Molecular Characteristics of Occult Hepatitis B Virus from Blood Donors in Southeast China

Quan Yuan,1† Shan-Hai Ou,2† Chang-Rong Chen,2*, Sheng-Xiang Ge,1 Bin Pei,2 Qing-Rui Chen,1 Qiang Yan,1 Yong-Cai Lin,2 Hong-Ying Ni,2 Cheng-Hao Huang,1 Anthony E. T. Yeo,1 James W. K. Shih,1 Jun Zhang,1* and Ning-Shao Xia1

National Institute of Diagnostics and Vaccine Development in Infectious Diseases, School of Life Science, Xiamen University, Xiamen, Fujian Province, China,1 and Xiamen Blood Service, Xiamen, Fujian Province, China2

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The characteristics of 30 carriers with occult hepatitis B virus (HBV) infection (OBI) were compared with those of 30 individuals diagnosed as being HBV carriers at the time of blood donation, 60 asymptomatic carriers, and 60 chronic hepatitis patients. The prevalence of genotype C was significantly higher in carriers with OBIs than in any other HBsAg-positive (HBsAg+) group (P < 0.001). Specific amino acid substitutions in the regions from amino acids 117 to 121 and amino acids 144 to 147 located in the major hydrophilic region of the S gene were associated with carriers with OBIs (P < 0.01 for carriers with OBIs versus HBsAg+ donors, carriers with OBIs versus HBsAg+ asymptomatic carriers, and carriers with OBIs versus HBsAg+ chronic hepatitis patients). GI45R was the major variation in the HBV isolates responsible for local occult HBV infections.

The introduction of screening for hepatitis B virus (HBV) surface antigen (HBsAg) has reduced the risk of overt HBV transfusion-transmitted infections (TTIs), although transfusion-transmitted infections continue to occur. In particular, occult hepatitis B virus infection (OBI) poses a threat to the blood supply. Individuals with OBIs are defined as those in whom viral DNA is detected in liver or blood by nested PCR or real-time PCR but in whom HBsAg is undetectable in serum by current commercial HBsAg assays. Occult HBV infection status can be associated with mutant viruses undetectable by current HBsAg assays (10, 11, 15, 17, 28), but it may also be due to the suppression of viral replication and gene expression and virus secretion (2, 7, 13, 23). The presence of an OBI can occur after recovery from an infection but anti-HBs remains present (21, 25, 29) or anti-HBC is the only marker (12), or an OBI may even be the state in which no antibody makers may be present. The presence of HBV is detectable only if a highly sensitive method is used (8). The viral load is mostly less than 10^10 IU/ml (4) and is often less than 200 IU/ml (24). Indeed, immuno-suppressed organ or bone marrow transplant recipients with anti-HBs or anti-HBc have been shown to be infectious. To prevent the transmission of such infections, screening for anti-HBc is not 100% effective. Additionally, screening of pooled blood donations, a common practice in blood banks, also decreases the sensitivities of the assays used (3). Finally, if an HBV nucleic acid test (NAT) is used, it needs to be extremely sensitive to eliminate HBV DNA-containing units (1).

In countries where HBV is endemic, such as China, research into OBI is limited by the lack of a suitable method for screening large numbers of samples. HBV NAT is procedurally cumbersome and incurs high costs. Thus, given these constraints, the use of an anti-HBc alone (serum antibodies against HBV core antigen in isolation) as a marker for OBI was investigated in the study described here, given that it may be a possible marker of infection (27), with one study citing an OBI detection rate of between 5 and 10% for patients who tested positive for anti-HBc as the sole marker of HBV infection (4). The clinical epidemiology of blood donor OBIs is not known in China, and the present study attempted to determine the clinical and molecular characteristics of these infections in the context of other cohorts of HBV carriers.

MATERIALS AND METHODS

Serological tests for study subjects. A total of 19,518 blood samples were collected from blood donors at the Xiamen Blood Service, Fujian Province, China, from 18 July 2007 to 27 August 2008. All samples initially tested negative by a rapid test for HBsAg. This was a colloidal gold immunoassay (Wantai Ltd, Beijing, China) with a lower detection limit of 2 IU. To minimize the occurrence of false-negative results, three different commercial assays (Murex [version 3] enzyme-linked immunosorbent assay [ELISA; Abbott Murex, Dartford, United Kingdom]; Wantai Company [Beijing, China] ELISA; Xinchuang ELISA [InTec, Xiamen, China]) were used for further screening for HBsAg. Samples with a positive result by any one assay were considered to be HBsAg positive. For specimens that were HBsAg negative, two additional commercial HBsAg assays (Hepanostika HBsAg Ultra [bioMérieux, Marcy l’Etoile, France]; Monolisa Ag, HBs Ultra [Bio-Rad, Marnes La Coquette, France]) were used to confirm the HBsAg status of the blood samples. The sensitivities of the five HBsAg assays ranged from 0.03 to 0.2 IU/ml. Testing for HBsAg, anti-HBs, and anti-HBc was performed by the Wantai ELISA. Anti-HIV, anti-Treponema pallidum (the syphilis spirochete), and anti-hepatitis C virus (anti-HCV) antibodies were detected by the use of the Murex ELISA products. All assays were performed according to the manufacturers’ instructions and were performed on an ELISASTARlet automated system (Hamilton, Bonaduz, Switzerland).

HBV DNA analyses. All specimens positive for anti-HBc alone were tested for HBV DNA. Viral DNA was extracted from 300 μl of plasma by using a QIAamp DNA blood kit (Qiagen, Hilden, Germany). Six different primers pairs (Table 1)
were utilized in the nested PCRs (which were performed by the procedures described in footnote a of Table 1), with samples from all positive cases being retested. Samples that had a confirmatory positive PCR result and a negative HBsAg result were determined to be from individuals with OBIs. The viral load was determined by real-time PCR (Kehua Company, Shanghai, China). The HBsAg result were determined to be from individuals with OBIs. The viral load was determined by real-time PCR (Kehua Company, Shanghai, China).

Control cohort. The control cohort consisted of a group of HBsAg-positive individuals matched to the study group only by age and gender. Among this control group, 30 were prospective blood donors diagnosed with HBV infection, 60 were asymptomatic carriers, and 60 were individuals with chronic hepatitis. All specimens were collected from the Xiamen Center for Disease Control and Prevention, Xiamen, China. The samples were analyzed for serologic markers for HBV, viral loads, and the HBV genotype. The genetic diversity of the major hydrophilic region (MHR) in the S gene in HBVs from the controls was assayed and was compared with that of the MHR in the S gene in HBVs from donors with OBIs.

Statistical evaluation. Statistical analyses were performed by the Mantel-Haenszel $\chi^2$ test and Fisher's exact test for categorical variables and by the Mann-Whitney analysis of variance test for continuous variables (Open Source Epidemiologic Statistics for Public Health [OpenEpi], version 2.3). Differences were considered to be statistically significant when the $P$ values were $\leq 0.05$. The demographic data collected included age, gender, and place of birth. The place of birth was included because the prevalence of HBV varies geographically (30).

RESULTS

Of the 19,518 samples tested, 19,360 tested negative for HBsAg (by one rapid test and three ELISAs) and 158 samples were confirmed as positive for HBsAg by one rapid test and three ELISAs. The viral load was determined by real-time PCR (Kehua Company, Shanghai, China). The HBsAg result were determined to be from individuals with OBIs. The viral load was determined by real-time PCR (Kehua Company, Shanghai, China).
The time of blood donation (HBsAg identified, the 30 individuals diagnosed as being HBV carriers at the time of blood donation (HBsAg donors) with OBIs were excluded from sequencing because 2 cases tested positive for HBV DNA load (log \( \log_{10} \text{copies/ml} \)). Samples from 4 of the 34 cases were positive for the S gene, to which primer set A is targeted. The results of sequencing with primer set P, and these two primer set did not target the S gene, from aa 110 to aa 123 than the samples from any other control group. The viral load was significantly lower among the donors with OBIs than among the asymptomatic carriers or patients with chronic hepatitis (\( P < 0.001 \) and \( P < 0.001 \), respectively), but the difference was insignificant when the results for the donors with OBIs were compared with those for the HBsAg donors (\( P = 0.065 \)).

Phylogenetic analysis indicated that 19 sequences (63.3% [19 of 30]; 95% confidence interval [CI], 43.7 to 80.1%) from donors with OBIs, 3 sequences (10.0% [3 of 30]; 95% CI, 2.1 to 26.3%) from HBsAg donors, 11 sequences (18.3% [11 of 60]; 95% CI, 9.5 to 30.4%) from asymptomatic carriers, and 15 sequences (25.0% [15 of 60]; 95% CI, 14.7 to 37.9%) from chronic hepatitis patients clustered with HBV genotype C. The remaining sequences clustered with HBV genotype B. The prevalence of genotype C was significantly higher than that of genotype B (\( P < 0.001 \)) in donors with OBIs.

The amino acid acid map of the S-protein MHR (amino acid [aa] 110 to aa 165) is shown in Fig. 1 and is stratified by HBsAg status. The average level of amino acid diversity in the a epitope (aa 124 to aa 147) was significantly higher in the OBI group than any other control group (\( P = 0.038 \) for donors with OBIs versus HBsAg donors, \( P = 0.011 \) for donors with OBIs versus asymptomatic HBV; \( P = 0.02 \) for OBI donors versus patients with chronic hepatitis, * statistically significant difference (\( P < 0.05 \)).

Table 2 compared the birthplaces, ALT levels, virus loads, HBeAg status, frequency of genotype C, and the mutations located in the MHRs of the S gene for the 30 individuals with OBIs identified, the 30 individuals diagnosed as being HBV carriers at the time of blood donation (HBsAg donors), the 60 asymptomatic carriers not from the blood donor pool, and the 60 hospitalized patients with chronic hepatitis. The viral load was significantly lower among the donors with OBIs than among the asymptomatic carriers or patients with chronic hepatitis (\( P < 0.001 \) and \( P < 0.001 \), respectively), but the difference was insignificant when the results for the donors with OBIs were compared with those for the HBsAg donors (\( P = 0.065 \)).

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Table 2. Comparison of characteristics between the occult HBV cases and other HBV groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Result for HBsAg-negative carriers with OBIs identified by testing (n = 30)</th>
<th>Result for HBsAg-positive (control) groups</th>
<th>( P ) value (( P^a ), ( P^b ), ( P^c )) ( ^d )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD age (yr)</td>
<td>30.4 ± 9.2</td>
<td>30.5 ± 8.7</td>
<td>Matched</td>
</tr>
<tr>
<td>Sex (no. of M:no. of F)</td>
<td>21:9</td>
<td>21:9</td>
<td>Matched</td>
</tr>
<tr>
<td>No. (% of cases with a local birthplace (Fujian Province))</td>
<td>28 (93.3)</td>
<td>28 (93.3)</td>
<td>Matched</td>
</tr>
<tr>
<td>Mean ± SD HBV DNA load (log no. of copies/ml)</td>
<td>2.3 ± 0.7</td>
<td>2.9 ± 0.8</td>
<td>Matched</td>
</tr>
<tr>
<td>ALT level (U/liter)</td>
<td>All below 40</td>
<td>All below 40</td>
<td>Matched</td>
</tr>
<tr>
<td>No. (% of cases HBeAg positive)</td>
<td>0 (0)</td>
<td>11 (18.3)</td>
<td>Matched</td>
</tr>
<tr>
<td>No. (% of cases infected with HBV genotype C)</td>
<td>19 (63.3)</td>
<td>15 (25.0)</td>
<td>Matched</td>
</tr>
<tr>
<td>a epitope aa 124-147</td>
<td>11 (36.7)</td>
<td>8 (13.3)</td>
<td>0.038, * 0.011, * 0.011</td>
</tr>
<tr>
<td>aa 124-aa 143</td>
<td>4 (13.3)</td>
<td>8 (13.3)</td>
<td>0.99, 0.99, 0.99</td>
</tr>
<tr>
<td>aa 144-aa 147</td>
<td>9 (30.0)</td>
<td>0 (0)</td>
<td>0.001, * 0.001, * 0.001</td>
</tr>
<tr>
<td>G145R in a epitope</td>
<td>7 (23.3)</td>
<td>1 (1.7)</td>
<td>0.005, * 0.003, * 0.001</td>
</tr>
<tr>
<td>aa 110-123</td>
<td>7 (23.3)</td>
<td>2 (3.3)</td>
<td>0.005, * 0.001, * 0.001</td>
</tr>
<tr>
<td>aa 117-aa 121</td>
<td>7 (23.3)</td>
<td>0 (0)</td>
<td>0.005, * 0.001, * 0.001</td>
</tr>
<tr>
<td>aa 148-aa 165</td>
<td>2 (6.7)</td>
<td>2 (3.3)</td>
<td>0.09, 0.02, 0.02</td>
</tr>
</tbody>
</table>

\( ^a \) Diagnosed as HBV carriers by testing at the time of blood donation.  
\( ^b \) \( P^a \), \( P \) value for OBI donors versus HBV carriers diagnosed by testing at the time of blood donation; \( P^b \), \( P \) value for OBI donors versus asymptomatic HBV; \( P^c \), \( P \) value for OBI donors versus patients with chronic hepatitis. * statistically significant difference (\( P < 0.05 \)).  
\( ^c \) M, males; F, females.  
\( ^d \) NC, not calculated.  

Examples tested positive for HBsAg. Of the 158 HBsAg-positive (HBsAg+) samples, only 52 samples were positive by all three assays, 30 samples were positive by any two assays, and 76 samples were positive by only one assay. Of the 19,360 HBsAg-negative samples, 995 were positive for anti-HBc alone and 34 of the 995 samples were HBV DNA positive but negative by all six commercial assays for HBsAg (one colloidal gold immunoassay, five ELISAs). Thus, these samples were from individuals with OBIs and were negative by all six assays for HBsAg. For all 34 individuals with OBIs, primer set A had the highest sensitivity; and use of this primer set revealed 30 of 34 positive cases (88%), while 16 cases (47%), 15 cases (44%), 8 cases (24%), 7 cases (21%), and 6 cases (18%) were positive with primer sets Vg, C, S, P, and Hc, respectively. All individuals with OBIs were clinically asymptomatic and HCV, HIV, and \( T. pallidum \) free and had alanine aminotransferase (ALT) levels of less than 40 U/liter. Samples from 4 of the 34 cases were excluded from sequencing because 2 cases tested positive by the use of primer set C and 2 cases tested positive by the use of primer set P, and these two primer set did not target the S gene, to which primer set A is targeted. The results of sequencing of the MHR of the S gene are shown in Table 1.
The specific amino acid substitutions in the viruses from donors with OBIs were concentrated from aa 117 to aa 121 ($P$ = 0.005 for donors with OBIs versus HBsAg + donors, $P < 0.001$ for donors with OBIs versus HBsAg + asymptomatic carriers, $P < 0.001$ for donors with OBIs versus HBsAg + chronic hepatitis) and aa 144 to aa 147 ($P = 0.001$ for donors with OBIs versus HBsAg + asymptomatic carriers, $P < 0.001$ for donors with OBIs versus HBsAg + chronic hepatitis), as indicated by a red box in Fig. 1. Seven mutants (23.3%; 95% CI, 9.9 to 42.3%) with single-point or multipoint G145R mutations were found in the OBI group, whereas none were found in the other groups ($P < 0.001$ for donors with OBIs versus asymptomatic carriers and patients with chronic hepatitis, $P = 0.005$ for donors with OBIs versus HBsAg + donors).

FIG. 1. Amino acid variability of S-protein MHR (aa 110 to aa 165) in individuals with occult HBV infection and controls. The consensus amino acid sequences of genotypes B and C were deduced from the sequences with GenBank accession numbers AB073826 and AF286594, respectively. Strains whose amino acid sequences were identical to the wild-type sequence are not represented in the figure. These nonrepresented strains included the following: OBI 9 to 11, 13 and 14, and 23; HBsAg + donors 1, 5 to 8, 10 to 14, 18 and 19, 21, 25, 27 and 28, and 30; asymptomatic chronic carriers (ASC) 2 and 3, 5 to 8, 12 to 14, 16, 19 to 23, 25 to 27, 29, 31 to 35, 37, 41, 43 to 45, 47 to 49, 51, 54, and 57 to 60; patients with chronic hepatitis 1, 3 to 7, 9, 11, 15, 17 to 20, 23, 25 to 27, 31 and 32, 34 to 40, 42 to 46, 49, 57, and 60 (genotype B, adw2); donors with OBIs 20, 22, 24, and 26 to 28; HBsAg + donors 15, 22, and 23; asymptomatic chronic carriers 4, 10, 11, 17, 24, 36, 39, 42, and 53; and patients with chronic hepatitis 22, 29, 33, 47 and 48, 51, 53, 55, and 59 (genotype C, adqrq positive).

DISCUSSION
The prevalence of occult HBV infections among non-A non-E chronic hepatitis cases is a function of several parameters. (i) The method of detection used (ELISA, PCR, or real-time PCR) affects the prevalence of occult HBV infections detected. (ii) If the PCR detection method is based, the primers selected will affect the sensitivity and the specificity of the test. In our study, six primer sets targeting different regions yielded sensitivities of between 18% and 88% for the OBI cohort. (iii) The population being studied affects the prevalence of occult HBV infections detected; e.g., the prevalence of blood donors with anti-HBc is likely to be very different from the prevalence of patients with chronic hepatitis positive for HBV DNA. (iv) Patients from areas where HBV is endemic are more likely to have occult HBV infections, if only because of the high numbers of infected individuals present. (v) The materials being tested, e.g., liver tissue or serum, affects the prevalence of occult HBV infections detected. Occult HBV infections are more likely to be found when liver tissue specimens than when serum specimens are tested.

The prevalence of OBIs varies greatly. A study from Hong Kong reported a prevalence rate of 6.9%, while the rate re-
ported in Italy was 11% (19). Among Canadian Inuits, the rate was 8.1% among subjects devoid of any HBV markers (22). In northeast China, the prevalence rates of OBs in IgG anti-HBc-positive subjects were 100% (45/45), 86.7% (85/98), and 33.3% (14/42) in patients with cryptogenic chronic liver disease, HBsAg-negative patients with hepatocellular carcinoma, and HBsAg-negative healthy people, respectively. In these cases, the viral load was low (<10^3 viral copies/ml) (9). In Taiwan, a study that used HBV NAT yielded 12 cases among 10,727 seronegative donations (0.11%) (18). The rate reported in this paper is 34 of 19,360 HBsAg-negative donations, or 0.18%. The rate of detection is thus dependent on a number of factors.

In our study, the average age of the donors with OBs was 30 years; and they had normal ALT levels, undetectable HBsAg, and very low viral loads. There were more males in the group that tested positive for anti-HBc only (n = 995; ratio of males to females, 1.8:1) than in either the HBsAg-negative donor group (n = 19,360; ratio of males to females, 1.2:1; P < 0.001) or the HBsAg-positive donor group (n = 158; ratio of males to females, 1:1; P = 0.008). The HBV genotype associated with clinical symptoms and disease progression was genotype C. This genotype is common in East Asian countries and has been linked with a higher risk of advanced hepatic disease, such as hepatocellular carcinoma (6, 16). The amino acid substitutions in the S-protein MHR were found to be different in strains from donors with OBs than in strains from HBsAg+ donors. Amino acid substitutions were detected in the regions from aa 117 to aa 121 and aa 144 to aa 147 in donors with OBs (P < 0.001) but not found in strains from HBsAg+ donors. It is interesting that these sequences are in the region (aa 119 to aa 124) which may interfere with hepatitis D virus (HDV) infectivity (14). HDV particles are coated with the same envelope proteins (large, middle, and small surface antigens) found in HBV. HDV is thus considered an occasional satellite of HBV, because its capacity to propagate depends on the envelope proteins of HBV. If the potential role of aa 119 to 124 in the infectivity of HDV is confirmed for HBV, the high frequency of mutations in this region might participate in the mechanism of occurrence of OBs. The amino acid residues from positions 120 to 123 were considered to be essential for the antigenicity of HBsAg in previous studies (26). Mutants with mutations from aa 144 to aa 148, such as GI45R and DI44A, were reported to be the common escape mutants that interfered with HBsAg detection and/or mutants that evaded vaccine-induced neutralizing antibodies (5, 20). Thus, mutations in these two regions may have played a crucial role in the occurrence of OBs.

In conclusion, HBV genotype C and specific mutations in the MHR of the S gene were associated with the occurrence of occult HBV infection. The clustering of substitutions in the regions that change the antigenicity of HBsAg and/or virus infectivity may play a key role in the establishment and/or maintenance of occult HBV infections.

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