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REVIEW

Genetic variation of occult hepatitis B virus infection

Hui-Lan Zhu, Xu Li, Jun Li, Zhen-Hua Zhang

Hui-Lan Zhu, Xu Li, Zhen-Hua Zhang, Department of Infectious Diseases, the First Affiliated Hospital, Anhui Medical University, Hefei 230022, Anhui Province, China

Jun Li, Zhen-Hua Zhang, School of Pharmacy, Anhui Medical University, Hefei 230032, Anhui Province, China

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Correspondence to: Zhen-Hua Zhang, MD, PhD, Department of Infectious Diseases, the First Affiliated Hospital, Anhui Medical University, 218 Jixi Road, Hefei 230022, Anhui Province, China. zzh1974cn@163.com
Telephone: +86-551-62922912
Fax: +86-551-62922912

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Abstract

Occult hepatitis B virus infection (OBI), characterized as the persistence of hepatitis B virus (HBV) surface antigen (HBsAg) seronegativity and low viral load in blood or liver, is a special form of HBV infection. OBI may be related mainly to mutations in the HBV genome, although the underlying mechanism of it remains to be clarified. Mutations especially within the immunodominant "α" determinant of S protein are "hot spots" that could contribute to the occurrence of OBI *via* affecting antigenicity and immunogenicity of HBsAg or replication and secretion of virion. Clinical reports account for a large proportion of previous studies on OBI, while functional analyses, especially those based on full-length HBV genome, are rare.

Key words: Hepatitis B virus; Occult; Variation; Hepatitis B surface antigen

Core tip: With error-prone reverse transcription during replication, hepatitis B virus (HBV) displays a high rate of mutation, and a single mutation may affect the biological properties of HBV. Occult HBV infection (OBI) is a special form of HBV infection and a frequent phenomenon. Many previous publications have explored the association of OBI with the "hot spots" mutations that occur within the immunodominant "α" determinant of S proteins. However, there are no reviews available that elaborate on the relationship between OBI and mutations throughout the entire HBV genome. This review attempts to provide a comprehensive summary of HBV genetic variants that have been associated with OBI.

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INTRODUCTION

Hepatitis B virus (HBV) is classified as a DNA virus, and it replicates *via* reverse transcription, which is an error-prone process, to generate an RNA intermediate. Thus, the mutation rate of HBV is in between those of RNA viruses and conventional DNA viruses[1]. The nucleotide mutation rate of HBV is estimated to be 10^{-5}-10^{-6} per site per year, about 10 fold higher than that of other DNA viruses, and this rate might increase 100 fold after liver transplantation[2,3]. Mutations are commonly encountered, especially in the basal core promoter (BCP), the pre-core region (Pre-C), the polymerase (P) gene, and the "α" determinant of S protein, and single or multiple mutations may affect the biological properties of HBV[4]. Occult HBV infection (OBI), a special form of HBV infection and a frequent phenomenon, was first reported in 1978 and has been studied further since then[5]. In 2008, the Taormina expert meeting defined OBI as the presence of HBV DNA in the liver of individuals negative for HBV surface antigen (HBsAg) testing with the amount of HBV DNA in the serum usually lower than 200 IU/mL or undetectable[6]. OBI is characterized by the persistence of low level viremia and HBsAg seronegativity and can be grouped into two types: seropositive [anti-hepatitis B core antigen (HBc) and/or anti-hepatitis B surface antigen (HBs) positive] and seronegative (anti-HBc and anti-HBs negative)[7]. OBI harbors the potential risk of HBV transmission through blood transfusion, organ transplantation, and hemodialysis as well as from occult infected or HBsAg-positive mothers to newborns[8,9]. Although the clinical and biochemical symptoms of most OBIs are not severe, there is still a risk of developing serious liver diseases, such as liver cirrhosis (LC) and hepatocellular carcinoma (HCC)[10]. In fact, a meta-analysis and other findings have revealed that OBI retains the pro-oncogenic properties of HBV and serves as a risk factor for the development of HCC[11-13]. Suppression of viral replication is reversible in the case of OBI, and reactivation of OBI and/or development of fulminant hepatitis failure (FHF) has been reported in patients following chemotherapy or immunosuppressive therapy and after transplantation as well as in patients co-infected with human immunodeficiency virus (HIV) or hepatitis C virus (HCV)[14-16].

PREVALENCE OF OBI

OBI is a health problem worldwide, and its prevalence is affected by several factors, such as the prevalence of HBV infection, the study population, and the sensitivity of diagnostic assays[17,18]. OBI is significantly associated with the endemicity of HBV infection but not restricted to countries highly endemic for HBV[10,17,18]. The prevalence of OBI in blood donors from China, South Korea, and Japan was reported to be 0.11%-0.13%, 0.016%, and 1.01%, respectively[19-22]. The incidence of OBI in anti-HBc positive donors was reported to be from 0% to 15%[23,24], and the detection rate of OBI in HIV infection was reported to be from 0% to 89%[25,26]. In the context of chronic HCV infection, the percentage of OBI ranged from 0% to 52%[27,28]. Previous studies conducted in hemodialysis patients indicated a prevalence of OBI from 0% to 36%[29,30]. OBI was detected in 32% of patients with cryptogenic LC in Hong Kong, an area with a high prevalence of HBV infection[31]. The prevalence of OBI was reported to be 70.4% among HBsAg-negative HCC patients in China[32].

The identification of OBI largely depends on the tests for HBsAg and HBV DNA. There are several commercially available HBsAg assays, which mainly depend on the use of anti-HBs antibodies, with different sensitivities and specificities. Some diagnostic HBsAg assays are based on monoclonal antibodies and recognize limited types of HBsAg variants. In comparison, assays that utilize polyclonal antibodies show higher sensitivity and specificity for the detection of various types of HBsAg mutants[33,34]. Because the level of viremia is low in OBI, a small amount of HBsAg below the detection limit of tests may not be reliably recognized. Although highly sensitive real-time quantitative polymerase chain reaction (PCR) is strongly recommended for HBV nucleic acid testing (NAT) for its capability to uncover low level of HBV DNA in serum, this method harbors a risk of false-positive results[35,36]. Anti-HBc screening can reduce the risk of HBV transmission but may possibly provide false-positive results as well. It is worth noting that the nature of the specimen tested (*i.e.*, blood sample or liver tissue), the amount of specimen, as well as contamination risks can also affect the detection of OBI.

Many studies have been conducted on OBI prevalence. However, cautions must be taken when results from different studies are compared, since differences in groups of people studied, areas selected, and diagnostic assays can significantly affect the results.

MECHANISM OF OBI

The mechanism of OBI is complicated and remains to be clarified. Both host and viral factors contribute to the occurrence of OBI. For example, the persistence of covalently closed circular DNA (cccDNA) in hepatocytes, suppression and detection failure of virion replication, and protein secretion are involved[37,38]. In addition, methylation of the HBV genome, microRNAs (miRNAs), and histone modification have been reported to affect the regulation of HBV replication and expression through epigenetic mechanisms, increasing

the possibility that epigenetic regulation may also be associated with OBI[39].

Viral factors

Genetic mutations may contribute to OBI. Mutations have an impact on the expression, secretion, and antigenicity of HBsAg and cause failure in detecting HBsAg by commercial diagnostic tests. HBV is prone to mutations and exists as quasispecies to facilitate viral survival. Point mutations and insertion as well as deletion mutations are commonly encountered in OBI, and although mutations in all open reading frames (ORFs) of HBV could directly or indirectly affect detection of HBsAg, most studies have focused on mutations in the Pre-S/S gene. Besides, OBI mutations are associated with the suppression of viral replication typical of OBI, which in turn affects HBsAg expression and secretion[40]. Circulating immune complexes consisting of HBsAg and anti-HBs may be associated with Pre-S/S gene mutations and impair the binding efficiency of HBsAg capture antibodies to HBsAg in serum, leading to immune escape[41,42].

Low levels of HBV replication may be correlated with OBI. Compared to HBsAg-positive infection, OBI always has lower (< 200 IU/mL) or even undetectable HBV DNA in serum[18]. Although HBV DNA from the liver is more reliable than circulating HBV DNA for recognition of OBI, the levels of intra-hepatic HBV DNA and cccDNA in OBI were also found to be lower than those in HBsAg-positive infection[38,43]. Thus, the low level of HBV replication may be related to OBI.

HBV genome methylation is related to the development of OBI. Hypermethylation of HBV has been observed in isolates from OBI patients, and HBV CpG islands 1 and 2 were more frequently methylated in HBsAg positive infections and OBIs, respectively[44,45].

Host factors

The alternation of host immunologic responses is associated with OBI. In patients with spontaneous and therapy-induced HBsAg clearance, residual HBV may present in the serum, liver, and lymphatic system for a long time[46,47]. Similarly, using a woodchuck model, the closet natural model of HBV infection, Michalak[48] and Mulrooney-Cousins *et al*[49] described the low-level but life-long persistence of woodchuck hepatitis B virus (WHV) DNA and cccDNA in both the liver and lymphatic system in animals recovered from acute WHV invasion. Based on these data, they suggested that the hard-to-eliminate cccDNA made the complete clearance of WBV difficult to accomplish and might contribute to WBV reactivation and further speculated that HBV cccDNA may act similarly to WBV cccDNA. Therefore, latency of HBV in liver and in extra-hepatic tissues and cells provide a favorable condition for the occurrence of OBI[50]. Reactivation of overt HBV infection and OBI has been frequently described in HBV infected patients with suppressed immune systems, suggesting that HBV was inhibited rather than cleared in the setting of immunosuppression. Colson *et al*[51] investigated 16 anti-HBc positive patients who developed HBV reactivation following chemotherapy and detected an increased variability within HBsAg and HBV reverse transcriptase (RT) in these patients. OBI has also been reported in vaccinated children from Taiwan and Iran[52,53]. It is speculated that the patients' immune response to HBV infection may be related to OBI by greatly controlling HBV activity, putting pressure on secretion of HBsAg and other HBV-secreted proteins, as well as promoting the screening of mutants.

In addition to altered host immunologic responses, host genetic factors are also linked to OBI. Related studies have focused on human leukocyte antigen (HLA), which plays an important role in the modulation of immune responses, and HLA polymorphisms are considered as vital factors in determining HBV infections outcome (persistence or clearance)[54]. Previous studies showed that HLA-A2 expression was correlated with protection against HBV infection, while significantly decreased intensity of HLA-A2 expression on peripheral blood mononuclear cells (PBMCs) was detected in some OBI patients compared to healthy controls, perhaps contributing to HBV resistance[55]. Serum level of interleukin (IL)-10 was markedly higher in some OBI patients than that in healthy controls, and polymorphisms of the IL-10 promoter were found with significant differences between OBIs and healthy controls, implying a relationship between IL-10 and OBI[56,57].

Host epigenetic modification may be related to OBI. miRNAs of host cells could regulate expression of some host genes and exert effects on HBV replication and expression[58]. A profile of HBV-specific serum miRNAs has been reported with a high accuracy to separate OBI cases from healthy controls (sensitivity: 99.9%, specificity: 99.8%), raising the possibility that they may serve as effective biomarkers in OBI detection[59]. Epigenetic modifications of cccDNA-bound histones may regulate HBV replication and transcription, with acetylation and methylation being the most common kinds of histone modification. Previous studies showed that HBV replication could be regulated by the acetylation status of cccDNA-bound H3 and H4 histones in chronic hepatitis B (CHB) patients, and this regulation has been confirmed by transfection experiments[60].

Other factors

Other factors may be connected with OBI, such as interference by other viruses. Concomitant presence of chronic HCV infection exerts a negative effect on HBV. HBV DNA levels are increased during interferon-ribavirin therapy of HCV but decreased after a viral breakthrough of HCV[61,62]. Moreover, *in vitro* studies have demonstrated the inhibitory effect exerted by HCV core protein on HBV replication and expression[63].

PRE-S/S MUTATIONS

Pre-S/S point mutation

Pre-S/S gene encodes three envelope proteins named large (L), middle (M), and small (S) protein, and S protein corresponds to HBsAg[64]. Pre-S/S has a relatively high mutation rate among all ORFs, and mutations of the Pre-S/S gene have been studied extensively. Point mutations that occur in the Pre-S/S gene may affect HBsAg in two different aspects, (1) mutations may affect antigenicity, immunogenicity, secretion, and/or expression of HBsAg, leading to detection failure of HBsAg[33,65]; (2) mutations may reduce or even abolish the replication and/or secretion of virion, exerting a negative effect on HBsAg[66,67]. Amino acid (aa) substitutions of HBsAg are mostly frequently clustered in the "α" determinant, which is located at aa124-147 of the S protein. The "α" determinant is rich in cysteine, and two loops are formed by disulfide bonds between C124 and C137 as well as C139 and C147 to maintain the conformational structure and antigenicity of HBsAg. The "α" determinant is a relatively conserved region within the major hydrophilic region (MHR) between aa100 to aa169, which serves as the most important antigenic determinant in all HBV strains and is essential to the detection of HBsAg and development of HBV vaccines[64,68]. Amino acids within the region between 120 to 123 were shown to be crucial for the antigenicity of HBsAg[69]. Therefore, single or multiple point mutations occurring within or adjacent to the "α" determinant may change the antigenicity and conformation of HBsAg, resulting in failure to detect HBsAg. Various point mutations of HBsAg have been observed in OBIs (Figure 1).

Amino acid mutations were detected mostly in the MHR in OBIs, and many previous reports have documented that the mutation rate in the MHR, especially in the "α" determinant, was higher in OBIs compared to that of HBsAg-positive patients[66,70]. A number of Pre-S/S mutations were observed in both OBIs and HBsAg-positive patients (Figure 1), while it has been speculated that they may not be associated with OBI. Clinically observed Pre-S/S mutations in OBIs summarized in Figure 1 indicate that: (1) Some mutations occurred in both OBIs and HBsAg-positive patients, while some mutations were only detected in OBIs; (2) Some mutations were found outside the "α" determinant and were located at the N-terminus or C-terminus of MHR or outside MHR; (3) Some mutations corresponded to amino acid substitutions in the P region; (4) Some amino acid substitutions were specific for genotypes, subgenotypes, or subtypes, while the clinical significance of them in OBIs remains to be determined; (5) Point mutations in the first loop of the "α" epitope (AA124-137) occurred more frequently in OBI isolates from unvaccinated patients[71], while point mutations in the second loop of the "α" epitope (AA138-147) were more frequently isolated from OBI patients after immune prophylaxis[72]; and (6) Apart from point substitutions, there were other types of mutations or combined mutations. However, further *in vivo* and *in vitro* studies are required to explore the effects of point mutations on the occurrence of OBI.

In order to elucidate the role of mutations in the occurrence of OBI, plasmids of Pre-S/S variants isolated from the sera and liver tissues of OBI patients were constructed by site-directed mutagenesis and transfected into cell lines or introduced into animal models. The antigenicity, immunogenicity, and secretion of HBsAg as well as the replication and secretion of virions were then analyzed. This strategy has been extensively applied to point mutations in the Pre-S/S gene (Table 1 and Figure 2). Similarly, these functional analyses also can be applied to research on other HBV-related mutations[73]. To date, most *in vivo* and *in vitro* studies have been limited to the Pre-S/S gene, and only a few functional studies have been performed with full-length, replication-competent HBV genomes. It is difficult to know whether some point mutations interfere with the replication of the virion and secretion of antigens or if they play a role in the occurrence of OBI[40,74].

Many studies have documented that substitutions of wild-type cysteines and G145R in the "α" determinant cause conformational and phenotypic changes in HBsAg (Table 1 and Figure 2)[33,66,71,78]. El Chaar *et al*[80] restored the cysteines at positions 124 and 137 in recombinant surface protein (rS protein) from OBIs and expressed them in yeast and showed that antigenic reactivity of HBsAg was improved. However, restoration of cysteine at position 147 and G145R did not improve the impaired reactivity of HBsAg. Y100C was commonly found in OBIs, but transfection with Y100C plasmid resulted in higher extracellular HBsAg levels than the wild-type plasmid, implying that the mutation alone could not explain the decrease of HBsAg secretion and/or affinity to antibodies[76]. *In vitro* studies have found that some point mutations could strongly influence the secretion of HBsAg and that these mutations may interfere with the secretion of HBsAg in some cases, leading to the failure of HBsAg detection[86]. Some point mutations were found to affect HBV replication in hepatoma cell lines and/or in mice and the vesicular transport of infectious virions of HBV (*i.e.*, Dane particles) from the cell[66].

Glycosylation, the most common kind being N-linked glycosylation, is required for crucial biological functions of many enveloped viruses, since it can impart various advantages to virus survival and virulence[88]. Point mutations can affect biogenesis, stability, and antigenicity of HBV through modification of the envelope protein glycosylation pattern[89,90]. The envelope protein of HBV possesses an N-glycosylation site at N146 of the "α" determinant, and the removal of this site decreased the production of virion without affecting the synthesis and stability of HBsAg (Table 1 and Figure 2)[78-80]. Several positions in the envelope

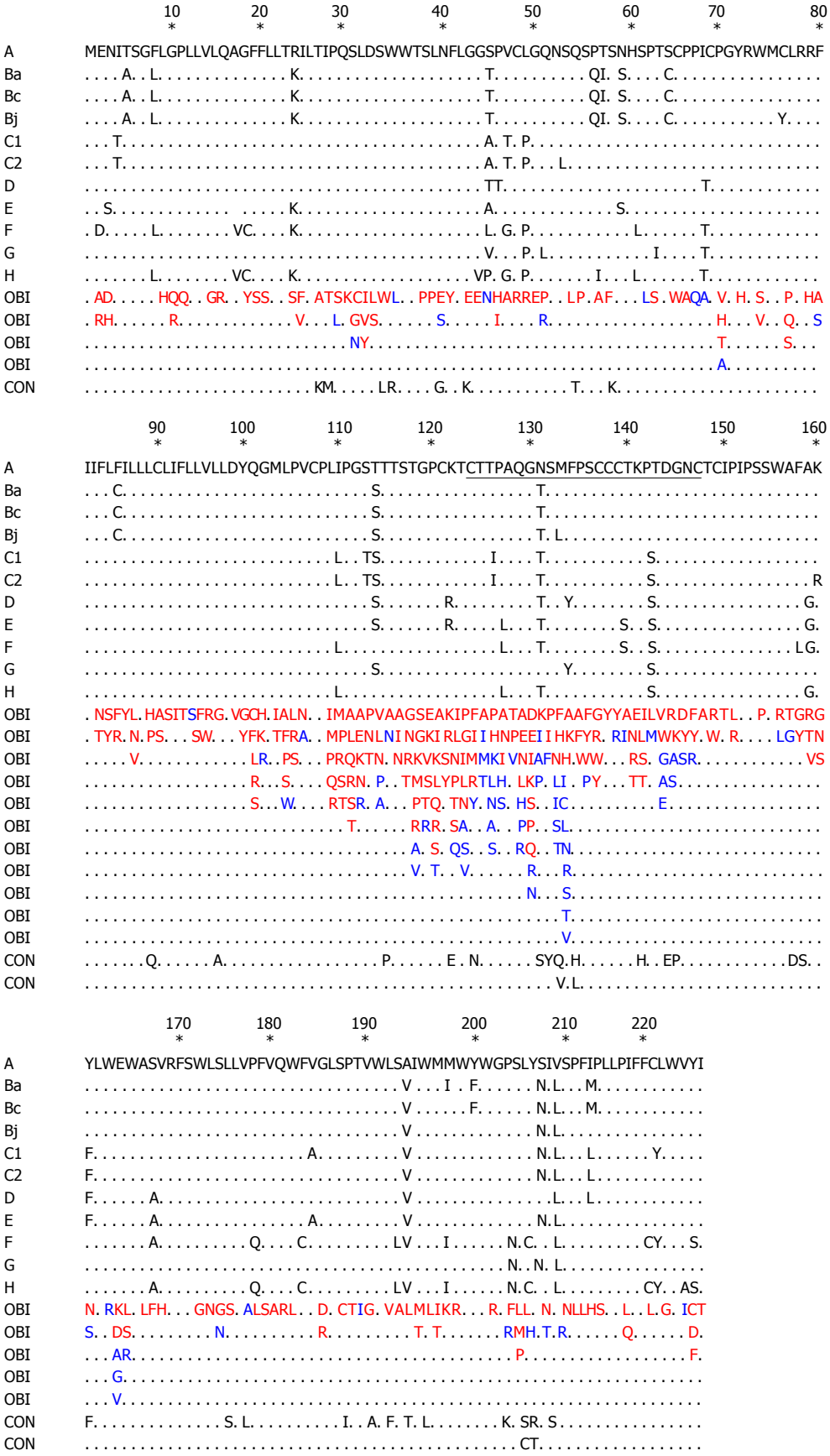

Figure 1 Comparison of the amino acid sequences of the 226AA hepatitis B virus, hepatitis B virus surface antigen between occult hepatitis B virus infections, and controls. Amino acid substitutions are indicated with one-letter codes. Mutations identified in occult hepatitis B virus infections group and control group are in red and black, respectively. Mutations detected in both groups are in blue. Black dots denote amino acid identity with any of the reference strains of genotype A to H retrieved from GenBank (accession numbers: A, X02763; Ba, D00330; Bc, GQ205440; Bj, AB073858; C1, KM999990; C2, KM999991; D, X02496; E, X75657; F, X69798; G, AF160501; H, AY090454). Genotype-specific substitutions are listed. CON indicates control.

Table 1 Summary of hepatitis B virus surface antigen variants involved in functional studies

Variants	Source	Model	Antigenicity	Immuno -genicity	HBsAg Intra-	HBsAg Extra-	Viral replication /secretion	References
W74[1]	CHB	Huh7	L		L	L		[65]
M75T		Huh7			↑	↓	↓[2]	[75]
Y100C	OBI	Huh7	↑					[76]
Y100S	OBI	Huh7	L					[77]
I110M		Huh7					L[3]	[78]
T114R		Huh7					↓[3]	[79]
T115A		Huh7					↓[3]	[79]
T116N	OBI	Huh7	↓					[77]
G119E		Huh7				↓	↓[3]	[78]
G119R	OBI	Huh7/mice	↓		↑	↓	↓[3]	[66]
P120T	OBI	Huh7/mice	↓					[66]
P120T		Huh7				↓		[79]
C121S		HepG2/Hela	↓		↓	↓		[69]
C121[1]	OBI	Pichia	↓ or L					[80]
C121A		Huh7				↓	↓ or L[3]	[79]
K122I		HepG2/Hela	↓		↓	↓		[69]
K122I		Huh7	↓	↓	↓	↓		[81]
K122I		Huh7/Hela	↓ or L	↓	↓	↓		[82]
R122P	OBI	Huh7	↓					[77]
T123N		HepG2/Hela	↓		↓	↓		[69]
T123N		Huh7/Hela	↓ or L				↓[3]	[82]
C124[1]	OBI	Pichia	↓ or L					[80]
C124A		Huh7				↓	↓ or L[3]	[79]
C124R	OBI	Huh7/mice	↓				↓[3]	[66]
C124Y	OBI	Huh7/mice	↓				↓[3]	[66]
T125A	CHB	Huh7	↓			↓		[65]
I126S	OBI	Huh7/mice			↑	↓	↓[3]	[66]
P127S		Huh7					↓[3]	[79]
Q129H		Huh7					↓[3]	[79]
Q129R	OBI	Huh7/mice			↑	↓	↓[3]	[66]
G130R		Huh7					L[3]	[79]
T131I		Yeast	↓					[83]
M133T[4]		Huh7						[78]
S136P	OBI	Huh7/mice			↑	↓	↓[3]	[66]
C137[1]	OBI	Pichia	↓ or L					[80]
C138Y		Huh7				↓	L[3]	[79]
C139R	CHB	HepG2	↓					[84]
C139R	OBI	Huh7/mice	↓				↓[3]	[66]
T140I	OBI	Huh7/mice			↑	↓	↓[3]	[66]
K141E	OBI	Huh7/mice	↓		↑	↓	↓[3]	[66]
K141E		Huh7				↓	↓[3]	[79]
K141E		Yeast	↓					[83]
P142S		Yeast	↓					[83]
P142S		Huh7				↓		[79]
P142A	KT	Huh7	↓					[85]
D144V	KT	Huh7	↓					[85]
D144A	OBI	Huh7/mice	↓		↑	↓	↓[3]	[66]
D144G		Huh7				↓	L[3]	[79]
D144E	LC	HepG2	↓					[84]
G145R		Yeast	↓					[83]
G145R		HepG2/Hela	↓		↓	↓		[69]
G145R	LT	Huh7					↓[3]	[67]
G145R		Huh7	↓	↓		↓		[81]
G145R		Huh7				↓		[79]
G145R	OBI	Huh7/mice	↓		↑	↓	↓[3]	[66]
G145A	OBI	Huh7/mice	↓					[66]
G145A	OBI	Huh7/HepG2	↓		↑	↓		[83]
G145A		Yeast	↓					[81]
G145W		Huh7		↓		↓		[81]
G145I		Huh7	↓	↓				[81]
G145P		Huh7	↓					[81]
G145N		Huh7	↓					[81]
G145D		Huh7	↓					[81]
G145E		Huh7		↓				[79]
N146S		Huh7					L[3]	[79]
C147A		Huh7					↓ or L[3]	[79]

C147R		Huh7				↓	↓[3]	[79]
C149A		Huh7				↓	↓ or L[3]	[79]
C149R		Huh7				↓	L[3]	[79]
A159G		Huh7/Hela	↓	↓			↓[3]	[82]
K160N		Huh7/Hela	↓	↑[5]			↓[3]	[82]
R169P/G		Huh7				↓	L[3]	[78]
Y100S + S143L	OBI	Huh7	L					[77]
M103I + G145A	OBI	Huh7/HepG2	↓		↓	↓		[86]
M103I + R122K + G145A	OBI	Huh7/HepG2	↓		↓	↓		[86]
T115I + T116N	CHB	HepG2	↓ or L					[87]
T116N + S143L	OBI	Huh7	↓					[77]
R122P + Q101R	OBI	Huh7	↓					[77]
R122P + S167L	OBI	Huh7	↓					[77]
R122K + G145A	OBI	Huh7/HepG2	↓		↓	↓		[86]
P142A + D144V	KT	Huh7	↓					[85]
A159G + K160N		Huh7/Hela	↓	↑[5]			↓[3]	[82]

[1]Stop codon mutation; [2]Viral replication; [3]Viral secretion; [4]Correct that I110M, G119E, G145R, N146Q/S, R169H mutations lead to the decrease of viral secretion; [5]Cell-mediated immunity. ↑: Increase; ↓: Decrease. Intra-HBsAg: Intracellular HBsAg; Extra-HBsAg: Extracellular HBsAg; L: Dramatic decrease; CHB: Chronic hepatitis B; OBI: Occult HBV infection; LC: Liver cirrhosis; KT: Kidney transplant; LT: Liver transplant; HBsAg: Hepatitis B virus surface antigen.

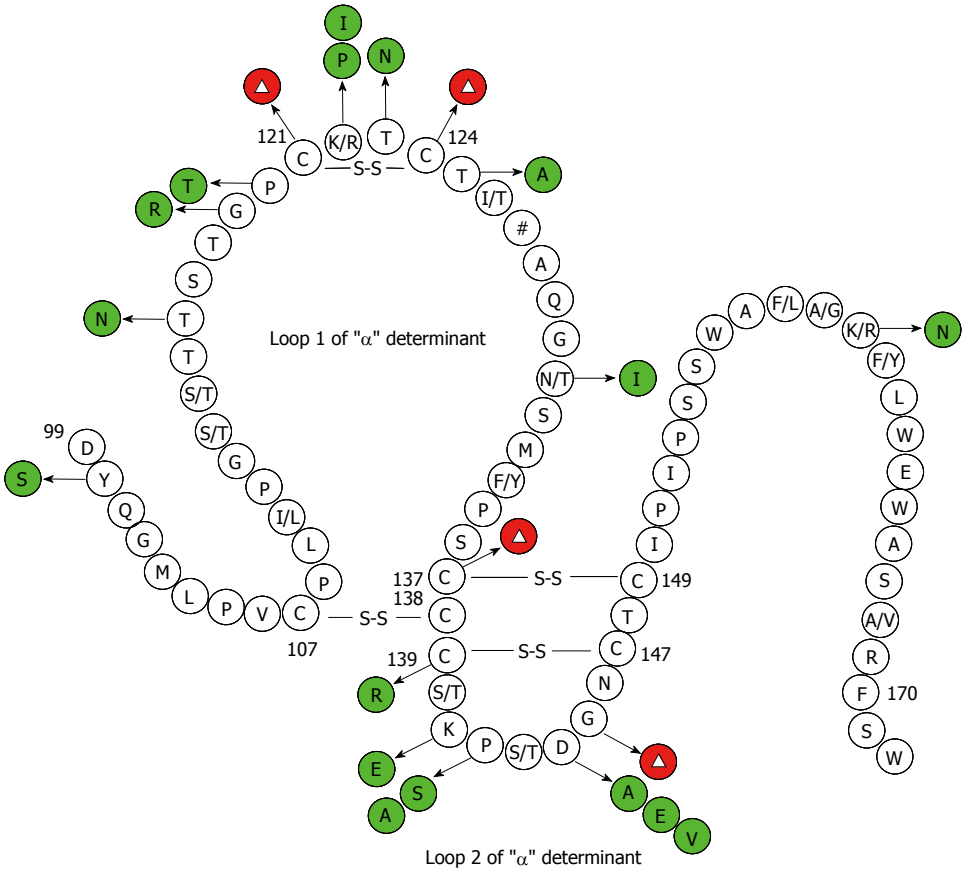

Figure 2 Secondary structure of the major hydrophilic region of the hepatitis B virus, hepatitis B virus surface antigen. Mutations have been confirmed by functional studies to exert negative effects on antigenicity of hepatitis B virus surface antigen (HBsAg) are shown in green and red circles. Red circles with the Δ symbol denote multiple kinds of amino acid substitutions. These have been substantiated to negatively correlate with HBsAg antigenicity at some sites. The # symbol indicates multiple genotype-specific substitutions. The numbers denote amino acid sites.

proteins can substitute for position 146 as the potential glycosylation site[78]. Previous studies reported that T123N and K160N were each capable of creating new glycosylation sites and causing a decrease of HBsAg antigenicity. These two point substitutions were found to mainly affect virion assembly and secretion without interfering with virion replication. K122I and A159G appeared to affect biological properties of HBsAg and facilitated glycosylation of HBV. A159G was shown to impair greatly the assembly and secretion of virion,

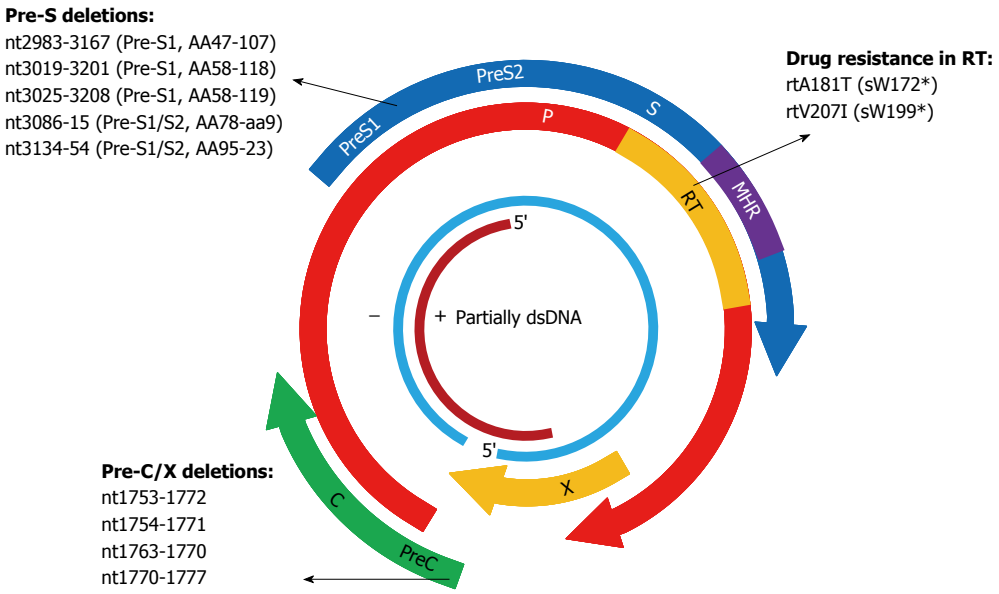

Figure 3 Mutations in X, P, and C genes of hepatitis B virus have been substantiated in association with the occurrence of occult hepatitis B virus infections by *in vivo* and *in vitro* studies. Plus and minus strands are denoted by + and -, respectively.

but K160N and A159G/K160N slightly reduced the virion production. Thus K160N could partially rescue the negative effect exerted by A159G on secretion of virion[82]. M133T mutation could create an additional glycosylation site ^{131}NST^{133} and partially rescued the impaired secretion of virion induced by some point mutations, such as I110M, G119E, G145R, N146Q/S, and R169H. Preventing glycosylation could destroy the effect of the M133T mutation[78,79,82]. M133T alone was not able to generate a glycosylation site, unless there was an N at AA131 (as in the reference sequences of HBV genotype A) The T131N/M133T double mutations were frequently observed in OBIs infected with non-A genotypes and changing T131N could also abrogate the secretion-rescue effect exerted by M133T[78,79].

Among OBI related mutations, some point mutations cause HBsAg secretion deficiency by affecting the start codons of Pre-S/S gene or causing amino acid substitutions[91]. A purine at the -3 position (*i.e.*, 3-nt upstream from the translational start site) of the AUG initiator codon is essential for protein expression, and substitution with a pyrimidine at this site makes translation more sensitive to changes at positions -1, -2, and +4 (*i.e.*, 4-nt downstream from the translational start site)[92]. Point mutations around the initiator codons of HBV S gene (*i.e.*, nt155-157), such as A152T and A152G/G158C double mutations, reduced HBsAg secretion by 70% and 30%, respectively, but maintained efficient virion secretion, while A152T combined with C154A or G158A completely abolished HBsAg secretion[91]. Some point mutations have an effect on RNA splicing. Hass *et al*[93] found that ntG458A inactivated 5' splice site of the Pre-S2/S mRNA and reduced the level of Pre-S2/S mRNA as well as HBsAg expression and caused a low-replication phenotype. Among some genotype D OBIs, ntG173T was able to activate in *cis* a previously inactive splice acceptor site at neighboring nt202 and promoted RNA splicing from nt2986 to 202. The novel splicing could abolish the L, M, and S proteins without affecting the functions of polymerase[94].

Pre-S/S deletion

Deletion mutations in Pre-S/S have been frequently reported in OBI and deletions covering the Pre-S/S promoters may affect RNA splicing and exert an effect on expression of one or more envelope proteins (Figure 3)[50,74]. In cases of OBI, in-frame 183-bp deletions (nt3019-3201 or nt2983-3167) were commonly detected in the Pre-S1 region. Functional analyses found that variants harboring the 183-bp deletion mutation did not express HBsAg or secrete viral particles when transfected in cell lines, although they appeared to be replication-competent[50,95-97]. A CCAAT element (nt3143-3147) and binding sites for SP1 transcription factor are located in the Pre-S2/S promoter (nt2960-3180), which are completely and partially overlapped by the 183-bp deletion mutation, respectively[98,99]. The CCAAT element is where cellular transcription factor NF-Y binds to and plays a key role in activating the S promoter and regulating the ratio of Pre-S and S mRNA. Mutations in the CCAAT element downregulate the level of Pre-S1 mRNA while upregulate that of Pre-S2/S mRNA, resulting in a decreased amount of L protein but increased amounts of M and S proteins[100]. The SP1 sites also regulate S promoter activity and production of Pre-S and S mRNA but exert a weaker effect than the CCAAT element. Mutations that occur at SP1 sites affect the ratio of Pre-S/S mRNA as well as expression of

envelope proteins[98,99]. The specific ratio of L protein and S protein is essential for assembly of the envelope particles, since an excessively high or low L/S proteins ratio could alter HBsAg assembly and secretion and reduce virion secretion[91,101,102]. Similarly, Xu and Yen[103] reported a 129-bp deletion within the Pre-S2/S promoter that affected Pre-S2/S mRNA production, leading to complete abolishment of HBsAg secretion. Chaudhuri *et al*[74] described several fragment deletions containing Pre-S2/S promoter in OBIs and demonstrated that full-length HBV genomes harboring these deletion variants could drastically decrease the expression of HBsAg by transfected HepG2 cells. Besides, the Pre-S1 region possesses a hepatocyte-binding site (AA21-47) that is vital to virion assembly and transport from the hepatocyte, and deletions covering this site may affect virion binding to and being secreted out of the hepatocyte[104].

In OBIs, deletions in Pre-S2 region were frequently detected at aa8-23, which have also been associated with progression of liver diseases[74,105]. These deletions overlap with the binding site for cross-linked human serum albumin (AA17-28) in the Pre-S2 region, which is involved in the attachment of HBV to the human hepatocyte membrane, and mutations affecting this site may decrease the infectivity of HBV[106]. Deletions in Pre-S2 region may influence expression of M protein, which is dispensable for formation and secretion of virion *in vivo* and *in vitro*[91,107]. Deletions in the Pre-S2 region may be related to OBI by affecting the production of L protein. Deletions in the Pre-S2 region shorten the gap between the Pre-S2/S promoter and the start codon of S mRNA, in turn leading to an overproduction of L protein and an alternation of L/S ratio, which may decrease the secretion of HBsAg[108,109]. Deletions in the Pre-S2 region may be associated with antiviral-resistance, which is in turn linked to OBI. Mutations corresponding to lamivudine (LMV)-resistance were commonly detected in OBIs[110], and Pre-S2 deletion mutants were frequently found in patients with antiviral-resistance and shown to play a supportive role in the replication of LMV-resistant viruses by transfection analysis[111].

Pre-S/S insertion

Two to 8 amino acid insertions have been observed between codons 121 and 124 located upstream of the "α" determinant in OBI patients from the Far East, and they caused detection failure of HBsAg by conventional assays utilizing monoclonal and polyclonal antibodies[112]. Insertions occurring at these sites strongly influence the binding efficiency of HBsAg antibodies and may affect the conformation of the "α" determinant as well as the subtype determinant d/y at aa122. In addition, insertions detected in the Pre-S/S gene in OBI patients may lead to frameshift mutations or stop codon mutations and interfere with HBsAg detection.

Pre-S/S stop codon

Stop codon mutations in the Pre-S/S gene result in truncations of envelop proteins and may affect the expression and/or secretion of HBsAg. A stop codon mutation at aa216 in the C-terminus of HBsAg was observed in occult HBV/HIV co-infected patients and displayed undetectable or extremely low level of HBsAg in transfection experiments[73]. Other stop codon mutations at amino acid positions 61, 69, 181 *etc*. were also detected in OBI patients. These mutations may be correlated with failure in HBsAg detection, but *in vivo* and *in vitro* studies are required to confirm this association[113]. Furthermore, the Pre-S/S gene overlaps with the RT region of the P gene, and mutations corresponding to antiviral-resistance in the RT domain commonly result in stop codon mutations in HBsAg and changes in the antigenicity and secretion of HBsAg[114].

C GENE MUTATIONS

A1762T/G1764A double mutations in the BCP region and G1896A in the Pre-C region are the most frequently observed point substitutions in the C gene. These mutations prevent the production of the hepatitis B e-antigen (HBeAg) and are linked to HCC[115]. Genotypes C and D of HBV have been reported to have relatively high mutation rates in the BCP region[116]. However, A1762T/G1764A and G1896A were not common in genotype D-infected OBI patients from Turkey and India[117,118]. A study from Taiwan conducted on OBI related HCC and HBsAg-positive HCC patients found that mutations commonly found in HBsAg-positive HCC, such as A1762T/G1764A, G1896A, *etc*., were not frequently detected in OBI related HCC patients[119]. Therefore, point mutations in BCP/Pre-C in CHB may be not associated with OBI and are required to be explored by further functional studies. Several deletion mutations in the BCP region, such as nt1753-1772, nt1751-1770, nt1754-1771, *etc*., have been reported in OBIs, and variants containing deletions of nt1754-1771 were shown to reduce virion replication and expression levels of HBsAg and HBeAg (Figure 3)[97]. The BCP region recruits initiators for the transcription of Pre-C mRNA and pregenomic (pg) RNA and is rich in AT content[120,121]. Deletion mutations in the BCP region may prevent transcription of pgRNA, leading to a reduction in HBV DNA replication and HBsAg expression. Moreover, BCP region overlaps with the X gene, and deletions in the BCP region lead to truncated X protein, which also has an effect on virion replication and antigen expression[122].

X GENE MUTATIONS

X gene is a regulatory gene and generates the X protein, which is made up of 146 to 154 aa. X protein acts as a transcription factor and trans-activates

gene expression of HBV and several other viruses and cells[123]. The X gene harbors enhancer elements and the C promoter, and deletion mutations covering the C promoter were frequently encountered in various HBV infections, such as OBI, HBeAg-negative infection, and so on[124]. The C-terminal end of X protein is essential for the trans-activation function of X protein[122]. Eight base pair (nt1763-1770) and 20-bp deletion (nt1753-1772) mutations at the C-terminal end of X protein were frequently observed in OBIs, and variants containing these two deletions led to truncation of X protein and a decrease in Pre-C promoter activity as well as secretion of HBsAg, HBeAg, and HBcAg in transfected cell lines (Figure 3)[125,126]. Eight nucleotide deletions (nt1640-1647 or nt1770-1777) were frequently detected in occult HBV/HCV co-infected patients, and they exerted a positive effect on HCV replication but a negative effect on HBV replication[127,128]. Besides, partial and large deletion mutations in the X gene have been reported in OBIs in renal dialysis and HBV-vaccinated thalassemia patients[129].

P GENE MUTATIONS

Since the P gene overlaps with the S gene, mutations that occur in one gene may affect the other gene. The RT region of the P gene completely spans the S region, thus mutations resistant to nucleos(t)ide analogues (NAs) in the RT region frequently lead to mutations of HBsAg (including stop codon mutations) and affect the antigenicity of HBsAg (Figure 3)[114,130]. Transient selection of a V542I mutant in the HBV polymerase has been described in a CHB patient with prolonged administration of famciclovir. The mutation corresponded to a stop codon mutation at aa199 in the S gene, and a transfection experiment with the full-length HBV genome harboring the mutation demonstrated a negative effect on replication capacity and failed to produce HBsAg[131]. The rtA181T variant was frequently found in patients with adefovir (ADV)-resistance, corresponding to a stop codon mutation at aaW172 in the S gene (sW172*) and leading to truncation of 55 aa at the C-terminal end of HBsAg. The variant not only resulted in secretion deficiency of the virion but also exerted a dominant negative effect on virion secretion despite co-transfection with wild-type isolates[132]. Other common antiviral-resistant mutations that lead to truncation of S proteins include entecavir (ETV)-resistant rtT184M corresponding to sL176*, rtM204I (YIDD) both resistant to LMV and telbivudine (LdT) corresponding to sW196*, and so on[114]. The rtM204V (YVDD), accompanied by rtV173L (corresponding to sI195M and sE164D) and/or rtL180M, were detected in both HBsAg-negative patients and HBsAg-positive patients untreated with LMV[133,134]. Variants harboring YIDD or YVDD caused a drastic reduction in virion replication capacity in transfection experiments[135]. Some studies found that LMV-selected HBsAg protein changes, including E164D, I195M, and E164D/I195M, significantly reduced the binding efficiency to anti-HBs, implying the potential to escape HBV vaccine like G145R[133]. Point mutations in the RNase H domain of P gene were observed in vaccinated children with OBIs[52]. *In vitro* studies demonstrated that the RNase H region is essential for both RNA packaging and DNA synthesis and that the P gene of HBV and duck hepatitis B virus (DHBV) have similar biological properties. Point mutations in the RNase H region of DHBV, such as L697Y, V719Y *etc*., led to deficiency of RNA packaging and significant reduction of DNA synthesis[136].

Deletion mutations in the P gene have been rarely reported. A 281-bp deletion (nt2068-2349) covering the start codon of the P gene was reported in genotype B infected OBIs, but the relationship remains to be confirmed by functional studies[137].

NO OBI-RELEVANT MUTATIONS

Mutations in the "α" determinant of the S protein are "hot spots" of OBI related mutations, but some clinical reports demonstrated that no mutation was detected in the "α" determinant or the mutation rate of the "α" determinant in OBI group was not significantly different from HBsAg-positive infections[31]. In some OBI patients, no mutation was detected in the Pre-S/S gene[117]. Moreover, no OBI-relevant mutation was found throughout the entire genome of HBV strains isolated from some OBI patients[138]. As no OBI-relevant mutation was encountered in some cases of OBI and some mutations occurred in both OBIs and HBsAg-positive infections, some reports concluded that there were no OBI-specific mutations[45]. Furthermore, *in vivo* and *in vitro* studies have demonstrated that some mutations identified in clinical reports did not interfere with HBV replication and expression or HBsAg secretion and were not associated with OBI. In a recent study, we investigated a number of OBI patients with a family history of HBV infection by cloning and sequencing the Pre-S/S region of HBV DNA isolated from serum samples. Sequence comparison between OBI patients and their family members with CHB failed to identify common genetic variations that were specific for OBI[139].

PROBLEMS

The mechanism of OBI is complex and remains to be clarified. Previous related studies have several limitations, as follows: (1) It is difficult to amplify the full-length HBV genome due to the extremely low viral load in OBI patients, and most clinical reports are limited to mutations in part of the viral genome (mainly the S gene coding for HBsAg). Studies based on full-length HBV genomes are rare; (2) Most previous

studies of OBI were clinical reports with limited case numbers and no comparable controls, and conclusions about some mutations were not consistent across reports; (3) Most mutations were not studied *in vivo* or *in vitro*, and some mutations occurred both in OBIs and HBsAg-positive infections; (4) Functional studies of mutations were mostly focused on the secretion, antigenicity, or immunogenicity of HBsAg, while only a few of them addressed virion replication and secretion. Opposite conclusions may be drawn by studies on the same mutations, depending on whether a fragment or the entire genome was used (data not available); (5) Most studies focused on individual mutations, and it remained to be determined whether multiple mutations interact with each other or whether there is secondary function; (6) HBV genotype and subgenotype were ignored in most studies; (7) There is a lack of suitable reference sequences for genotype and subgenotype of HBV, and it is difficult to determine whether some amino acid substitutions were genotype-specific or OBI-related[140]; and (8) The levels of serum HBsAg in some OBI patients were fluctuant, and it remains to be elucidated whether HBV methylation, glycosylation, acetylation, or miRNA plays a role in this phenomenon.

The ability to screen for OBI is strongly affected by the assays used for detecting HBsAg and HBV DNA. For instance, the Abbott assay is highly sensitive for G145R detection. It is our opinion that multiple assays should be performed to verify the accuracy of the screening results and to reduce false positive or false negative results. NAT of HBV is highly sensitive and able to detect extremely low levels of HBV DNA but has a potential risk of false-positive results due to quality-control problems. HBV NAT is still important for recognition of OBI in HBsAg-negative blood donations worldwide especially in high-endemic countries. However, NAT has not been implemented in most of these countries due to its high cost[141]. In these countries, blood donations are screened by serological testing of HBsAg alone or combined with anti-HBc. In low-endemic regions of HBV or high-endemic regions of anti-HBc, anti-HBc testing may be not as effective as NAT[142]. Anti-HBc screening is not capable to detect pre-seroconversion window period infections and anti-HBc assays based on radioimmunoassay or enzyme immunoassay may suffer from false-positive results[143,144]. Although liver biopsy is the best way for detecting OBI, it is hard to obtain liver tissue and standardized assays for it are not yet available[145].

CONCLUSION

OBI is a special and complex form of HBV infection with worldwide distribution. Its reported prevalence significantly varies depending on study locations, detection assays used, and study population. The underlying mechanism of OBI is complex and unclear. Genetic variants may be relevant but future research is still need both *in vivo* and *in vitro*.

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