pre-S/S gene may affect the antigenicity, immunogenicity, expression, and secretion of HBsAg, leading to detection failure of HBsAg. It may also reduce or even abolish the virion’s replication and/or secretion, exerting a negative effect on HBsAg presentation.

Unlike HCV and some other hepatitis viruses, their antibodies could not provide protection and were the only evidence of infection. The anti-HBs were the neutralizing antibodies for HBV and the markers of recovery from acute infection or immunity from vaccination. The coexistence of HBsAg and anti-HBs seemed contradictory, but it was reported in many populations of different ages, sex, and immune situations [4, 6–8]. The mechanism behind anti-HBs (+) OBI is still unclear, and the research mainly focuses on the differences in OBI rates may be due to the different prevalence rates of HBV infection in North and South China [14].

The distribution of HBV genotypes presents clear differences in geographical regions, ethnicities, and clinical outcomes [15]. Genotypes B and C are prevalent in Southeast Asia and China. In particular, genotype C circulates mainly in North China, Korea, Japan, and Thailand, and genotype B is predominant in South China and Southeast Asia [10, 16]. Several studies have shown the enhanced infectivity of genotypes A and C, and genotype C is associated with more severe liver diseases, including cirrhosis and HCC [17, 18]. It was reported that HBV genotypes had a significant influence on the occurrence of OBI [5]. In our study, the mutation rates of pre-S1, pre-S2, and S in genotype C were higher than in genotype B, but no statistical differences were found. In addition, the mutation rates in S protein of anti-HBs (−) OBI were higher than anti-HBs (+) OBI samples (4.40% vs. 2.43% in genotype B, 6.81% vs. 3.47% in genotype C, P < 0.05). Controversially, Wang JW et al. reported that the number and frequency of site substitutions in the S protein from anti-HBs (−) OBI strains were higher than those associated with anti-HBs (−) OBIC strains, which suggested that the presence of anti-HBs might more effectively select variants in the S regions in genotype C than in genotype B OBI strains [4]. However, our results indicated that nonsynonymous mutations may occur randomly and were not selected by anti-HBs pressure. Further studies should be conducted based on larger samples and a strict control group to investigate whether the amino acid substitutions in HBV strains are significantly associated with clinical features and OBI occurrences in anti-HBs positive or negative patients.

Unlike HCV and some other hepatitis viruses, their antibodies could not provide protection and were the only evidence of infection. The anti-HBs were the neutralizing
of the S region were found with high frequency (9.1–45.5%) in OBI strains (Table 4). The mutations psN40S and psK122R were found in 27.3% and 45.5% of anti-HBs (−) OBI strains, respectively. The other 11 mutations were detected in anti-HBs(+) and anti-HBs(−) OBI populations. Regarding the distribution of the substitutions in genotypes of HBV, psN40S was uniquely observed in OBIb carriers, 3 mutations were observed in OBIc strains, and the other 9 mutations were detected in both OBIb and OBIc strains. SK122I appeared to affect the biological properties of HBsAg and facilitated glycosylation of HBV [26], and sK122R was reported in OBIb strains in both anti-HBs (+) and anti-HBs (−) OBI strains [4]. In our study, sK122R was uniquely found in anti-HBs (−) OBI strains, suggesting that sK122R may occur not due to anti-HBs pressure.

Several substitution sites were detected in this study, including sN40S, sY100S/C, sK122R, sI126T/M, sQ129R/L, sM133T/I, sP134I/L/R, sK160R/S/K, sD144E/N/A, and sV177A, have been reported to be associated with OBI [19]. The classical immune escape mutations sG145A/K were found in both anti-HBs (+) and anti-HBs (−) OBIc strains with low mutation frequency, suggesting that this classical mutation might be better detected due to the improvement in HBsAg detection reagents, and the mutation may occur randomly regardless of the pressure of anti-HBs.

5. Conclusion

The mutations in the pre-S/S region of OBI strains were analyzed and compared among different HBV genotype groups (OBIb and OBIc) and serological groups (anti-HBs positive and anti-HBs negative). The amino acid substitutions occurred more frequently in OBI than in wt-HBV, providing possible explanations for the occurrence and existence of OBI. The amino acid mutation rate in the S protein of anti-HBs (−) OBI samples was higher than anti-HBs (+) OBI samples, suggesting that the mutations in the S region are not selected by anti-HBs pressure resulting from vaccination or a natural host immune response to HBV. Our study provided insights into the underlying mechanisms by which the OBI occurs and the anti-HBs present in OBI carriers. However, the numbers of OBI strains that could amplify the pre-S/S region were small, and all the samples came from Shaanxi. The source of HBV strains was relatively single. Further work should be conducted based on a multicenter and large sample size, and more accurate results could be obtained. Furthermore, functional analysis of relevant genetic variants should be performed to elucidate the mechanism of OBI with anti-HBs positive in the future.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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