Review

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Occult hepatitis B infection and hepatocellular carcinoma: Epidemiology, virology, hepatocarcinogenesis and clinical significance

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Summary

Occult hepatitis B infection (OBI) refers to a condition where replication-competent HBV DNA is present in the liver, with or without HBV DNA in the blood, in individuals with serum HBsAg negativity assessed by currently available assays. The episomal covalently closed circular DNA (cccDNA) in OBI is in a low replicative state. Viral gene expression is mediated by epigenetic control of HBV transcription, including the HBV CpG island methylation pathway and post-translational modification of cccDNA-bound histone, with a different pattern from patients with chronic HBV infection. The prevalence of OBI varies tremendously across patient populations owing to numerous factors, such as geographic location, assay characteristics, host immune response, coinfection with other viruses, and vaccination status. Apart from the risk of viral reactivation upon immunosuppression and the risk of transmission of HBV, OBI has been implicated in hepatocellular carcinoma (HCC) development in patients with chronic HCV infection, those with cryptogenic or known liver disease, and in patients with HBsAg seroclearance after chronic HBV infection. An increasing number of prospective studies and meta-analyses have reported a higher incidence of HCC in patients with HCV and OBI, as well as more advanced tumour histological grades and earlier age of HCC diagnosis, compared with patients without OBI. The proposed pathogenetic mechanisms of OBI-related HCC include the influence of HBV DNA integration on the hepatocyte cell cycle, the production of pro-oncogenic proteins (HBx protein and mutated surface proteins), and persistent low-grade necroinflammation (contributing to the development of fibrosis and cirrhosis). There remain uncertainties about exactly how, and in what order, these mechanisms drive the development of tumours in patients with OBI.

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Background

A consensus meeting was held in Taormina, Italy in 2018 by a group of experts who reviewed and updated the recent development of occult hepatitis B infection (OBI). The published report focused mainly on the diagnosis, transmission and clinical implications of OBI, namely hepatocellular carcinoma (HCC), HBV reactivation, and antiviral therapy.[1] The aim of the present review was to focus attention on the basics – the epidemiological, pathological and mechanistic aspects of OBI-related HCC.

Definitions of OBI

"Overt" hepatitis B (chronic hepatitis B [CHB]) infection is characterised by the detection of HBsAg and viral genomic materials in the serum, resulting from active replicative and transcriptional activities. In contrast, "occult" hepatitis B infection refers to a condition where replication competent HBV DNA is present in the liver, in the presence or absence of HBV DNA in the blood, in individuals with serum HBsAg negativity assessed by currently available assays.[1] In OBI, the HBV DNA – in the form of episomal covalently closed circular DNA (cccDNA) – is in a low replicative state owing to host immune or epigenetic control. Therefore, when serum HBV DNA is detectable, it is invariably in a low viraemic range, *i.e.* <200 IU/ml, and may only be detected intermittently.[2]

Serology of OBI

In practice, serum markers of HBV exposure are used to define different types of OBI, which can be classified as seropositive or seronegative. Seropositive OBI accounts for 80% of all OBI cases,[3] where antibody to HBV core antigen (anti-HBc) and/or antibody to HBsAg (anti-HBs) are detectable in the serum (Fig. 1). Conversely, the absence of both antibodies in seronegative OBI leaves serum HBV DNA as the only detectable marker, making diagnosis more challenging. Concerning this serologic aberration, it may be of interest to discover that primary seronegative occult infection has been described in the woodchuck model of hepatitis

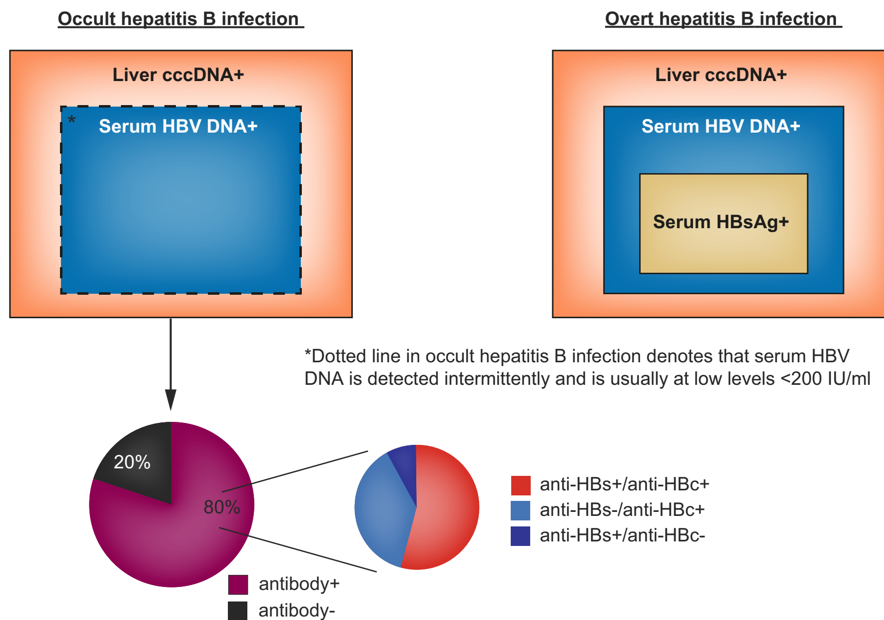


Fig. 1. Definition of occult hepatitis B infection. cccDNA, covalently closed circular DNA.

following inoculation with 10–100 virions of woodchuck hepatitis virus (WHV).[4] The probability of detecting positive serum HBV DNA is highest in individuals who are anti-HBc positive/anti-HBs negative.[2]

Patient origins in OBI

OBI mainly originates from a few groups of patients. The largest group includes patients who have had CHB infection for decades before achieving HBsAg seroclearance. Another group are patients who have recovered from self-limiting acute HBV infection. These are usually adult immunocompetent patients who have mounted a strong immune response to HBV upon exposure, inducing rapid viral control. These patients are often seropositive for anti-HBs as also observed in the woodchuck model.[5,6] There is an additional group of patients with HBV pre-S/S variants[7] or HBsAg escape mutations, either as acquired mutations or vaccine breakthrough infection. However, it remains controversial whether this last group of patients should be classified as OBI, since the serum HBV DNA level is often as high as found in CHB infection. Vaccinated children – born to HBsAg-positive mothers – who subsequently present with OBI are of particular interest. These children are often anti-HBs positive, anti-HBc nonreactive, with pre-S/S variants, and may harbour higher levels of serum HBV DNA than the first 2 groups of patients.[8–10] However, the presence of anti-HBs following HBV vaccination (alone or in combination with hepatitis B immunoglobulin) may eventually neutralise HBV and lead to resolution of OBI.[11] The infectivity and risk of liver-related complications, such as HCC, cirrhosis and HBV reactivation, differ significantly between these different groups (Fig. 2). This is probably due to differences in the level of immune exhaustion found in different scenarios of OBI.[12–14] However, for children born to HBsAg-positive mothers with vaccine breakthrough OBI, the risk of liver-related complications is unknown, given that some children will go on to clear the virus.

Diagnosis of OBI

To determine whether an individual with undetectable serum HBsAg has OBI requires the availability of highly sensitive and ultra-specific assays. Established commercial assays for HBsAg (*i.e.*, Abbott Architech) have a lower limit of detection (LLOD) of 50 mIU/ml. In contrast, recently developed HBsAg assays have LLODs that are 10 to 100-fold lower (Table 1) and will likely change the diagnosis of OBI in these cases into overt CHB infection.[15,16] To enhance the detection of surface escape variants, anti-HBs probes targeting multiple epitopes of HBsAg in the presence or absence of anti-HBs should be mandatory.[1]

Based on the current definition of OBI, analysis of liver tissue for replication-competent HBV DNA appears to be the most direct approach for diagnosis. However, liver tissue is not always available, and there is no standardised assay with internal and external validity. The diagnosis of OBI excludes detection of integrated HBV DNA for a variety of reasons (see section on 'Practical recommendations for the approach to case identification'). In a study involving 90 patients with cryptogenic HCC, 62 (69%) were found to have OBI. Only about half of them had detectable cccDNA in liver tissue, while

Key point

OBI is a difficult-to-diagnose liver disease with potential for development of hepatocellular carcinoma.

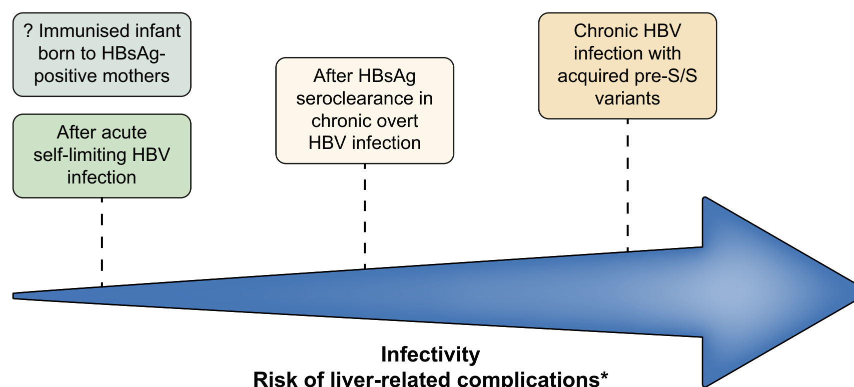


Fig. 2. Conceptual diagram illustrating the infectivity and potential risk of liver-related complications in different scenarios of occult hepatitis B infection. *Cirrhosis, hepatocellular carcinoma, HBV reactivation.

Table 1. Common quantitative serum HBsAg assays.

Name of assay	Manufacturer	Lower limit of detection
Architect HBsAg QT	Abbott Laboratories	0.05 IU/ml
Elecsys HBsAg II	Roche Diagnostics	0.05 IU/ml
Liaison XL Murex HBsAg Quant	DiaSorin	0.03 IU/ml
Lumipulse HBsAg-Quant	Fujirebio, Inc.	0.005 IU/ml
Ultrahigh sensitive HISCL HBsAg	Sysmex Co. Ltd.	0.0005 IU/ml

integrated HBV DNA was detected in almost 90% of patients with undetectable cccDNA.[17]

A more common (but less sensitive) approach to diagnose OBI is the detection of HBV DNA in the blood. The optimal standard is the analysis for HBV DNA extracts from plasma performed by real-time, nested PCR techniques. The PCR primers should span at least 3 HBV genomic regions, while validation should allow detection from at least 2 genomic regions. The sensitivity of the assay is crucial to find cases of OBI since the levels of HBV DNA in the blood are usually quite low (<200 IU/ml). The lower limit of quantification of most commercially available assays is 5–20 IU/ml although detection without quantification can be observed at lower levels. In the setting of blood donations, a highly sensitive assay is preferred for nucleic acid testing (NAT) on the donated samples, mostly in the range of 2–4 IU/ml of HBV DNA. Notably, the window period of acute HBV infection is not regarded as OBI, since these individuals usually have high serum HBV DNA and will eventually develop seropositivity for HBsAg.[18]

Detection of anti-HBc in the blood indicates prior HBV infection, but does not imply a diagnosis of OBI, and HBV DNA analysis should be further pursued. Moreover, a subset (20%) of patients with OBI are seronegative for anti-HBc. Therefore, anti-HBc screening identifies most, but not all, individuals with prior HBV infection and specifies which patients might benefit from HBV DNA testing to confirm the diagnosis of OBI. Within this cohort, those with isolated anti-HBc (negative for anti-HBs) with high antibody levels are more likely to be reactive for HBV DNA.[19]

Epidemiology of OBI

The worldwide prevalence of OBI is quite variable across patient populations and is higher in areas of the globe where hepatitis B is endemic. As reviewed by G. Raimondo *et al.*[1] and others,[20–22] OBI has been detected in patients coinfected with HIV (10–45%) or HCV (22–73%), in those with cryptogenic chronic liver disease (5–40%), in apparently healthy blood donors (see below), among people who inject drugs (PWID) (45%), individuals with thalassemia and haemophilia (5–51%), patients on haemodialysis (0–58%), orthotopic liver transplant patients (42–64%), other immunosuppressed groups, and even individuals that are free of apparent liver disease (1–34%). Reasons for this disparity include different geographic locations, demographic factors, endemicity, vaccination status, characteristics of the assays, host response, risk factors (*i.e.*, injection drug use, transfusion requirements and viral load), severity of liver disease (*e.g.*, cirrhosis, high alanine aminotransferase [ALT] levels), and coinfection with HCV or HIV.

Since transfusion is a major route of transmission in developing and resource-poor countries,[23] it is reasonable to examine the global prevalence of OBI and transfusion safety, as reviewed by Clive R. Seed and an assembly of international experts.[24] In this forum, a recent multicentre survey of almost 11 million donations worldwide found lower OBI NAT yield rates that varied from 1 in 3,900 to 1 in 59,000, with even higher rates of 1 in 1,000 donations found in regions where genotypes B, C or E prevail (Asia and Western Africa).[25,26] As previously discussed,

detection of OBI is directly correlated with increased anti-HBc reactivity which also correlates with the level of cccDNA.[27] The estimated rate of transmission of HBV from donors with OBI to recipients of blood components varies widely (2–48% based on lookback studies).[28–33] Risk factors include viral load and the absence of anti-HBs. It is estimated that the risk of transmission is highest for plasma, then packed red blood cells and/or platelets,[28,30,33] with estimated minimal infectious doses ranging from 100 IU to ~3 IU per unit transfused based on volume.[28,33] These levels, converted from genomic equivalents (geq) to IU,[34] are similar to those previously reported in chimpanzee transmission experiments (CID_{50}) for genotypes A at 169 geq, C at 3 geq, and D at 78 geq.[35] NAT was implemented globally between 1999 and 2010 but the practice is not widespread. Cases of HBV transmission post-NAT testing in these selected blood facilities yielded a lower residual risk ranging from 1 in 52,000 (Hong Kong) to 1 in 7.5 million (Canada).

Virology, epigenetics and immunology of OBI

OBI is characterised by the persistence of HBV cccDNA in the nucleus of infected hepatocytes. It is generally believed that the undetectability of HBsAg in individuals with OBI, despite cccDNA persistence, is due to the suppression of viral replication as a result of epigenetic or immune control of gene expression.

Studies have shown that some patients with OBI harbour a higher proportion of mutations in the preS/S region than patients with CHB, which may result in a reduced antigenicity for HBsAg detection or impairment in HBsAg production or secretion.[7,12,36–41] The mutations in pre-S1 may also alter the B and T epitopes that affect immune recognition.[7] However, HBV DNA isolated from individuals with OBI is fully replication competent *in vitro*,[42] and the virus can also be transmitted via blood transfusion or organ transplantation.[43,44] In addition, reactivation of HBV is often seen in patients with OBI under immunosuppression.[45–47] These findings suggest that host factors may play a more important role than viral factors in OBI.

OBI is mostly associated with a low level of cccDNA in the hepatocyte nucleus which results in a low level of HBV transcription and protein expression, contributing to the undetectability of HBsAg.[48,49] The persistence of transcriptionally muted cccDNA in patients with OBI suggests that gene expression can be mediated by epigenetic control of HBV transcription. However, direct experimental data on the epigenetic regulation of HBV transcription in patients with OBI are scarce. One study has identified different methylation patterns in the HBV CpG islands between patients with occult and overt chronic infection, suggesting that OBI and CHB may have different epigenetic control mechanisms.[40] Specifically, patients with OBI have a high methylation density at HBV CpG island 2[40]; high methylation density has been shown to be associated with low HBV replicative activity in HBsAg-positive, HBeAg-negative patients with CHB.[50] Despite evidence that a negative correlation between HBsAg level and cccDNA methylation status exists,[51] further investigation is required.

Another mechanism affecting the regulation of HBV transcription is the post-translational modification of cccDNA-bound histones.[52] Like eukaryotic chromosomes, cccDNA is arranged as nucleosomes, with histones as well as other cellular or viral proteins forming a minichromosomal structure.[53] It has been demonstrated, both *in vitro* and *in vivo*, that HBV transcriptional activity and viral load are affected by the degree of acetylation of cccDNA-bound histones (H3/H4) and the association between cccDNA and histone-modifying enzymes.[54] A number of cccDNA-associated proteins, including the hepatitis B core and X proteins (HBc and HBx, respectively), transcription factors such as cAMP response element binding protein (CREB), signal transcription factors 1 and 2, chromatin modification proteins such as histone deacetylase 1, p300/CREB-binding protein, protein arginine methyltransferase 1 and 5, and sirtuin 1, have been demonstrated to regulate the transcriptional activity of HBV.[52,54–61] In theory, these cellular and viral epigenetic factors can facilitate a robust control of viral replication, leading to very low HBV DNA and undetectable HBsAg in patients with OBI.[62] However, to date, direct evidence of the involvement of these epigenetic factors in OBI has yet to be reported.

The role that immune control plays in OBI is largely supported by evidence of HBV reactivation in patients with OBI receiving immunosuppressive therapy or haematopoietic stem cell transplantation.[45–47] Early studies have shown that, in some patients with ostensibly self-resolved acute HBV infection, there exists a long-lasting HBV-specific T cell response which controls HBV replication.[13,14] This HBV-specific T cell response is also observed in OBI donors at a level higher than that detected in HBsAg-positive patients with CHB.[12] The profiles of HBV-specific T cell responses are also different between seropositive and seronegative individuals with OBI. While both groups showed a very low frequency of circulating HBV-specific T cells *ex vivo*, HBV-specific T cells in seronegative patients did not readily expand upon stimulation *in vitro*, suggesting the existence of different immune-control mechanisms between seropositive and seronegative OBI.[63] This has also been seen in WHV infection, in which resolution of acute WHV infection results in the life-long persistence of seropositive occult WHV infection.[5,64] However, exposure to a very low dose of WHV, even with multiple doses,

Key point

OBI is the combined result of host immune control and different genomic expressions of the virus, which lead to a virological quiescent state.

results in occult WHV infection without any serological markers.[4,65,66] These woodchuck studies suggest the possibility that a very low-dose infection may be insufficient to mount a protective cellular memory response.

In addition to cellular immunity, humoral immunity is likely to play an important role in the control of HBV replication in OBI, as demonstrated by the occurrence of HBV reactivation in patients with B-cell depleting, immune-suppressive anti-CD20 therapy such as rituximab and ofatumumab.[45,47,67] Innate immunity may also contribute to the control of OBI, as suggested by a recent study in the woodchuck model that demonstrates a differential expression of toll-like receptors 1–10 in acute, chronic, and occult woodchuck hepatitis infection.[68] However, partly due to the scarcity of adequate animal or *in vitro* models for OBI study, current data have, by necessity, been based on inferences generated from experiments designed to control HBV replication using *in vitro* systems approximating CHB infection.[54–61]

Key point

OBI is more commonly found in patients with chronic hepatitis C virus infection, those with cryptogenic or known liver disease.

Association of OBI and HCC

Woodchuck model of OBI

The woodchuck model of OBI is an excellent experimental system to study the pathogenicity of occult hepadnaviral persistence and its role in the development of HCC.[69] Investigators reported that woodchucks infected with low doses of WHV developed asymptomatic, seronegative, molecularly evident persistent occult hepatitis with a low viral load. This led to HCC in 2 out of 10 animals and viral integration in hepatocytes and the lymphatic system in 9 of the animals. Virus recovered from infected livers could transmit the infection to healthy animals resulting in hepatitis and HCC.

Patients with HBsAg seroclearance

It is assumed that for OBI arising from patients with CHB who clear their HBsAg, the risk of HCC is not eliminated, but may be reduced. One study showed that 2.34% (7/298) of patients with HBsAg seroclearance developed HCC over a median follow-up of 9 years,[70] depending on gender and the age at which HBsAg seroclearance occurred.[70,71] Moreover, free HBV genomes and HBx mRNA were expressed in the liver tissues of patients with HCC, which is evidence for persistent viral transcription and replication.[70] On the other hand, there are no data on the risk of HCC solely in patients with OBI following resolved acute HBV infection. It will be difficult to assess this risk, as many of these patients may have had asymptomatic acute HBV infection, in which case such history is not usually elicited.

Key point

Chronic liver conditions co-existing with OBI increase the risk and aggressiveness of hepatocellular carcinoma.

Patients with a cryptogenic cause of HCC

OBI is reported to play an important role in the progression of cirrhosis and the development of HCC in several epidemiological and molecular studies. For example, in retrospective studies involving Asian or European patients with cryptogenic HCC, 60–70% were found to have OBI in their liver tissues.[17,48,49,72] In a prospective study involving 82 Japanese patients with cryptogenic cirrhosis followed for a median of 5.8 years (range 0.1–34.8 years),[73] the rate of HCC was 100% with and 17.6% without OBI at 10 years (p = 0.008; hazard ratio 8.25). Multivariate analysis confirmed that OBI was an independent risk factor for hepatocarcinogenesis in patients with cryptogenic cirrhosis in this prospective study. Finally, in a meta-analysis of 16 studies involving 3,256 individuals, both retrospective (n = 8) and prospective studies (n = 8) showed an increased risk of HCC in individuals with OBI (odds ratio 6.08 and 2.86, respectively).[74]

Patients with or without coinfection with HCV

Between 2000 and 2013, several published studies evaluated the role of OBI in the development of HCC in patients with or without HCV infection. In a systematic review of 8 retrospective studies from Asia, South Africa, Italy and Egypt,[21] the overall mean prevalence of OBI in 631 non-HCV-infected patients with HCC was 59.4% (median of 69.8%). In 4 of these studies where comparisons could be made,[48,72,75,76] the prevalence of OBI in the anti-HCV negative patients with HCC was significantly higher (40.5–70.4%) than the prevalence of OBI in anti-HCV negative patients with chronic hepatitis (26.3%) and/or healthy controls (9.0%) without HCC (p <0.001). In the 4 remaining uncontrolled retrospective studies, high rates of OBI ranging from 69.2-76.2% were found in anti-HCV negative patients with HCC, but no control populations were included for comparison.

In the same systematic review,[21] the prevalence of OBI was found to be 47.9% (range 22–73.3%) in 292 treatment naïve, HCV-infected patients in 7 retrospective studies. In 3 of these studies where a comparison could be analysed,[48,77,78] the prevalence of OBI was significantly higher in patients with HCC than in patients without HCC or in healthy controls (p <0.001).

In other studies from Asia and Europe, the prevalence of OBI in patients with chronic HCV infection was 15–49% in those without HCC compared to 73% in those with HCC.[79–81] However, this association was not observed in studies performed in the USA[82] and Taiwan.[83] For instance, in the USA cohort of patients with advanced chronic HCV (>50% had cirrhosis), without HBsAg seropositivity, OBI was detected in 10.7% of patients with HCC and 23.6% of controls without HCC (p = 0.18).[82] Paradoxically, according to another

Table 2. Prospective studies evaluating the cumulative incidence of HCC in HCV-infected patients with and without occult hepatitis B.

Study	Year	Country	Sample for HBV DNA detection	OBI in follow-up patients, %	Incidence of HCC in patients with OBI, %	Incidence of HCC in patients without OBI, %	Follow-up duration, months	*p* value
Squadrito *et al.*[85]	2006	Italy	Liver	40.3 (50/124)	14.0 (7/50)	1.4 (1/74)	Median 82.8 (±32.6)	<0.002
Squadrito *et al.*[86]	2013*	Italy	Liver	39.4 (37/94)	35.1 (13/37)	8.8 (5/57)	Median 132 (range 60–228)	<0.003
Miura *et al.*[87]	2008	Japan	Serum	5.7 (8/141)	50.0 (4/8)	21.8 (29/133)	Mean 81.8 (±48.5)	<0.004
Matsuoka *et al.*[88]	2008	Japan	Serum	43.6 (204/468)	14.2 (29/204)	3.4 (9/264)	Mean 80.4**	<0.0001

HCC, hepatocellular carcinoma; OBI, occult hepatitis B infection.
*The Squadrito *et al.* 2013 study is an extension of the 2006 study with median follow-up of 11 years *vs.* 6.9 years previously.
**Information on the standard deviation of follow-up duration was not available.

study, the risk of HCC was higher in populations with OBI that were not coinfected with HCV than in those that were coinfected with HCV (odds ratio 10.65 and 2.83, respectively).[74]

More potentially conflicting evidence comes from a recent Egyptian study involving 50 patients with HCV-related HCC who underwent resection or liver transplantation. Of these patients, 25 (50%) had co-existing OBI. While there were no clinical differences between patients with and without OBI (gender, serum transaminases, platelet count, albumin, bilirubin, prothrombin time, alpha-fetoprotein, Child-Pugh score), those with OBI were younger (48.4 year-old for OBI compared to 51.4 year-old for non-OBI), and were associated with more advanced histological grades of HCC (odds ratio 3.69).[84] This supports the premise that OBI may play a synergistic role in the occurrence of HCC in HCV coinfected patients, especially in patients with advanced fibrosis and cirrhosis.

Apart from all the aforementioned cross-sectional studies, at least 7 prospective studies evaluating the cumulative incidence of HCC in HCV-infected patients with and without OBI coinfection have been published, with systematic analysis of these data discussed elsewhere.[21] Four of these prospective studies are shown in Table 2. Two of these investigations[85,86] utilized the same cohort of patients, whose numbers declined from 124 during a median follow up of 6.9 years (in the first study) to 94 over a median follow-up of 11 years (in the second study). Both cirrhotic and non-cirrhotic patients were included in the analysis. The incidence of HCC in HCV-infected patients with OBI was significantly higher than among HCV-infected patients without OBI. In those without HCC, OBI coinfection was associated with a significant worsening of liver disease and shorter survival. The 2 Japanese studies listed in Table 2 included HCV-infected patients with and without OBI who were unsuccessfully treated with interferon regimens. In the Miura *et al.*[87] study that excluded cirrhotic patients, the rate of HCC development increased progressively over time from 8.9% at 5 years, to 25.7% at 10 years, and 53.7% at 15 years. Importantly, HCC was more than twice as common in the coinfected patients as in the HCV-monoinfected group. The fourth study[88] also found that the risk of HCC was significantly greater in OBI/HCV-coinfected patients, who had significantly more fibrosis and necroinflammation than HCV-monoinfected patients, regardless of whether or not they were treated with interferon. Three other prospective studies were excluded from this analysis because a plethora of confounding variables made interpretation problematic.[21]

Pathogenetic mechanisms of OBI-related HCC

A body of evidence indicates that OBI maintains the pro-oncogenic properties attributed to CHB infection. HBV exerts its hepatocarcinogenic activity by direct and indirect pathogenetic mechanisms, which may schematically be related to: (a) the capacity of the viral DNA to integrate into the host's genome; (b) the production of proteins with potential transforming properties; and (c) the induction of necroinflammation within the liver progressing toward cirrhosis, which is the most important risk factor for HCC development.[89,90] Fig. 3 summarises the potential hepatocarcinogenic mechanisms involved in OBI-related HCC.

Direct pro-oncogenic mechanisms

A number of studies performed in the early 80s, based on hybridisation technology, revealed the presence of integrated forms of viral DNA within the host genome of patients with HBsAg-negative HCC.[91] Subsequent studies performed with more advanced molecular approaches – in particular, the PCR-based assays and, more recently, the newly developed high-throughput sequencing approaches[92–96] – confirmed this observation, and provided further relevant information in terms of possible mechanisms by which HBV (even in the OBI phase) may contribute to hepatocyte transformation. The use of these more sensitive PCR-based methods revealed the presence of integrated HBV genomic sequences in over 60–75% of HCCs from HBsAg-negative patients,[17,94] a prevalence very similar to that reported in HBsAg-positive patients with HCC.[97,98] Moreover, by applying the ALU-PCR assay, it was shown that, in analogy to what occurs in HBsAg-positive cases, HBV integrants frequently include sequences corresponding to the X gene, the preS/S genomic

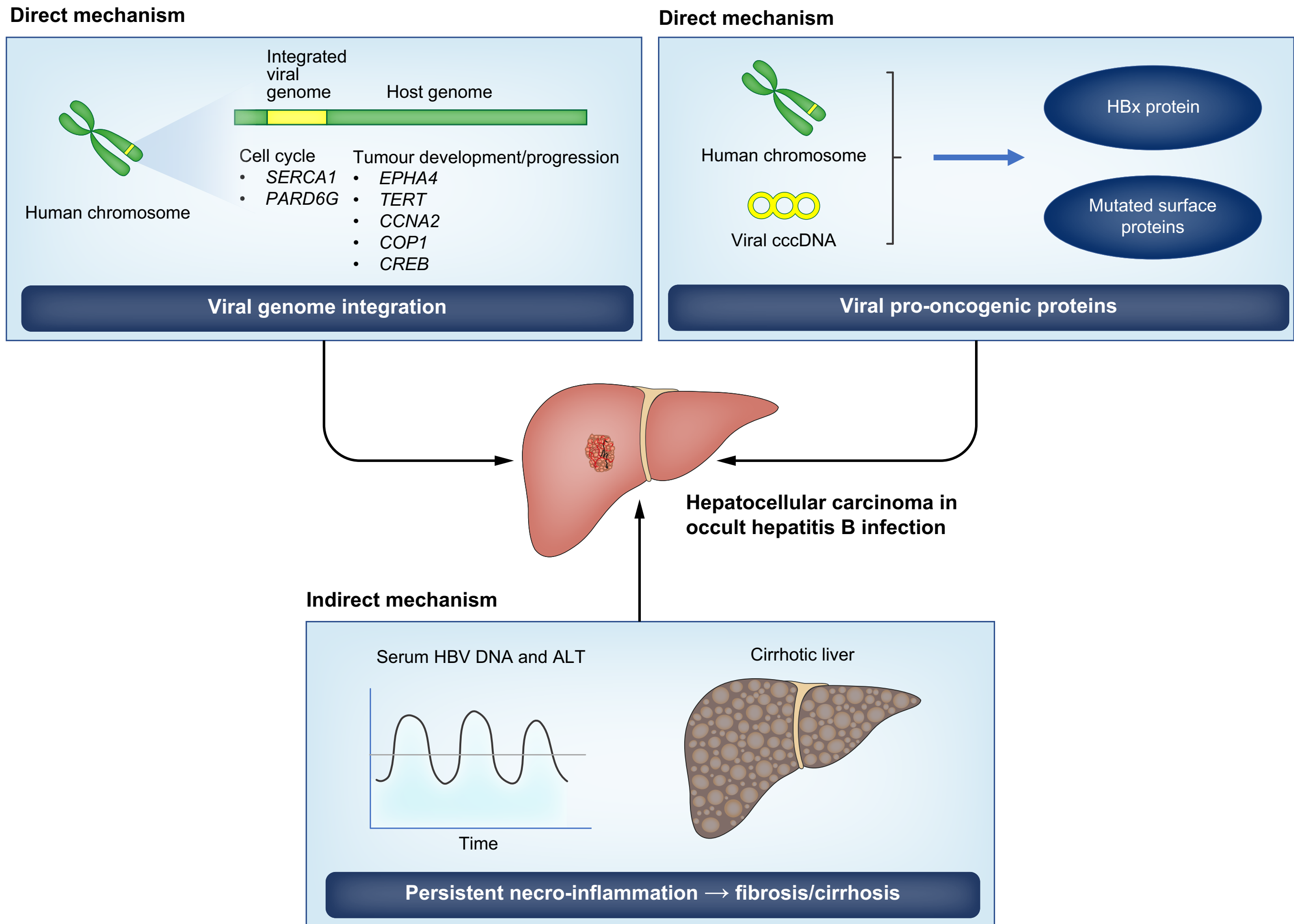


Fig. 3. Potential pathogenic mechanisms in occult hepatitis B infection-related HCC. ALT, alanine aminotransferase; cccDNA, covalently closed circular DNA; HCC, hepatocellular carcinoma.

region, and viral regulatory elements. In addition, viral integration often targets host genes that are involved in cell cycle, cell survival and immortalisation,[17,94] as well as genes that have been reported to play a role in HCC development or progression, such as *EPHA4*, *TERT*, *CCNA2*, *COP1*, and *CREB*.[17] Importantly, several studies have shown that HBV integration is also frequently detected in HBsAg-negative patients with HCC but without cirrhosis,[17,96,99–103] further corroborating the direct role of HBV integration on liver cancer development in patients with OBI. Additionally, cases of cis-activation of cellular genes, such as *SERCA1* (the gene encoding sarco/endoplasmic reticulum Ca^{2+}-ATPase which pumps calcium from the cytosol to the endoplasmic reticulum) and *PARD6G* (the partitioning-defective-6-homolog-gamma gene encoding for a protein that is part of the Par6 complex, which is involved in the establishment of cell polarisation and in the polarised migration of cells) have also been reported in HCC from non-cirrhotic HBsAg-negative patients following HBV DNA integration.[101,102] One study showed that the HBx gene promoter cis–activates chimeric HBx/*SERCA1* transcripts causing dimerisation and accumulation of chimeric proteins in the tumour of an HBsAg-negative individual. These chimeric proteins localise to the endoplasmic reticulum (ER) and may control cell viability.[101] In another study, HBV integration involving a 5′-deleted HBx gene with an intact enhancer-II/basal core promoter region was identified in tumour tissue from a non-cirrhotic patient with HFE-haemochromatosis and HCC. The HBV integrant was located upstream of the *PARD6G* gene, which was highly overexpressed in tumour compared to adjacent non-tumour liver tissue and normal liver controls.[102]

Key point

Hepatocarcinogenesis of OBI is mechanistically mediated through virus-to-human DNA integration, oncogenic effects of viral proteins and the insidious and low-grade liver inflammation leading to advanced fibrosis and cirrhosis.

Integrated HBV is frequently associated with deletions, loss of heterozygosity, duplications, and translocation at the site of viral insertion.[97] Besides inducing cis-activation of the host's genes and the production of fusion transcripts, HBV integration in HBsAg-negative HCC has also been associated with alterations of tumour suppressor genes, p53 mutations and loss of heterozygosity,[104,105] replication errors, and genomic instability.[106] In particular, p53 missense mutations have been associated with the presence of HBx gene sequences both in

HBsAg-positive and HBsAg-negative patients with HCC from specific geographic areas of Africa and China.[105] Accumulation of HBx transcripts in HCC from HBsAg-negative patients was reported in several studies.[48,103,107] In HBsAg-negative tumours and adjacent non-tumour tissue, HBx transcripts can be produced both from free replicative and from integrated viral DNA. One study demonstrated the selective expression of HBx genes from integrated HBV DNA in HBsAg-negative HCCs,[100] providing evidence of production of HBx RNA and protein (but not of S and core products) in patients with OBI. This study also showed that the HBx gene was modified (3′-terminally truncated or highly mutated) in the majority of tumours compared with the surrounding non-tumour tissues, suggesting that the selection of HBV genomes carrying interrupted or mutated HBx genes may play a role in malignant transformation of hepatocytes. Indeed, deletion of the C-terminal region of HBx, and specific amino acid mutations observed in several HBx mutants isolated from HCC tissues,[42,100,108,109] may lead to the abrogation of HBx-dependent transactivation, cell cycle arrest and apoptosis inhibition and promote the transforming capacity of HBx.[110,111]

Finally, there is evidence of a high prevalence of pre-S2 variants in HCC tissues from patients with OBI.[42,112] These variants are known tumourigenic factors in CHB infection because they induce an imbalance in the synthesis of surface proteins, leading to their retention within the hepatocyte ER. The accumulation of mutated surface proteins may cause ER stress, consequently inducing oxidative DNA damage, genomic instability, and increased risk of cancer development.[113] These genetic mutants may also exert a pro-oncogenic role in OBI when the transcriptional activity of HBV persists.

Indirect pro-oncogenic mechanisms

Patients with CHB who achieve HBsAg seroclearance can still develop HCC, especially if cirrhosis had already developed during the overt phase of the infection. The risk of HCC development is maintained although probably at a reduced level.[70,114–117] Among immunocompetent individuals, OBI may not always indicate active, progressive, inflammatory liver disease of an infectious nature. However, in the presence of other factors associated with liver injury, such as HCV infection, non-alcoholic steatohepatitis and alcohol abuse, OBI could intensify the course of the underlying disease and facilitate progression of fibrosis and the development of HCC. That said, this topic is widely debated.[1] Certainly, the strong suppression of HBV replication and gene expression may prevent any clinical impact in the vast majority of OBI cases. However, some scholars have suggested that OBI developing after recovery from self-limited acute hepatitis may be associated with a modest but long-lasting persistence of hepatic necroinflammation on histological analysis in some patients, despite the absence of any clinical or biochemical signs of liver damage.[6,118–120] These individuals show a high level of specific anti-HBV cytotoxic T lymphocyte (CTL)-responses even decades after clinical recovery and anti-HBs seroconversion, likely due to the continuous stimulus exerted by the minute amounts of viral protein produced.[13,14] Finally, there is evidence that detectable serum HBV DNA may periodically reappear in patients with OBI, alongside a rise in ALT values.[121,122] These observations might suggest that the HBV suppression is not stable in the course of an occult infection, and phases of viral reactivation may transiently occur over time, provoking very modest but histologically detectable liver damage despite the prompt control exerted by the CTL-response. This minimal damage might be persistent, and it is tempting to speculate that the usually innocuous OBI might play a pathogenetic role as a co-factor of liver disease when it is present together with other major causes of liver injury.

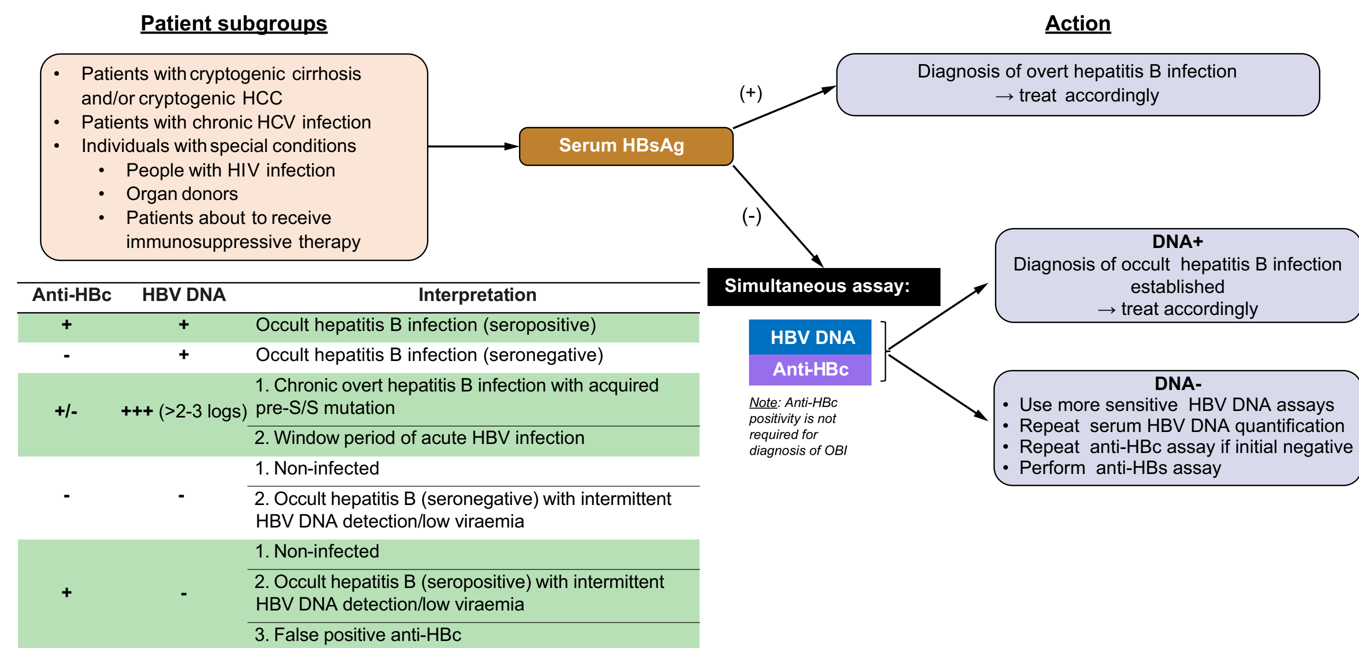
Practical recommendations for the approach to case identification

A selective approach should be adopted in deciding who to screen for OBI. At this juncture, population screening to identify individuals with OBI cannot be recommended because of the lack of data on the benefits of such an approach. Therefore, only selected patient subgroups that are at risk of accelerated liver damage or HBV reactivation should be screened for OBI (Table 3). These include patients with cryptogenic cirrhosis and/or cryptogenic HCC, chronic HCV infection, HIV infection, organ donors, and patients about to receive immunosuppressive therapy. After confirming seronegativity for HBsAg, they should be tested for serum HBV DNA. Although anti-HBc is not required for the diagnosis of OBI (but rather, for classification into seropositive or seronegative group), it is often the first assay to be performed and this practice is currently widely adopted. For those with detectable serum HBV DNA, the diagnosis of OBI is established, and they should be treated accordingly. For those with negative serum HBV DNA, one should consider using a more sensitive HBV DNA assay, or repeating the HBV DNA assay at a later time to identify those with intermittent viraemia (Fig. 4 and Table 3). Diagnosis of OBI in patients at increased risk of accelerated liver disease warrants close HBV monitoring and enhanced surveillance for liver-related complications, including HCC. Occasionally, the management of the underlying condition will be adjusted due to co-existing OBI. For instance, for patients with HIV infection with coexisting OBI, antiretroviral agents that also effectively suppress HBV can be chosen. In patients

Table 3. Practical recommendations for screening and diagnosing occult hepatitis B infection in individuals with negative serum HBsAg.

Patients	Recommendation	Rationale
Patients with cryptogenic cirrhosis and/or cryptogenic HCC Patients with chronic HCV infection Individuals with special conditions (HIV infected, organ donors*, patients about to receive immunosuppressive therapy)	Simultaneous assay for anti-HBc and HBV DNA • If DNA −ve, perform follow-up tests • If DNA +ve, treat accordingly	These patients, if they have co-existing OBI, are at risk of accelerated liver damage including higher risk of HCC or HBV reactivation, compared to those without co-existing OBI
Healthy blood donors (no information on HBsAg status)	Blood for nucleic acid testing • If DNA −ve, no follow-up actions • If DNA +ve, not allowed to donate blood and perform follow-up tests	Viraemia is the mechanism for transmission of HBV via blood products Detection of viraemia helps to prevent blood-borne transmission of HBV and identify infected subjects for proper medical care
Other patient groups at risk of HBV infection** (*e.g.,* PWID, men who have sex with men, haemodialysis patients, health care workers etc)	Not recommended for routine anti-HBc and HBV DNA assay	No data on the benefits of screening for OBI in these groups
General population	Not recommended for routine anti-HBc and HBV DNA assay	No data on the benefits of population-based screening for OBI

HCC, hepatocellular carcinoma; OBI, occult hepatitis B infection; PWID, people who inject drugs.
*Risk of HBV reactivation in the organ recipients (as they will be on immunosuppressive therapy).
**See AASLD 2018 guidelines (Terrault NA *et al.*, Hepatology 2018; 67:1560–99).[123]



Anti-HBc	HBV DNA	Interpretation
+	+	Occult hepatitis B infection (seropositive)
-	+	Occult hepatitis B infection (seronegative)
+/-	+++ (>2-3 logs)	1. Chronic overt hepatitis B infection with acquired pre-S/S mutation
		2. Window period of acute HBV infection
-	-	1. Non-infected
		2. Occult hepatitis B (seronegative) with intermittent HBV DNA detection/low viraemia
+	-	1. Non-infected
		2. Occult hepatitis B (seropositive) with intermittent HBV DNA detection/low viraemia
		3. False positive anti-HBc

Fig. 4. Proposed algorithm for diagnosing occult hepatitis B infection in selected patient subgroups. HCC, hepatocellular carcinoma.

at risk of HBV reactivation, prophylactic or pre-emptive antiviral therapy is often required.

For healthy blood donors, serum HBV DNA detection by NAT is the appropriate approach to identify HBV-infected individuals for proper medical care and to prevent blood-borne HBV transmission via blood products. If NAT is negative, no further testing for OBI is required (Table 3). Other groups at high risk of HBV infection,[123] *e.g.,* PWID, men who have sex with men, haemodialysis patients, health care workers, *etc*., should be tested for serum HBsAg and anti-HBs to identify overt HBV carriers and vaccine non-responders, so that they can receive proper medical care and vaccination, respectively. As for the general population, the purpose of identifying OBI cases in these patient groups is unclear and is currently not recommended (Table 3).

The use of liver biopsies, and novel HBV biomarkers (such as hepatitis B core-related antigen, HBV RNA) are merely for research purposes at this juncture. In special circumstances where liver histology is available as part of clinical management (*e.g.* hepatectomy for cryptogenic HCC), HBV DNA or cccDNA can possibly be isolated from the liver tissue to establish the diagnosis of OBI, if laboratory

support is feasible. Although integrated HBV DNA is also present in patients with OBI, detection of integrated HBV DNA is purely a research tool at this juncture. Moreover, since the implications of OBI entail both accelerated risk of liver damage including HCC, and risk of HBV reactivation, the presence of replication-competent HBV DNA is required to establish the diagnosis of OBI. Integrated HBV DNA, although directly pro-oncogenic, is not replication competent. Therefore, it is not practical to include the presence of integrated HBV DNA in the definition of OBI.

Future perspectives

Over the past two decades, considerable research and speculation have begun to define the role of OBI in the development of HCC. HBV DNA testing in the serum or liver is required to delineate OBI in virtually all cases. This poses a challenge to refine highly sensitive HBV DNA tests in the serum. In addition, repeated testing at different time points should be used to diagnose the condition in selected patient subgroups as discussed. While these requirements have often not been met by those who publish on this topic, expansion of our knowledge evokes optimism, and we are beginning to see light at the end of the tunnel. We eagerly await further data on the true prevalence of OBI and its contribution to HCC development.

Concerning the exact pathogenetic mechanisms leading to OBI status and its role in hepatocarcinogenesis, there are plenty of uncertainties. The available evidence suggests that they are multifactorial with additive or synergistic effects. Further studies are required to delineate the sequence of virus and host events and the major drivers among the possible mechanisms leading to HCC development.

With advances in HBV diagnostic technology and increasing case-finding efforts in different liver disease areas, the entity of OBI has undoubtedly been revealed as a significant cause of HCC worldwide, especially in those with underlying predisposing conditions. Although the treatment of HCC may not be different from those with HCC due to other causes, enhanced surveillance and early diagnosis is still the key for better patient outcomes. Therefore, clinicians should always be cognisant of this entity in at-risk patient groups.

Abbreviations

ALT, alanine aminotransferase; cccDNA, covalently closed circular DNA; CREB, CAMP response element binding; ER, endoplasmic reticulum; HCC, hepatocellular carcinoma; LLOD, lower limit of detection; NAT, nuclei acid testing; OBI, occult hepatitis B infection; PWID, people who inject drugs; WHV, woodchuck hepatitis virus.

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

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Authors' contributions

LYM, DKHW, TP, GR and FBH were responsible for data review, writing and critical revision of manuscript. MFY was responsible for study concept, data review, writing, critical revision and overall supervision of manuscript.

Supplementary data

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Author names in bold designate shared co-first authorship

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