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Review

Hepatitis B virus-related hepatocarcinogenesis: Molecular oncogenic potential of clear or occult infections

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ABSTRACT

Chronic viral infection is the most important oncogenetic factor, and hepatitis B virus (HBV) plays an important role in the development of hepatocellular carcinoma (HCC).

HBV-related carcinogenesis is a multi-step process, encompassing the combination of different, not mutually exclusive effects such as the induction of chronic liver inflammation and regeneration, its integration into the hepatocyte genome and the transactivating and transforming activity of several viral proteins (HBx and truncated Pre-S2/S) that may stimulate cellular oncogenes or suppress growth-regulating genes. Data related to the influence of different hepatitis B virus genotypes and the emergence of selective variants as biomarkers of HCC development still remain controversial. At last, recent studies on occult HBV infection, as defined by serologically undetectable hepatitis B surface antigen (HBsAg–), despite the presence of circulating HBV DNA, suggest that the occult viral strains, maintaining the transcriptional activity and the pro-oncogenetic assets of the clear HBV infection (HBsAg+), may harbour a potential risk for liver cancer development.

1. Introduction

1.1. *HBV molecular structure and replicative cycle*

HBV is an enveloped, partly double-stranded (minus- and plus-strand) DNA virus of approximately 3200 nucleotides that contain overlapping open reading frames (ORFs), efficiently encoding for proteins. The viral translational products include large, middle and small envelope proteins (Pre-S/S gene), pre-core and core (Pre-C/C gene), polymerase (P gene) and the transcriptional regulator X protein that are encoded by overlapping gene (Fig. 1).[1] In particular, HBV envelope proteins display morphogenetic and regulatory functions, and play a central role in the diagnosis of HBV infection. The Core gene encodes for HBc protein that is involved in viral encapsidation and DNA replication. The Pre-Core domain is upstream of the core and results in the secretion of a heterogeneous population of proteins, serologically defined by HBe antigen.[2] HBeAg has relevant clinical importance, because its persistence in infected patients is associated with active replication and high infectivity, and represents a potential index of chronic evolution of the infection.[3,4] A single mutation at the end of the pre-C gene, resulting in a translational stop codon, leads to the loss of HBeAg synthesis. In these cases, active viral replication may persist despite the elimination of HBeAg and seroconversion to anti-HBe.[5] How this mutation, alone or in combination with other mutations, affects the clinical course of HBV-related liver disease, is still a

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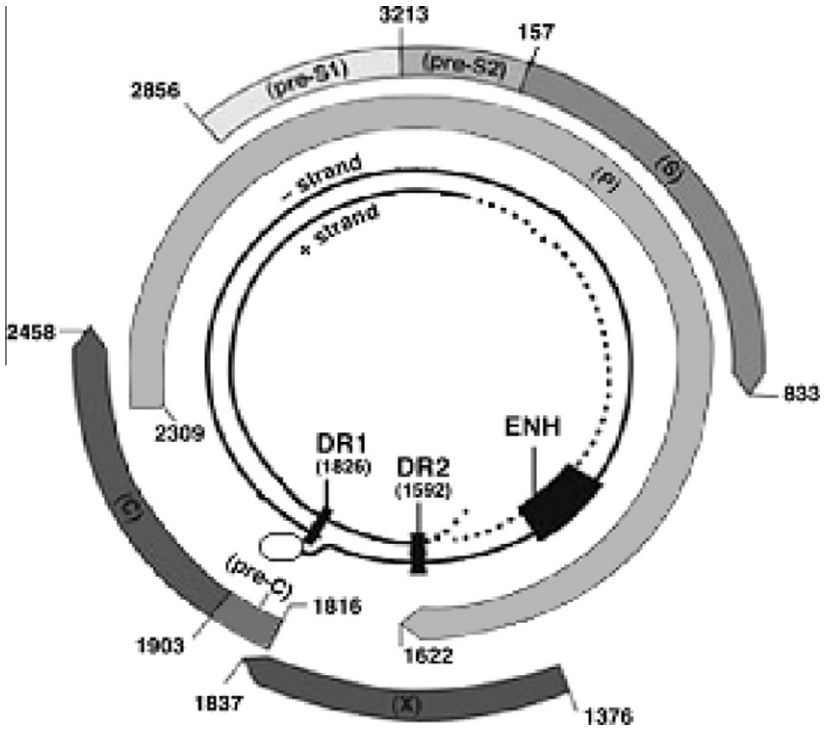

Fig. 1 – Schematic molecular organisation of the HBV genome. The four open reading frames (ORF), encoded by the viral genome and designed as Pre-S1/Pre-S2/S (surface), C (core), P (polymerase) and X, encompass the minus (–) and plus (+) DNA strands. The nucleotide numbers represent the limits of each ORF on the genomic map. The map positions of the viral direct repeats (DR1, DR2) and the enhancers (ENH I-II) are also indicated.

matter of debate. The Pre-S2/S genes encode for C-terminally surface proteins that exhibit a regulatory function. HBx protein is a multifunctional regulator of viral and cellular genes interfering with viral replication and proliferation.[2] The regulatory HBx and Pre-S2/S proteins, generated from integrated delete viral sequences, are involved in hepatocyte transformation. In fact, HBx and truncated Pre-S2/S have been shown to be efficient transactivators of cellular and viral genes implicated in signal transduction pathways, cell cycle control and transcriptional regulation.[6,7] Finally, the viral DNA polymerase-reverse transcriptase, encoded by the P gene, has a strategic importance for viral replication and has recently been identified as a target for antiviral drugs.[8]

After hepatocyte infection, viral nucleocapsids are transported into the nucleus, where the relaxed circular DNA (rcDNA) of HBV genome is converted into a covalent closed circular DNA (cccDNA). The cccDNA molecules serve as transcriptional templates for synthesis of new pregenomic RNA (pgRNA). The replication cycle of HBV involves the transcription of the pgRNA intermediate from the cccDNA by host cell RNA polymerase II, followed by reverse transcription of the pgRNA template within the nucleocapsid. Then, the nucleocapsid, which also contains the core and polymerase proteins, is coated with the surface antigen envelope proteins and released from the hepatocyte (Fig. 2).[9]

Functional regulatory elements, placed along the entire genome, are involved in the expression of each HBV gene. Thus, HBV genomic expression is regulated by two enhancers (Enh I and II), four promoters (BCP, Pre-S1, Pre-S2/S and X) and negative regulatory elements (NRE). The promoters control the corresponding four mRNA differing in the extent: 3.5 Kb (Pre-C/C and pg RNA), 2.4 Kb (Pre-S1), 2.1 Kb (Pre-S2/S), 0.7 Kb (X), whereas the genomic structures, involved in the regulation of the viral replication, are the post-transcriptional regulatory elements (PRE), the polyadenylation signal and the encapsidation signal (epsilon).[2]

1.2. *HBV infection and liver carcinogenesis*

Chronic HBV infection is the most common cause of hepatocellular carcinoma (HCC) worldwide. It has been estimated that more than half of HCC is related to HBV, and patients carrying HBsAg have a higher risk to develop HCC as compared to non-infected people.[10] HBV exerts its oncogenic potential through a multi-factorial process, which includes both direct and indirect mechanisms that likely act synergistically.[11] Liver cirrhosis itself, resulting from sustained inflammatory damage and hepatocyte regeneration, has been considered as a pre-neoplastic condition.[12] HBV DNA sequences are frequently found to be integrated in the host genes encoding for proteins related to the control of cell signalling, cellular proliferation and viability. This leads to a cascade of interactive events that ultimately transforms normal hepatocytes into malignant cells.[13] The transactivating potential of several viral oncoproteins, such as HBx and the truncated Pre-S2/S, on the regulatory cellular pathways is a further crucial oncogenic consequence of the integration process (Fig. 3).[7]

Due to spontaneous errors in viral reverse transcription, variations along HBV genome occur naturally. These mutations that arise during the course of HBV chronic infection

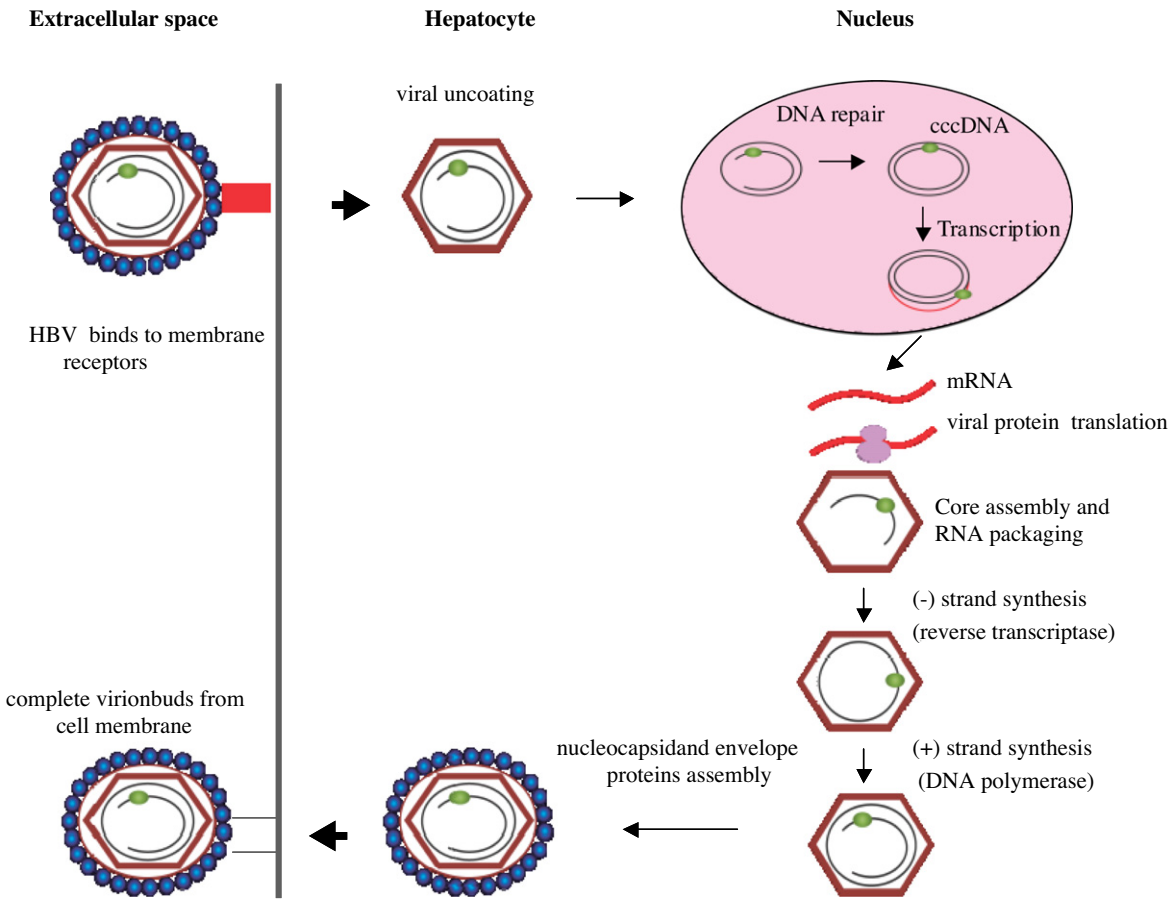

Fig. 2 – HBV replication pathway.

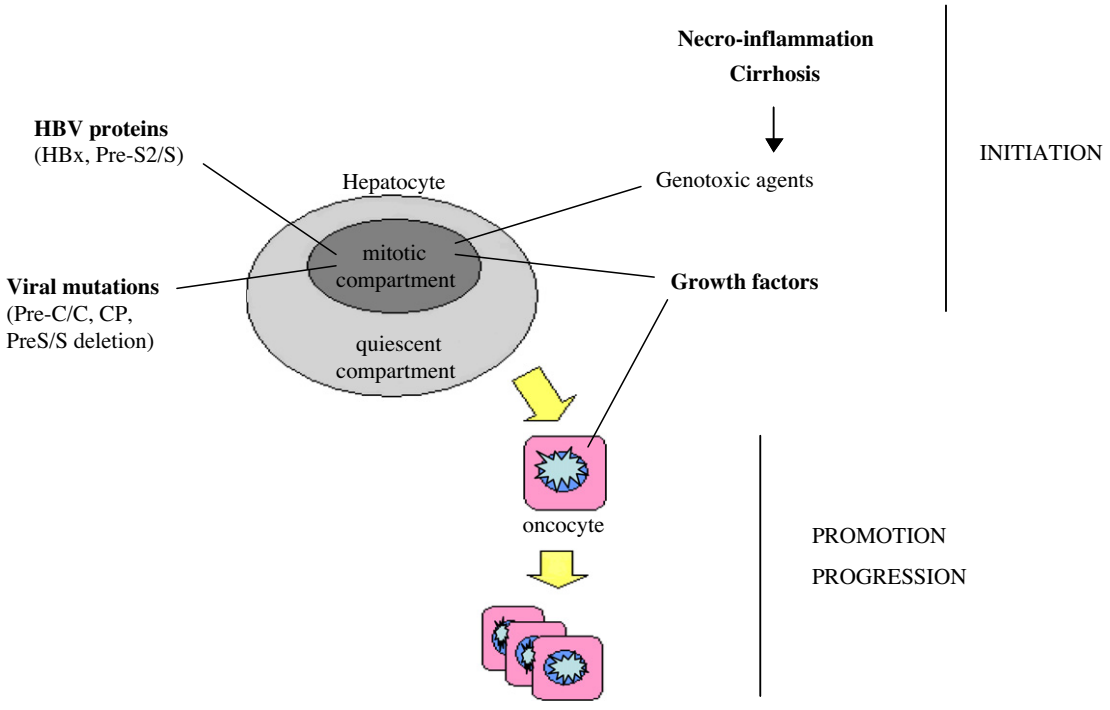

Fig. 3 – Schematic representation of long-term process of liver carcinogenesis (initiation, promotion and progression): interacting effects of viral factors and host cell genome.

have consequences at both clinical and epidemiological level. In fact, several HBV mutant strains including mutations in pre-C/C, core promoter and deletion in pre-S/S genes are involved in the pathogenesis of progressive liver disease and HCC development (Fig. 3).[14]

From phylogenetic analyses, eight HBV genotypes (A–H) have been identified based on the variation of the entire genome.[15] There is growing evidence suggesting that viral genotypes may influence the clinical outcome of HBV infection including its persistence, viral replication and HCC risk.[16]

The occult HBV infection, characterised by the presence of HBV DNA in serum and/or liver, despite undetectable circulating HBsAg with or without HCV co-infection, occurs in a spectrum of clinical settings.[17] The virological features and the clinical evolution of occult HBV infection are still not completely defined, but recent data showed that this condition may have an intriguing oncogenetic potential.[18]

2. Cirrhosis: indirect pathway to liver cancer

In Western countries, the majority of HCC arise in the context of liver cirrhosis, clearly suggesting that cirrhosis is the most important risk factor of HBV-related HCC.[19] There is convincing evidence that the incidence rate of HCC is about fivefold higher among infected patients with cirrhosis than in HBV asymptomatic carriers, suggesting that cirrhosis is a pre-neoplastic condition *per se*.[20] The strong association between cirrhosis and HCC suggests a hepatocarcinogenic process that is largely mediated by inflammation, leading to repeated cycles of cell death and regeneration that increase hepatocyte proliferation turnover.[21] The sustained stimulation of liver cell to progress towards the cell cycle can ultimately overcome DNA repair mechanisms in the presence of mutational events. The accumulation of critical variants in the host genome may heavily contribute to transformation of hepatocytes into malignant clones and the cells, designed to the elimination through the apoptosis program or immune response, will become fully transformed.[22] Concurrently, liver fibrosis disrupts the architecture of hepatic structure. As a consequence, cell-to-cell interactions are modified, and this ultimately leads to loss of control over cell growth. Thus, the persistent inflammatory changes, caused by chronic infection, promote liver cancer development through an integrated multi-step process (Fig. 4).

3. Viral factors and host genome: interacting effects in liver carcinogenesis

HBV-related HCC can also arise in the absence of significant liver damage. This mainly occurs in the Asian world, and indicates that cirrhosis, despite its crucial importance, does not represent an absolute requirement for HCC development, and suggests a direct effect by the virus or viral products on liver carcinogenesis.[23,24]

3.1. *HBV DNA and host genome interaction: oncogenetic role of HBx and truncated Pre-S2/S viral proteins*

It is well known that the HBV DNA genome is able to integrate into the cellular chromosomal DNA, causing both viral and host genome rearrangements.

Several studies have shown that HBV DNA insertion into cellular genes is frequent and can occur in genes encoding for proteins that are crucial for the control of cell signalling, proliferation and apoptosis.[25] HBV DNA integration also enhances the host chromosomal instability, leading to large inverted duplications, deletions and chromosomal translocations.[26] Many of these chromosomal segments contain key players in liver carcinogenesis such as p53, Rb, Wnt/β-catenin, cyclins A and D1, TGFβ and Ras signalling.[27] Up to now, however, a preferential integration site for HBV DNA into the infected cell genome has not been identified.

Another oncogenetic mechanism following HBV DNA integration is represented by the transactivation ability of a family of regulatory proteins, HBx and Pre-S2/S. The X gene encodes for 154 amino acid protein well conserved among hepadnaviruses. The x protein has been regarded as a multifunctional viral regulator, which is able to modulate a wide

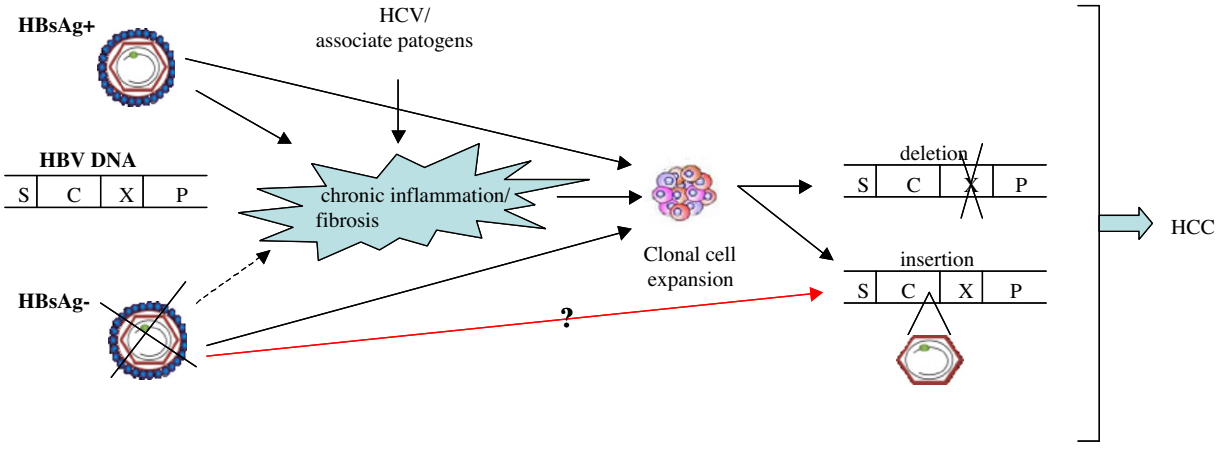

Fig. 4 – Oncogenetic potential of HBV genome in clear (HBsAg+) and occult (HBsAg–) infections. Both conditions contribute to HCC development through the clonal cell expansion and genome rearrangements (deletions), whereas, in occult infection, the low replicative activity of HBV not seems directly involved in chronic inflammation and liver fibrosis, as well as, viral integration into liver cells seems a rare event.

variety of host functions. The biological properties of HBx, as a transcriptional regulator of viral and host promoters, have been demonstrated both *in vitro* and *in vivo*.[28,29] The exact function of HBx during HBV replication is still undefined. However, even though it does not seem to be crucial for HBV viral life cycle, it plays a relevant role in viral replication by up-regulating the expression of HBV genes through the transactivation of both viral promoters and cellular genes.[30]

HBx interferes with signalling cascades upstream from the transcription complex including Ras-Raf-mitogen-activated protein kinase (Ras-Raf-MAPK), extracellular signal-regulated kinase (ERK), JAK/STAT, activator protein-2 (AP-2), nuclear factor kappa B (NF-κB) and cellular calcium signalling pathway.[31–35] Furthermore, it acts as a transactivator to a wide range of cellular and viral genes, including promoters, enhancers, proto-oncogenes (c-*myc*, c-*jun/fos*) and cytokine-encoding genes (including TNF-α, TGF-β) is able to modulate apoptosis, cell proliferation and DNA damage repair.[36,37]

Experimental evidence has been provided that the functional inhibition of the tumour suppressor protein p53 has been involved in cell transformation. In fact, tumour development correlates with HBx binding to p53 in transgenic mice expressing HBx.[38] Such binding resulted in a functional p53 inactivation during the oncogenetic process. Thus, the carcinogenic potential of the inhibition of p53 transcription may consist of failure in the transcription of genes involved in cell cycle block and apoptotic pathway.[39] HBx also inhibits the caspase-3 and anti-Fas antibody-dependent apoptosis and interferes with the DNA repair mechanism. At last, it modulates the degradation of cellular and viral proteins by inhibiting serine protease inhibitors and proteosome complexes.[30]

The other group of HBV regulatory proteins is represented by the middle surface proteins (MHBs) encoded by pre-S2 and S genes combined. To generate the transactivating forms of the MHBs, a deletion of at least 87 C-terminal amino acids is required.[40] Specific binding and activation of mitogen-activated protein kinases (PKC) signalling by the truncated pre-S2/S protein results in a permanent activation of the c-Raf-1/MEK/ERK (extracellular signal-regulated kinase) signal transduction cascade, which can ultimately exert a tumour promoter-like function by enhancing hepatocyte proliferation.[41] Furthermore, the overproduction of HBV envelope proteins results in their intracellular accumulation, and may provoke a cellular stress potentially leading to hepatocyte transformation.[42]

3.2. *Viral factors: oncogenetic potential of viral load, genotypes and genomic mutations*

Increasing attention has recently been addressed on the impact of viral load, HBV genotypes and genomic heterogeneity on the evolution of chronic liver disease. The serum viral load correlates with the risk of progression to cirrhosis, and high HBV viremia and serum aminotransferases may favour HCC development through a sustained inflammatory activity.[43] A recent report on a large cohort of HBV patients from China showed that high serum HBV DNA levels strongly predicted the development of HCC, irrespective of HBeAg seropositivity, serum aminotransferases and presence of cirrhosis. Interestingly, the spontaneous decline of viremia was associated with a reduced HCC risk with respect to patients who maintained high viral load.[44]

Other HBV genomic characteristics such as genotypes, protein expression and selected mutations, appear to exert an additional effect on cancer promotion. Hepatitis B virus is classified into eight genotypes (A–H), based on an intergroup divergence of 8% or more in the complete nucleotide sequence, which show variant geographical and ethnic distribution. Genotypes A and D are ubiquitous, even though they frequently occur in Africa, Europe and India, while genotypes B and C are prevalent in Asia. Genotype E is found in sub-Saharan Africa, as well as in France and Britain, likely due to immigration. Genotypes F and H are mainly found in South and Central America. Little is known about the distribution of genotype G.[15]

Naturally occurring mutations in different genotypes have been identified in both structural and non-structural genes, as well as regulatory sequences of the virus. In the past few years, it has been suggested that HBV genotypes may influence the severity and the outcome of liver disease, the HBeAg seroconversion rates, the mutational patterns in the pre-core and core promoter regions, and the response to antiviral therapy.[45] The results of these studies, however, are controversial. In fact, several reports suggested that genotype A more often leads to chronic disease than genotype D,[46] while a Spanish group described a significantly higher spontaneous viral clearance in patients infected by this genotype than in carriers of other genotypes.[47] A study from Japan suggested that genotype C would progress more rapidly to cirrhosis and HCC than genotype B, but the mechanisms through which the infecting HBV lineage influences HCC evolution remain unknown.[48]

On the other hand, a relationship between HBV genotypes and mutations in the pre-core/core promoter regions has been clearly demonstrated. The most common pre-core mutation consists of a G to A substitution in position 1896 leading to a premature stop codon that abolishes or reduces the production of 'e' antigen.[4,5] It evolved in genotypes B, C and D but not in genotype A, and this accounts for the high prevalence of HBeAg-negative chronic hepatitis in Southern Europe and Asia. The background for the genotype-dependent selection of the pre-core G1896A mutation is related to the need to maintain the base pairing of the stem loop structure of the pregenome encapsidation sequence.[49,50]

In the multi-factorial process through which HBV infection contributes to the development of HCC, viral genome heterogeneity introduces a further complex variant. Like the RNA viruses, HBV utilises a reverse transcriptase step in the replication of viral genome. Because of the occurrence of spontaneous errors in viral reverse transcriptase, chronic HBV infection is associated with the emergence of mutations that randomly occur along the viral genome, and generate different viral populations. The potential for HBV to alter its genome is substantial. Several genetic variants have been found to be associated with HCC, and this may imply that such variants can influence the mechanisms underlying hepatocarcinogenesis. Selective mutations in the HBV pre-C region have been found in tumorous liver tissues, suggesting that HBV variants play a role in the persistence of the virus in tumoral cells and favouring HCC development.[51] These mutations

affect the production of HBeAg and lead to the emergence of HBV strains that have a translational stop codon mutation at codon 28 in position 1896 (from TGG to TAG) of the Pre-Core gene. This position contains the epsilon signal structure which is essential for pregenome encapsidation and the start of HBV DNA synthesis.[52–54]

Further mutations in regulating HBV genome sequences and important loci of overlapping genes related to viral function have been identified. There are convincing data that support an association between core promoter variants and more severe liver disease. In fact, the prevalence of core promoter mutations was higher among chronic hepatitis B patients who developed complications of cirrhosis and HCC.[55] The double mutation exhibited in the basal core promoter (BCP) at positions A1762-G1764 is associated with a reduction of the Pre-C/C mRNA, and leads to a significant decrease of HBeAg levels and enhanced viral replication.[14] The BCP double variant, alone or in addition to other pre-core mutations, may increase the risk of liver disease progression and HCC development for both genotypes B and C infection.[56] Thus, the association of high viremia levels, viral genotype and pre-C/C and BCP heterogeneity may play a synergistic role in the oncogenic process.

In addition to these common mutations, other nucleotide variants, that is C1653T in the Enhancer II (Enh II) region and the T to C/A/G at position 1753 in the core promoter region, have recently been identified as potential biomarkers of HCC development.[57] A synonymous T1936C mutation was found in HBV/HCV co-infected HCC cases, suggesting that HCV co-infection is associated with specific HBV variants that potentially accelerate the progression to HCC.[50] Furthermore, because the HBV core promoter region overlaps with X sequence, mutations in this domain not only involve the regulatory elements that control viral replication, but also potentially enhance its transactivating ability. In addition, all deletions/insertions in the BCP shift the X gene frame and lead to the production of truncated x proteins. The active replication of the shortened HBV variants might also be implicated in liver carcinogenesis.[58] When HBx deleted mutant plasmids were transfected into murine and human cell lines, an accelerated cell cycle progression and a synergic promotion of the *ras* and *myc* transforming capacity were found. Therefore, a COOH-terminal deletion may alter the balance of HBx functional domains in regulating cell proliferation, apoptosis, viability and transformation.[59]

Finally, the intracellular accumulation of viral variants, likely occurring during continuous cell cycle division, and offering an advantage to the fitness of the virus, may lead to malignant transformation of some hepatocytes through a multistage process.[60]

4. Occult HBV infection: putative role in liver oncogenesis

A peculiar aspect of chronic HBV infection is represented by the persistence of HBV genomes in the absence of circulating HBs antigen. This so called 'occult' infection can occur not only in individuals with anti-HBs and/or anti-HBc antibodies but also in those who are negative to HBV markers.[61] Extensive studies have demonstrated that occult HBV infection represents an entity with clinical relevance as risk of transmission through organ transplantation, blood transfusion, perinatal transmission, acute exacerbation or even fulminant hepatitis after immunosuppression or chemotherapy.[62,63] Occult HBV infection has a worldwide diffusion, and its distribution is related to the prevalence of HBV infection in different geographical areas. Its prevalence is also high in certain patient populations, such as those who are chronically infected by HCV and those affected by cryptogenic liver disease and HCC.[64,65]

The awareness of occult HBV infection emerged following the development of molecular sensitive technology that was able to detect very low levels ($<10^3$ viral genomes per millilitre) of HBV DNA in the serum samples and/or in the liver. However, the detection of HBV genomes in the liver tissue remains the most truthful way to identify the occult infection, and this may strongly limit the impact of viral persistence on real incidence, viral viability and pathogenic consequences.[66] Despite a low replication rate, silent HBV detection can be associated with increased cytonecrotic activity and advanced liver diseases. The mechanisms leading to occult HBV infection remain poorly understood. Occult infection results from a multi-factorial process, likely involving both host and viral factors. For instance, the host immune response may play a role in maintaining low levels of intrahepatic HBV replication and transcription.[63] Among the viral factors, mutations which may alter HBsAg production and negatively influence viral DNA multiplication, co-infection with HCV and presence of circulating deleted viral particles might affect the replication rate of HBV. The HBV DNA variants could act by modifying the antigenicity of viral proteins. Namely, rearrangements in the pre-S1 and S genes have been associated with reduced HBsAg expression, changes in the X gene might affect viral replication fitness, mutations in the overlapping Core promoter region may influence the low replicative potential and variants in the and Pre-core/Core sequences may reduce HBV replication efficiency through the epsilon signal functional structure that is essential for pregenomic encapsidation and starting HBV DNA synthesis.[53,67–69]

Over the last years, several reports have indicated that occult HBV infection has a clinical impact. In fact, it seems to accelerate the progression of liver disease and the development of cirrhosis.[64] At present, it is widely accepted that occult HBV persistence is an important risk factor for liver cell clonal expansion and HCC development. This association was suggested by epidemiological and molecular studies, and supported by animal models. The HBV genome has been detected in tumour tissue of HBsAg negative patients with HCC in a prevalence ranging from 30% to 80%.[70,71] Studies on the rodent models showed that the HBV infection has an increased risk of developing HCC, despite the apparent clearance of the virus by serological tests.[72]

Moreover, a high proportion of HCV-related HCC cases showed occult HBV infection suggesting that an interplay underlying HCV/HBV occult co-infection might contribute HCC development, as it occurs in clear HCV/HBV co-infection.[50,71,73] *In vitro* experiments revealed that HCV core proteins suppress HBV expression in cell cultures, thus potentially favouring the occult status (Fig. 4).[74]

Occult HBV strain populations harbour a genetic heterogeneity in viral regions (Pre-S/S, Pre-Core/Core; X, Polymerase) and regulatory elements (Core promoter, Enhancer I and II) potentially involved in viral replication and/or gene expression. However, point variations or deletions, a particular genotype or a pattern of changes able to predict oncogenic transformation, remain to be identified. At first, the mechanism by which occult HBV infection may promote HCC development would seem to depend on the viral DNA integration into the hepatocyte genome. However, the intrahepatic persistence of HBV cccDNA replicative intermediate suggests that the occult status has to be referred to a low replication rate rather than integration capacity.[75]

On the other hand, in a recent study carried out in Taiwan, multiple genetic variants in the Pre-S2 (M1I and Q2K) and in Enhancer II (G1721A) domains of viral genome have been found among HCC carriers of HBV occult infection compared to HCC with clear HBV infection. This pattern of mutations, distinctive of the occult status, was proposed as viral marker for HCC in occult HBV carriers that may aid in the identification of the cases HBsAg – with progressive chronic hepatitis and high risk of HCC development.[76]

5. Conclusion

The pathobiological effects of HBV infection on HCC development is indisputable but there is still a long way in understanding the process behind liver carcinogenesis. The biological heterogeneity of HBV, the complex interactions between the virus and host cells and the random occurring of the oncogenetic events slow down the identification of key molecular mechanisms that lead to liver cell transformation. The occult HBV infection, represented by the persistence of the viral DNA in absence of circulating hepatitis B surface antigen (HBsAg), is an underhand factor for liver cell clonal expansion. Since the absence of substantial genomic differences in the infecting strains between occult and clear HBV infections in patients with HCC suggested that occult virus maintains the oncogenetic properties implicated in the hepatocyte transformation.

In spite of the plethora of data on the potential diagnostic markers or pre-emptive target of hepatocarcinogenesis, studies should continue in the search of functional analysis of mutated HBV strains, viral and cellular genes and proteins. This knowledge is essential to better understand the mechanisms that influence the progression of liver disease to HCC promoted by both clear or occult infections.

Conflict of interest statement

None declared.

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