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Occurrence of occult hepatitis B virus infection associated with envelope protein mutations according to anti-HBs carriage in blood donors

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ABSTRACT

Objectives: Occult hepatitis B virus infection (OBI) carries a risk of hepatitis B virus (HBV) transmission and hepatocellular carcinoma. As previous studies have had a limited sample size, the characteristics of OBI with genotype B and C (OBI_B and OBI_C) mutations relating to hepatitis B surface antibody (anti-HBs) elicited by vaccination or a limited host immune response to HBV have not been fully explored.
Methods: In this study, the occurrence of OBI_B or OBI_C strains associated with envelope protein (pre-S/S) amino acid substitutions obtained from 99 blood donors stratified according to anti-HBs carriage were characterized extensively.
Results: According to the presence of anti-HBs within each genotype, the number and frequency of substitution sites specific for anti-HBs(−) OBI_B were higher than those specific for anti-HBs(+) OBI_B strains (67 vs 31; 117 vs 41), but the reverse pattern was found in OBI_C strains (3 vs 24; 3 vs 26). Mutations pre-s_1T68I and sQ129R/L were found uniquely in 15–25% of anti-HBs(+) OBI_B carriers and mutation pre-s_1A54E was found preferentially in anti-HBs(+) OBI_C, while 17 substitutions were found preferentially in 11–38% of anti-HBs(−) OBI_B strains. In the major hydrophilic region (MHR) region, mutations sS167 in OBI_B, sT118 in OBI_C, and sA166 in both genotypes were possibly immune-induced escape mutation sites.
Conclusions: Several mutations in pre-S/S of OBI appeared to be associated with carrier anti-HBs pressure, which might be risk factors for potential reactivation of viruses under anti-HBs selection in OBI carriers.
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Introduction

Occult hepatitis B virus infection (OBI) is a low replication form of hepatitis B virus (HBV) infection. OBI refers to the detection of HBV DNA without detectable hepatitis B surface antigen (HBsAg) outside the pre-seroconversion window period, most being associated with hepatitis B core antibody (anti-HBc) (Allain, 2004; Raimondo et al., 2008, 2019). Published data suggest that OBI is infectious through direct blood contact, particularly in transfusions, and might rarely infect the general population through sexual contact; it is thought to be potentially associated with severe liver damage and hepatocellular carcinoma (HCC) (Zacharakis et al., 2008; Chemin et al., 2009; Wong et al., 2011). The transmission of OBI to blood recipients has confirmed a significant risk of transfusion-transmitted HBV infection (Gerlich, 2006; Levicnik-Stezinar et al., 2008). Therefore, OBI is a challenge for several preventative and therapeutic strategies, including blood donation screening, clinical diagnosis, vaccination, and treatment of chronic HBV infection.

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At present, a two-round HBsAg screening is implemented in blood donations in China, and HBV nucleic acid testing (NAT) is progressively being adopted in pilot blood centers, leaving untested DNA-containing blood units at risk of occult HBV transfusion-transmission. Shenzhen is one of the pilot cities. This city has a population derived from migration from most of the provinces of China, making it representative of the whole situation in China to a certain extent (Shang et al., 2007; Ye et al., 2013).

Previous studies conducted in Shenzhen and in other areas have explored the prevalence and characteristics of OBI with genotype B or C (OBI_B and OBI_C) in China (Yuan et al., 2010; Zheng et al., 2011). However, as previous studies have had a limited sample size, the characteristics of OBI_B and OBI_C mutations associated with hepatitis B surface antibody (anti-HBs) elicited by vaccination or the host immune response to HBV have not been fully explored. These amino acid substitutions might be one of the mechanisms behind the occurrence of OBI associated with anti-HBs pressure. In this study, a large number of HBsAg-negative/HBV DNA-positive (HBsAg(−)/HBV DNA(+)) blood samples from blood donors, which were classified as OBI, were identified. The characteristics of envelope protein (pre-S/S) amino acid substitutions were analyzed extensively, which were further correlated to the potential emergence of variant viruses selected by anti-HBs presence in OBI carriers.

Materials and methods

Blood sample collection and screening

A total of 310 167 blood samples were collected from donors between April 2010 and December 2012 in Shenzhen Blood Center; 114 761 (37%) of these samples were from first-time donors and 195 406 (63%) were from repeat donors. All plasma samples were screened for HBsAg and alanine aminotransferase (ALT) during pre-donation screening using rapid tests with colloidal gold strips, and were further screened for HBsAg and antibodies to hepatitis C virus (HCV), HIV, and syphilis using two different enzyme immunoassays (EIA) (domestic and imported), as described previously (Zheng et al., 2011). Plasma samples confirmed negative by EIA were subsequently screened for HBV DNA, HCV RNA, and HIV-1 RNA with a triplex NAT (Novartis TIGRIS, USA) (Zheng et al., 2015). One hundred and twenty-one HBsAg(−)/HBV DNA(+) samples were followed up, including 37 from patients who had one to six follow-up samples obtained 14 to 690 days after collection of the index sample. They were confirmed by chemiluminescent microplate immunoassay (CMIA) for HBsAg and with a highly sensitive in-house nested PCR and real-time PCR (QPCR) for HBV DNA (Zheng et al., 2015).

HBV DNA detection and sequencing

HBsAg(−)/HBV DNA(+) plasma samples were quantified for HBV DNA load by QPCR. For samples with sufficient volume, viral DNA was extracted from 400 μl of plasma using a High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Mannheim, Germany), as instructed by the manufacturer. For samples collected from the donation plasma bag, 2.5 ml of plasma were used for extraction by High Pure Viral Nucleic Acid Large Volume Kit (Roche Diagnostics GmbH, Mannheim, Germany). The extracted DNA was quantified by QPCR (Zheng et al., 2011) with a sensitivity of 5 IU/ml and amplified with a combination of nested PCRs targeting the full genome, the pre-S/S region (nt 2808–710) or the S region (nt 215–710), as described previously (Zahn et al., 2008). All quantitative measurements were performed in triplicate. Amplicons were sequenced commercially (Huada Gene, Shenzhen, China).

Phylogenetic analysis

Phylogenetic trees were constructed with MEGA version 7.0 (www.megasoftware.net) using the neighbor-joining method in alignment with 124 genotype B (subtype B1–B7) and 95 genotype C (subtype C1–C7) reference sequences of the S region from HBsAg-positive blood donors from China, Thailand, and Malaysia (Candotti et al., 2012).

Mutation analysis of occult HBV strains

The envelope protein pre-S/S sequences (pre-S1, pre-S2 and S) or S regions of OBI were analyzed. Deduced amino acid sequences of each region were compared to the corresponding genotype B or C consensus sequences of HBV wild-type strains (Zheng et al., 2011).

Statistical analysis

The statistical analysis was performed using SPSS software (version 13.0; SPSS Inc., Chicago, IL, USA). The Pearson Chi-square test was used to compare the differences in number or frequency of substitution sites of pre-S/S amino acid sequences between OBI and wild-type strains. The Mann–Whitney *U*-test was used for the comparison of continuous variables for the different genotypes and HBV DNA loads between OBI and HBsAg-positive samples. Statistical significance was defined as $p < 0.05$ (two-tailed).

Results

Classification of HBsAg-negative/HBV DNA-positive samples

A total of 121 HBsAg(−)/HBV DNA(+) plasma samples were identified by EIA and NAT from 310 167 blood samples, and were further characterized (Table 1). Among these, six samples were classified as false-positive when re-tested by QPCR and nested PCR. Four donors became HBsAg-positive in follow-up samples and were classified as having window period (WP) infections. Twelve

Table 1
Classification of HBsAg-negative/HBV DNA-positive samples.

Anti-HBV	OBI	Window period	False-positive	Unclassified	Total
S+/C+	31	0	1	0	32
S+/C−	12	0	4	0	16
S−/C+	53	2	0	0	55
S−/C−	3	2	1	12	18
Total	99	4	6	12	–
Age (median), years	20–56 (35)	27–38 (35)	21–45 (27)	19–41 (23)	–
Male/female	72/27	1/3	3/3	8/4	–
ALT (median), U/l	6–39 (17)	10–22 (17.5)	8–25 (15)	8–30 (19)	–
VL (median), IU/ml	0–2122 (27.9)	20.56–156.1 (85.04)	0–1205 (18.8)	0–2782 (68.9)	–

HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; OBI, occult hepatitis B virus infection; S, hepatitis B surface antibody (anti-HBs); C, hepatitis B core antibody (anti-HBc); ALT, alanine aminotransferase; VL, viral load. Number of samples for each group is listed.

samples remained unclassified or indeterminate as non-reactive to all five serological markers of HBV, and follow-up samples were unavailable. OBI status was confirmed for 99 samples with follow-up testing and/or serological markers of HBV (Table 1). Follow-up samples were available for 29 donors and were collected 14 to 690 days after the index samples. The approximate frequency of OBI in Shenzhen blood donors was 1:3133 (99/310 167).

Among the 99 OBI donors (Table 1), there were nearly three times more males than females. All had normal ALT levels (<40 U/l). The median age of the OBI donors was 35 years. Their viral load was low (median 27.9 IU/ml). The OBI were further classified with antibody markers: 84 were anti-HBc-positive of which 31 also contained anti-HBs (36.9%); 12 samples contained anti-HBs without detectable anti-HBc (12.1%). Samples from three donors contained no serological markers (3.0%) and were classified as primary OBI.

Amplification and sequencing of envelope protein genes of OBI

A total of 20 full-genomic sequences (17 OBI_B and 3 OBI_C), 29 pre-S/S (24 OBI_B and 5 OBI_C), and 18 S (15 OBI_B and 3 OBI_C) sequences were obtained (67/99 OBI samples or 67.7%). Phylogenetic analysis classified 56 (84%) strains as OBI_B and 11 (16%) as OBI_C (Figure 1). No significant difference was found according to sex, age, or viral load between OBI_B and OBI_C (p = 0.224–0.924); however ALT levels were higher in genotype C (p = 0.034), although all were within the normal range of <40 U/l.

Diversity of envelope protein sequences in OBI

Deduced amino acid sequences of envelope proteins (50 pre-S/S and 67 S) of OBI were aligned with the corresponding consensus sequences from 121 genotype B and 92 genotype C wild-type HBV strains, respectively. The mean number of amino acid substitutions for each protein (pre-S1, pre-S2 and S) was analyzed and compared between OBI and wild-type strains (Supplementary material Table S1). Compared with HBsAg-positive HBV strains, the mean number of amino acid mutations in pre-S/S proteins of OBI was significantly higher (p = 0.003 to $p < 0.0001$). Only pre-S2 appeared similarly conserved in both OBI and HBsAg-positive HBV strains (p = 0.941).

Identification of envelope protein substitutions in OBI according to anti-HBs carriage

The site substitutions and their frequency within envelope proteins of OBI are presented in Table 2. In 41 pre-S1/S2 and 55 S sequences of OBI_B strains, the substitutions ps_2D14N (29.3%), sE2G/D/A (20%), sA5S/V/T (21.8%), sK24R (27.3%), sQ101R/K/H (23.6%), sM103I (25.5%), sP105R/L (21.8%), sG130R/A (20%), and sF134I/S/L (30.9%) occurred significantly more frequently than in the HBsAg-positive comparator group (p = 0.033 to $p < 0.0001$). A higher frequency of substitutions ps_1A54E (44.4%), ps_1N56H/Q (55.5%), and ps_1V60A (33.3%) were found in 9 pre-S1/S2 and 12 S OBI_C strains (p = 0.037–0.003).

According to the anti-HBs seromarker, 16 pre-S1/S2 sequences (11 OBI_B and 5 OBI_C) and 21 S sequences (14 OBI_B and 7 OBI_C) from 43 anti-HBs(+)/anti-HBc(+/−) OBI carriers were obtained, while 21 pre-S1/S2 sequences (19 OBI_B and 2 OBI_C) and 26 S sequences (24 OBI_B and 2 OBI_C) from 56 anti-HBs (−)/anti-HBc(+/−) were identified. Site mutations in the pre-S/S proteins of OBI according to genotype and the presence of anti-HBs were aligned, as shown in the Supplementary material (Table S2).

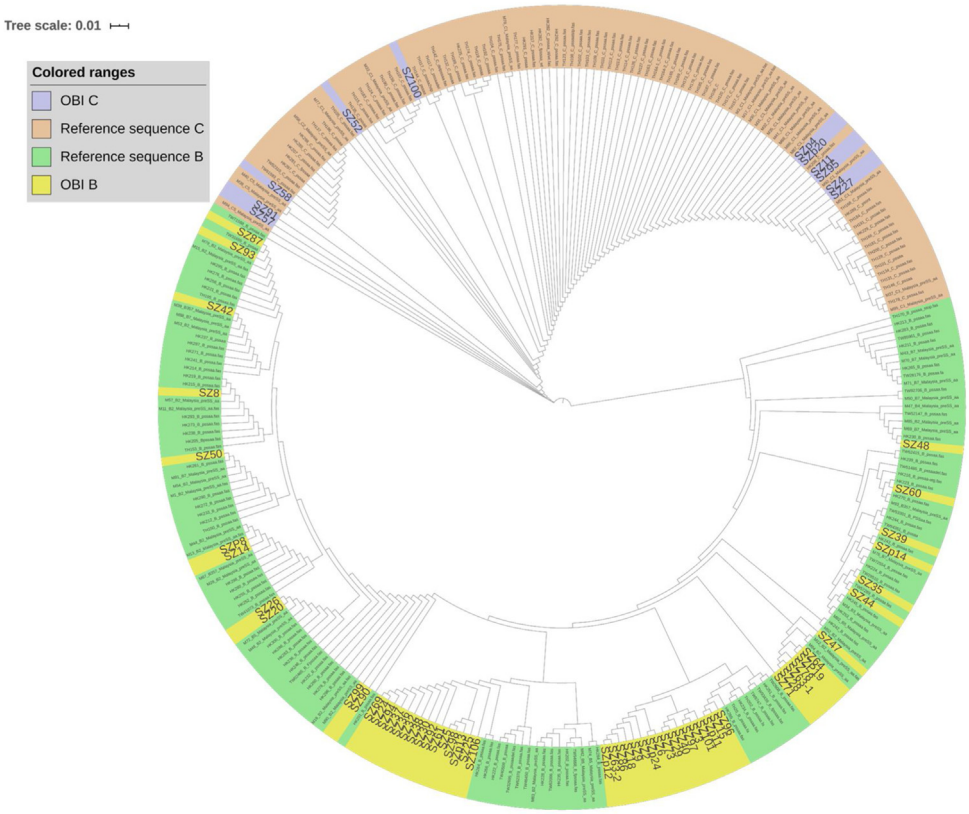


Figure 1. Phylogenetic relationship of OBI S region sequences. The S sequences of OBI_B and OBI_C stains were aligned with the reference strains from 124 genotype B and 95 genotype C HBsAg-positive blood donors, as indicated by the different colors. The bar on the left side of the tree denotes genetic distance. (OBI, occult hepatitis B virus infection; OBI_B, genotype B; OBI_C, genotype C; HBsAg, hepatitis B surface antigen).

Table 2
Frequency of envelope protein substitutions in OBI sequences aligned with 121 genotype B or 92 genotype C reference amino acid sequences.

Region	Mutation	Frequency in OBI blood donors[a]	Frequency in HBsAg-positive blood donors	*p*-Value (OBI vs HBsAg-positive)
Gt-B				
PreS1	F67I	4/41 (9.8%)	5/121 (4.1%)	0.714
	T68I	4/41 (9.8%)	33/121 (27.2%)	0.021
	A81T/S	4/41 (9.8%)	0/121 (0%)	0.004
	N98T/K	3/41 (7.3%)	4/121 (3.3%)	0.275
PreS2	Q2K	6/41 (14.6%)	5/121 (4.1%)	0.021
	D14N	12/41 (29.3%)	0/121 (0%)	<0.0001
	F22L	3/41 (7.3%)	6/121 (5%)	0.569
	I42T	3/41 (7.3%)	2/121 (1.7%)	0.103
S (s)	E2G/D/A	11/55 (20%)	0/121 (0%)	<0.0001
	A5S/V/T	12/55 (21.8%)	12/121 (10%)	0.033
	K24R	15/55 (27.3%)	0/121 (0%)	<0.0001
	N40S	10/55 (18.2%)	20/121 (16.5%)	0.787
	G44E	3/55 (5.5%)	21/121 (17.4%)	0.033
	I92T	5/55 (9.1%)	2/121 (1.7%)	0.031
	Y100S/C	7/55 (12.7%)	4/121 (3.3%)	0.017
	Q101R/K/H	13/55 (23.6%)	2/121 (1.7%)	<0.0001
	M103I	14/55 (25.5%)	2/121 (1.7%)	<0.0001
	P105R/L	12/55 (21.8%)	6/121 (5%)	0.001
	I110L	5/55 (9.1%)	6/121 (5%)	0.294
	S113N	9/55 (16.4%)	0/121 (0%)	<0.0001
	K122R	7/55 (12.7%)	15/121 (12.4%)	0.951
	G130R/A	11/55 (20%)	2/121 (1.7%)	<0.0001
	F134I/S/L	17/55 (30.9%)	3/121 (2.5%)	<0.0001
	Y161S/F	10/55 (18.2%)	8/121 (6.6%)	0.019
	V168A	8/55 (14.5%)	4/121 (3.3%)	0.006
	L175S	7/55 (12.7%)	0/121 (0%)	<0.0001
	V177A	10/55 (18.2%)	3/121 (2.5%)	<0.0001
Gt-C				
PreS1	G27D	3/9 (33.3%)	28/92 (30.4%)	0.857
	Q51H	3/9 (33.3%)	13/92 (14.1%)	0.132
	A54E	4/9 (44.4%)	14/92 (15.2%)	0.029
	N56H/Q	5/9 (55.5%)	12/92 (13%)	0.003
	V60A	3/9 (33.3%)	9/92 (9.8%)	0.037
	S62A	3/9 (33.3%)	21/92 (22.8%)	0.48
PreS2	S6T/N	4/9 (44.4%)	18/92 (19.6%)	0.084
S	E2D	2/12 (16.7%)	0/92 (0%)	0.012
	S3N	3/12 (25%)	14/92 (15.2%)	0.389
	L53S	3/12 (25%)	18/92 (19.6%)	0.659
	I68T	2/12 (16.7%)	3/92 (3.3%)	0.101
	I126T	3/12 (25%)	19/92 (20.7%)	0.729
	S154P	2/12 (16.7%)	0/92 (0%)	0.012
	R160K	2/12 (16.7%)	4/92 (4.3%)	0.085
	S174N	2/12 (16.7%)	0/92 (0%)	0.012

OBI, occult hepatitis B virus infection; HBsAg, hepatitis B surface antigen.
[a] Aligned with 121 genotype B or 92 genotype C reference amino acid sequences[15], the frequency of site substitutions in the pre-S/S region was calculated for 41 PreS1/S2 or 55 S sequences of genotype B, or 9 PreS1/S2 or 12 S of genotype C of OBI strains from blood donors in Shenzhen, China.

Comparison of envelope protein amino acid substitutions between OBI carriers with and without the presence of anti-HBs

The amino acid substitutions in the pre-S/S proteins of OBI from individuals with and without anti-HBs were examined (Figure 2). The number and frequency of substitution sites were calculated according to anti-HBs and genotype of the OBI strains (Table 3). We first concentrated on the number of substituted sites common to strains associated with both anti-HBs(+) and anti-HBs(−), and then specific to either anti-HBs(+) or anti-HBs(−) in genotype B or C of OBI. The number and frequency of site substitutions in the pre-S/S protein from anti-HBs(−) OBI_B strains were higher than those associated with anti-HBs(+) OBI_B strains (67 vs 31 for number, and 117 vs 41 for frequency), but the reverse pattern was found in OBI_C strains (3 vs 24 for number, and 3 vs 26 for frequency). The sequences presented a large difference in the number of substitution sites between genotypes B and C (144 vs 37), but a small difference between anti-HBs(+) and anti-HBs(−) OBI (55 (31 + 24) vs 70 (67 + 3)).

According to the anti-HBs status of genotype B or C OBI carriers, the high frequency of pre-S/S substitutions at individual sites was classified as preferentially associated with either the anti-HBs(+) or anti-HBc(−) OBI population (Figure 3A), or as common to both populations (Figure 3B; highlighted in green, yellow, or blue in Figure 2). In anti-HBs(+) OBI carriers, substitutions ps_1T68I and sQ129R were uniquely found in 14–25% of OBI_B strains (Figure 3A), while ps_1A54E was found uniquely in 19% of OBI_C strains. In anti-HBs(−) OBI carriers, substitutions ps_1E39A/G, ps_1L45F/N, ps_1S109T, ps_2D14N, sE2G/D, sA5S/V, sG10R/E/K, sY100S/L, sM103I, sI110L, sS113N, sT126P/I/A, sA128V, sQ129R/L, sG130A/R, sS154R/L, sE164G/L, and sW165S/L were found uniquely or preferentially in 12–38% of OBI_B strains (Figure 3A). Thirteen mutations ps_2Q2K, sK24R, sN40S, sQ101H/K/R, sP105R/L, sK122R, sP127T/L/H/S, sM133L/S/I/T, sF134L/S/I, sY161F/S, sV168A, sL175S, and sV177A were commonly detected in 21–60% of OBI_B strains in both anti-HBs(+) and anti-HBs(−) populations (Figure 3B). The well-known vaccine-related substitutions sG145R/A/V in the MHR region were observed at relatively low frequency (4.8% for anti-HBs(+) and 9.5% for anti-HBs(−)) in OBI_B carriers, clearly not preferentially associated with circulating anti-HBs at the time of sample collection (Figure 2).

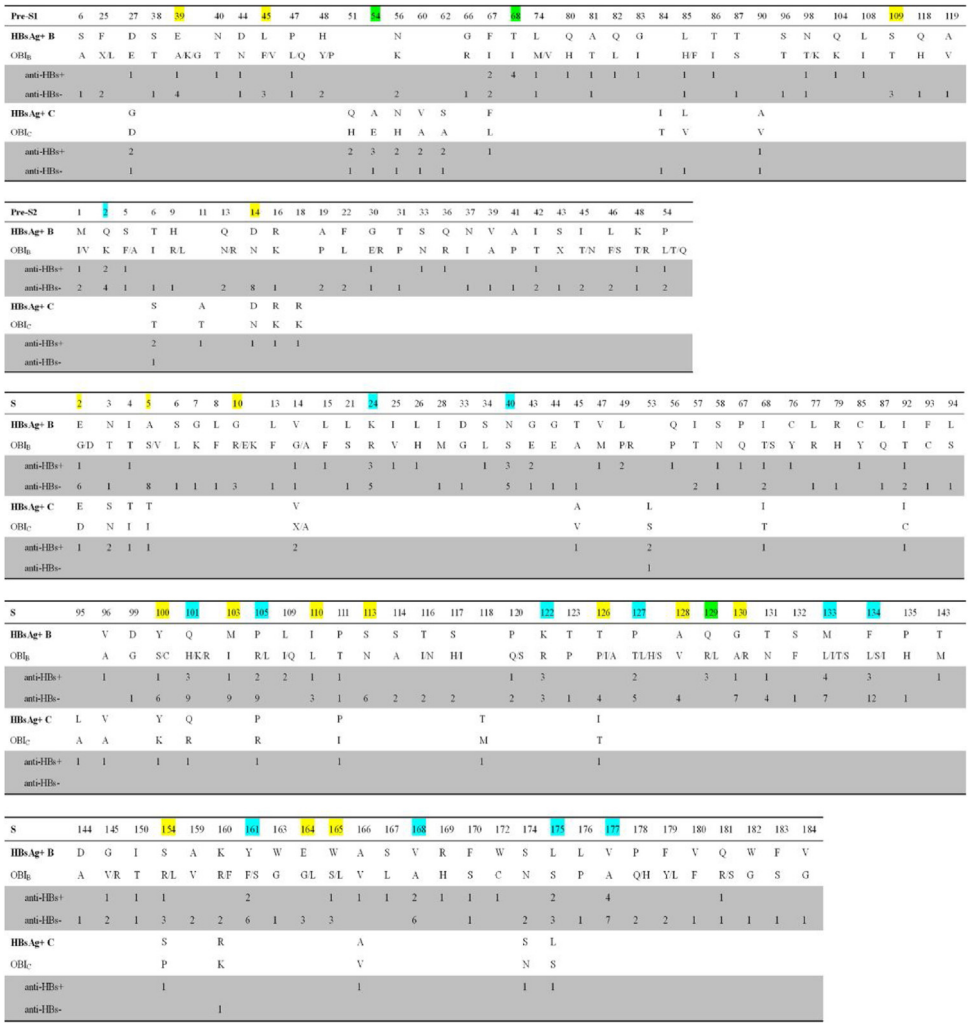


Figure 2. Alignment of amino acid substitution frequency observed in envelope proteins of anti-HBs(+) and anti-HBs(−) OBI sequences. Substitutions highlighted in green, yellow, and blue were observed preferentially in anti-HBs(+), anti-HBs(−), and in both anti-HBs(+) and anti-HBs(−) OBI carriers, respectively. The numbers indicate the number of substitutions at each site out of the 41 pre-S1/S2 and 55 S proteins from OBI_B or the 9 pre-S1/S2 and 12 S proteins from OBI_C sequences. (Anti-HBs, hepatitis B surface antibody; OBI, occult hepatitis B virus infection).

Substitutions in the MHR occurred frequently in OBI_B strains (Figure 2). When comparing the amino acid substitutions in MHR with those in other published reports (El Chaar et al., 2010; Candotti et al., 2012; Huang et al., 2012; Wang et al., 2015) and aggregating the current data with these data (Figure 4), the mutations sM103, sS113, sS114, sG130, sS132, and sK160 appear specific to OBI_B (highlighted in green) and sD102 and sW165 appear specific to OBI_C (highlighted in pink), while sT118, sP135, and sS154 appear common to both OBI_B and OBI_C (in blue). Since OBI is defined as a lack of detectable HBsAg in plasma and a low viral load, specific substitutions in OBI might affect the production and detection of HBsAg or might lead to escape from anti-HBs recognition. Further, when stratifying OBI between anti-HBs(+) and anti-HBs(−) carriers, specific substitutions in anti-HBs(+) OBI might be key adaptive substitution sites selected under immune pressure. Therefore, sS167 in OBI_B, sT118 in OBI_C, and sA166 in all genotypes of OBI appear to be immune-induced escape substitutions.

Discussion

In this study, the yield of OBI was 1:3133 in Shenzhen blood donors between 2010 and 2012, which shows an apparent increase compared with previously published data (1:7517) from the years 2003–2009 (Zheng et al., 2011). This is likely due to the use of a more sensitive NAT applied to individual donations (ID-NAT). The reported frequency of OBI in blood donations from various countries ranges between 1:2000 and 1:15 000 (Brojer et al., 2006; Candotti et al., 2008; Allain et al., 2009). Here we report a yield of OBI considerably lower than that reported in East Asia and West Africa (1:100–1000), but higher than that reported in Western Europe, North America, and Australasia (<1:5000) (Allain, 2017; Li et al., 2017). In the blood donor population of Shenzhen, those with OBI are predominantly male (72% male vs 27% female). However, this is in part related to the 2:1 male:female ratio in the Shenzhen blood donor population.

The genotype distribution of HBV presents clear geographical and ethnic differences, and different clinical outcomes (Chu et al., 2003). Genotypes A and D are dominant in North and West Europe and the United States (Chu et al., 2003; Candotti et al., 2008). Genotype D is associated with more severe clinical outcomes, and its clearance rate for HBV DNA, hepatitis B e antigen (HBeAg), and HBsAg is lower than that of genotype A (Chu et al., 2003). Genotypes B and C are prevalent in Southeast Asia and China; genotype C circulates mainly in North China, Korea, Japan, and Thailand, and genotype B is predominant in South China and South

Table 3
Comparison of envelope protein substitution sites in genotype B or C of OBI according to the presence or absence of anti-HBs.

Protein	Number of amino acids	Substituted sites	Genotype B Number of substitution sites (%)/frequency	Genotype C Number of substitution sites (%)/frequency	Sites common B/C Number of substitution sites/ frequency
Pre-S1	119	Total	30 (25.2)/56	11 (9.2)/31	5/22
		Common S +/S−	8/21	8/28	
		Anti-S+ specific	10/16	1/1	
		Anti-S− specific	12/19	2/2	
Pre-S2	55	Total	23 (41.8)/49	5(0.1)/7	3/15
		Common S +/S−	7/21	1/3	
		Anti-S+ specific	2/2	4/4	
		Anti-S− specific	14/26	0/0	
S	226	Total	91 (40.3)/281	21 (9.3)/25	19/98
		Common S +/S−	31/186	1/3	
		Anti-S+ specific	19/23	19/21	
		Anti-S− specific	41/72	1/1	
Pre-S/S	400	Total	144 (36.0)/386	37 (9.25)/63	27/135
		Common S +/S−	46 (31.9)/228	10(27.0)/34	
		Anti-S+ specific	31 (21.5)/41	24 (64.9)/26	
		Anti-S− specific	67 (46.5)/117	3 (8.1)/3	

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

East Asia (Yuan et al., 2010; Zheng et al., 2011; Zheng et al., 2015). Several studies have shown that genotype C is associated with more severe liver disease than genotype B (Kao et al., 2000; Orito et al., 2001), including cirrhosis and HCC (Yuen, 2017). The difference between OBI_B and OBI_C remained irrespective of sex and age, while higher diversity was found in most regions of functional proteins of OBI_B compared to OBI_C (Table 2; Supplementary material Table S1). A considerable difference in the number of pre-S/S substitution sites between OBI_B and OBI_C was found (144 vs 37) (Figure 2; Table 3). In pre-S1, pre-S2 and S, the number of substitution sites was higher in OBI_B than in OBI_C (30 vs 11, 23 vs 5, 91 vs 21, respectively). When anti-HBs was present, the reverse pattern was observed in pre-S2 (2 vs 4) and S (19 vs 19), but the pattern remained in pre-S1 (10 vs 1) between OBI_B and OBI_C. It is possible that the presence of anti-HBs might more effectively select variants in the S/MHR regions in genotype C than in genotype B OBI strains.

In the pre-S1 domain, frequent insertion, deletion, or amino acid substitutions could result in the S promoter being typically affected and disrupted, altering the L/S protein ratio (Huang et al., 2013), which would lead to intracellular retention of the L form of HBsAg (Zhou et al., 2009). In this study, we found a significantly more frequent pre-S1 substitution ps_1A81T/S in 10% of OBI_B strains compared with HBsAg-positive strains, while a high frequency of other substitution sites (ps_1A54E, ps_1N56Q/H, ps_1V60A, and ps_1S62A) was observed in 33–56% of OBI_C strains (Table 2). A few frequent pre-S2 mutations ps_2Q2K and ps_2D14N in 15–30% of OBI_B and no statistically frequent mutations of OBI_C strains were detected, in which some presented aspects of immune escape (Pumpens et al., 2002). The most common substitution at the start codon affecting pre-S2 was found previously to prevent the synthesis of the corresponding protein and to be associated with fulminant hepatitis and HCC (Pollicino et al., 1997; Takahashi et al., 1998), which in this study was found in 14.3% of OBI_B samples (Figure 2; Supplementary material Table S2).

Infection with HBV S gene variants that produce a modified HBsAg possibly not recognized by the host immune system or not detected by commercially available screening assays is one of the possible mechanisms leading to OBI (Candotti et al., 2012). In this study, 17 individual substitutions of the S sequences were found in a high frequency (5–50%) of OBI strains (Figure 3). Only two mutations ps_1T68I and sQ129R/L were uniquely observed in 15–25% of anti-HBs(+) OBI_B carriers; a single mutation ps_1A54E was found preferentially in anti-HBs(+) OBI_C, while 17 substitutions were found preferentially in 11–38% of anti-HBs(−) OBI_B (Figure 3A). Thirteen other substitutions in OBI_B strains were detected at high frequency in both anti-HBs(+) and anti-HBs(−) OBI individuals (Figure 3B). Substitutions were mostly found in the S domain of anti-HBs(−) OBI carriers with no preferential anti-HBs selection, suggesting that non-synonymous mutations were randomly occurring. The mutation sQ129R has previously been described as associated with escape from vaccination and as reducing extracellular HBsAg (Ueda et al., 2011; Huang et al., 2012), while sR122K/T/P has been found in genotype D of OBI strains irrespective of anti-HBs and sK122R in genotype A2 anti-HBs(+) OBI carriers (Candotti et al., 2008; Svicher et al., 2012). This observation suggests that these anti-HBc-only patients are those who over time lose detectable anti-HBs. In the present study, sK122R was reported in genotype B OBI strains irrespective of anti-HBs pressure.

A number of substitution sites detected in this study have been reported to be associated with occult HBV infection (Candotti et al., 2008; Allain et al., 2009; El Chaar et al., 2010; Huang et al., 2012; Svicher et al., 2012; Biswas et al., 2013), including anti-HBs(−) preferential mutations sY100S/C, sS113N, sT126P/I/A, and sQ129R/L, and common mutations sN40S, sQ101H/K/R, sP105R/L, sK122R, sP127T/K, sM133S/I/T, sF134L/I, and sV177A of anti-HBs(+/−) OBI

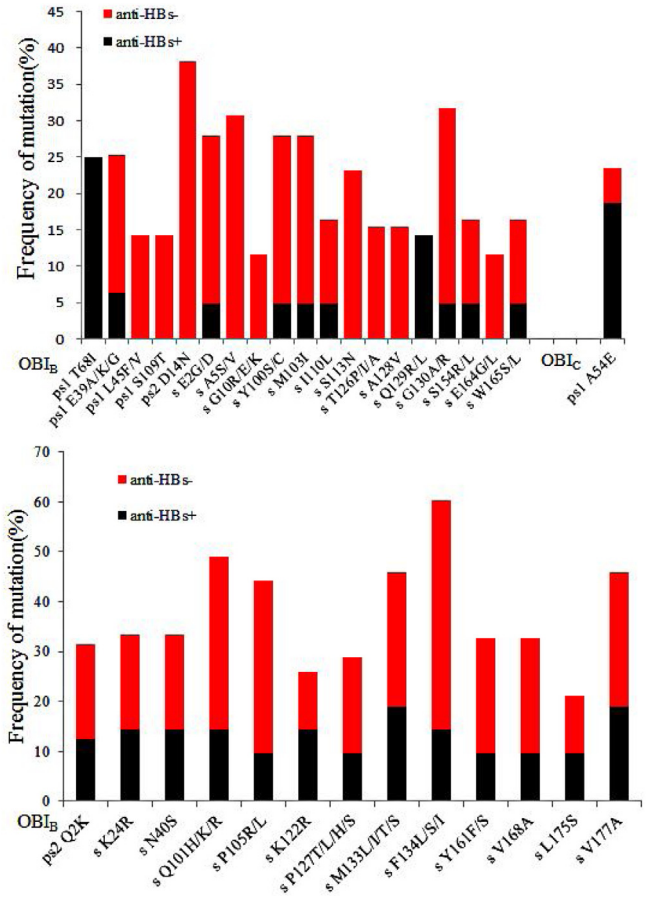


Figure 3. Frequency of pre-S/S amino acid substitutions in the population of OBI carriers with and without the presence of anti-HBs. (A) High frequency mutations preferentially found in the anti-HBs(+) (black bars) and anti-HBc(−) (red bars) OBI populations. (B) High frequency substitutions common to both populations. (OBI, occult hepatitis B virus infection; anti-HBs, hepatitis B surface antibody; anti-HBc, hepatitis B core antibody).

strains. By comparing the amino acid substitutions in MHR with other published reports (El Chaar et al., 2010; Candotti et al., 2012; Huang et al., 2012; Wang et al., 2015) and aggregating our data with their data (Figure 4), sS167 in OBI_B, sT118 in OBI_C, and sA166 in all genotypes of OBI appear to be immune-induced escape substitutions. In this study, vaccine-induced escape substitution sG145R was more frequently found in HBV-infected vaccinees (Hawkins et al., 1996). However, this site mutation appeared in 4.8% of anti-HBs(+) and 9.5% of anti-HBs(−) OBI_B blood donors, but not in OBI_C strains in this study.

Amino acid substitutions within the HBV surface protein could disrupt the protein structure and lead to escape, vaccine-induced anti-HBs (Kreutz, 2002), or breakthrough HBV infection (Chang, 2010; Li et al., 2017). Among anti-HBs(+) OBI blood donors, these substitutions may occur under pressure of protective immunity induced by HBV vaccines. Other rarely detected mutations were present as well (Figure 2), which might have a lower 'replication fit' compared to the wild-type virus, providing a possible explanation for the low viral load in these OBI samples (median ≤27.9 IU/ml). However, reactivation of OBI might be emerging in immunodeficient carriers and cause overt HBV infection (Raimondo et al., 2019).

In summary, 121 HBsAg(−)/HBV DNA(+) blood samples were detected among 310 167 blood donors at Shenzhen Blood Center. Of the 121 selected samples, 99 were identified as OBI, in which OBI_B was predominant. A total of 20 whole genomes, 29 pre-S/S, and 18 S sequences from OBI samples were characterized. Amino acid sequence analyses suggested that the amino acid substitutions occurred more frequently in OBI than in wild-type HBV, providing possible explanations for the occurrence and existence of OBI. Among OBI carriers, mutations ps_1T68I and sQ129R/L were observed only in anti-HBs(+) OBI_B carriers, suggesting selection by antibody pressure related to vaccination or a natural host immune response to HBV. Mutations ps_1E39A/K/G, ps_1L45F/V, ps_1S109T, ps_2D14N, sE2G/D, sA5S/V, sG10R/E/K, sY100S/C, sM103I, sI110L, sS113N, sT126P/I/A, sA128V, sG130A/R, sS154R/L, sE164G/L, and sW165S/L were found uniquely or preferentially in anti-HBs(−) OBI_B carriers and mutation ps_1A54E was found preferentially in anti-HBs(+) OBI_C carriers. Other high frequency substitutions such as 13 OBI_B pre-S/S mutations were not preferentially detected in either anti-HBs(+) or anti-HBs(−) OBI carriers, suggesting random

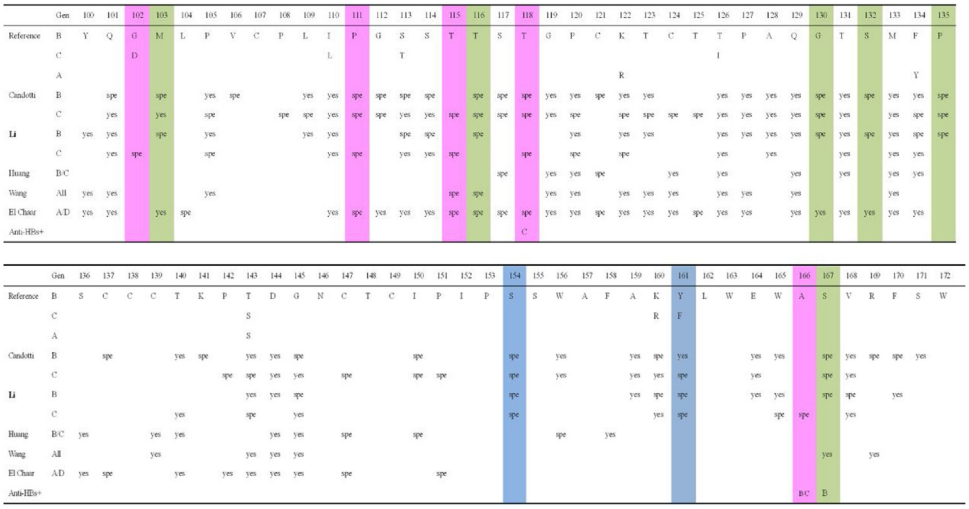


Figure 4. Comparison of amino acid substitutions in the MHR of OBI variants in this study and four published reports. 'Yes' indicates substitutions present in OBI also found in HBsAg-positive control strains. 'Spe' means substitutions apparently specific to OBI since not reported in HBsAg-positive controls strains. Amino acid sites highlighted in green, pink, and blue are specific for OBI_B, OBI_C, and all genotypes of OBI. (MHR, major hydrophilic region; OBI, occult hepatitis B virus infection; HBsAg, hepatitis B surface antigen).

occurrence. In the MHR region, sS167 in OBI_B, sT118 in OBI_C, and sA166 in all genotypes of OBI were quite possibly immune-induced escape substitution sites. This suggests that some mutations in pre-S/S of OBI were associated with the carrier's anti-HBs selection, and more substitutions were associated with HBV genotype. The results obtained from this study might be helpful for understanding the occurrence of OBI and preventing the reactivation of OBI strains.

Author contributions

C.L. and T.L. designed the research; J.W., P.Z., J.Z., P.D., X.Z., and Y. F. performed the research; T.L., P.D., P.Z., X.Y., D.C., J.P.A, and W.Z. analyzed the data; T.L., C.L., D.C., and J.P.A. wrote the paper.

Ethical considerations

This study was approved by the SZBC ethics committee. All individuals involved in this study signed an informed consent.

Conflict of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijid.2019.12.026.

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