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REVIEW

Hepatocellular carcinoma: the point of view of the hepatitis B virus

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Hepatitis B virus (HBV) infection is the main risk factor for hepatocellular carcinoma (HCC) development, as suggested by many epidemiological and molecular studies (1–13) and as dramatically confirmed by data from Taiwan where the universal childhood vaccination program against HBV determined a striking reduction of new infections in infancy and a parallel decrease of liver cancer incidence in childhood (14). Despite the availability of a very efficacious vaccine, however, HBV infection is still a major health problem worldwide, with an estimate of ∼400 million chronic carriers of the HBV surface antigen (HBsAg), many of whom suffer from progressive forms of liver disease and show a high propensity to develop HCC. Furthermore, when other risk factors for HCC development—such as hepatitis C virus (HCV) infection, Aflatoxin B_1 exposure, alcohol abuse and metabolic factors as obesity and diabetes—coexist with HBV infection, a considerable increase of the relative risk for cancer development occurs, probably due to a synergic pro-oncogenic effect of the different factors (13,15–19). Consequently, the World Health Organization includes HBV in 'group 1' human carcinogens classifying it among the most important oncogenic agents after tobacco smoking.

HCC development underlies complex and multifactorial pathogenetic mechanisms. Much evidence indicates that HBV exerts its pro-oncogenic properties playing a role in many of these mechanisms. Moreover, this virus seems to maintain its pro-oncogenic role also in cases with persistence of viral genomes in the liver of individuals who are negative for circulating HBsAg (namely, 'occult' HBV infection) (20). Here, we review the different aspects of HBV involvement in heapatocarcinogenesis.

Virological aspects

HBV belongs to the Hepadnaviridae family, comprising hepatotropic DNA viruses able to infect mammalian (orthohepadnaviruses) and avian (avihepadnaviruses) hosts and sharing with HBV most of the genetic structure and replicative characteristics (Table I) (21–24). HBV is one of the smallest viruses in nature and its genome presents a highly compact genetic organization. It consists of a partially double-stranded relaxed circular DNA of ∼3200 nucleotides in length and contains four partially overlapping open-reading frames (ORF): *preS/S*, *preC/C*, *P* and *X*. The *preS/S* ORF encodes the three viral surface proteins: the preS1 (or Large), the preS2 (or Middle) and the S (or small) that corresponds to HBsAg. The *preC/C* ORF encodes the core antigen (HBcAg) and the soluble antigen 'e' (HBeAg). The P ORF encodes the terminal protein and the viral polymerase that possesses DNA polymerase, reverse transcriptase and RNaseH activities. The X ORF encodes the regulatory X protein, which is essential for virus replication and is capable of trans-activating the expression of numerous cellular and viral genes (25) (Figure 1).

Abbreviations: BCP, basal core promoter; DNMT, DNA methyltransferase; ER, endoplasmic reticulum; HBV, hepatitis B virus; HBsAg, hepatitis B virus surface antigen; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IGF, insulin-like growth factor; GGH, ground-glass hepatocytes; miR, microRNA.

The replication cycle of HBV presents very peculiar characteristics that are schematically summarized in the Figure 2 (26). HBV—as well as some plant viruses like Cauliflower mosaic virus—has been classified as a pararetrovirus because of some similarity with retroviruses. In fact, HBV—although a DNA virus—replicates through the reverse transcription of a pregenomic RNA representing its intermediate replicative form (27,28). Similarly to retroviruses, HBV DNA can integrate in the genome of the host hepatic cells, an event considered to have a primary role in the pro-oncogenic activity of the virus (29–31). However, unlike what happens for retroviruses, integration has no role in the replicative cycle of HBV, which does not produce any protein with integrase enzymatic activity, the integrative process being most probably mediated by the activity of the cellular topoisomerase I (32).

The complex and peculiar HBV life cycle, its strong replication activity (up to 100 billions virions per day) and the lack of proofreading properties of the viral polymerase lead to the higher genomic variability of HBV compared with other DNA viruses (33) Furthermore, since HBV—and particularly, its covalently closed circular DNA—can persist for decades (perhaps indefinitely) in an infected individual, it is clear that a considerable amount of genetic mutations, either spontaneously occurring during viral replication, selected under the host's immune pressure or therapeutically induced by immunoprophylaxes (vaccine, anti-HBs immunoglobulins) or by specific antiviral therapies, may accumulate in the HBV genome, determining the emergence of viral strains with new biological characteristics and different replicative and pathogenetic abilities (34).

HBV variability and HCC

Eight different HBV genotypes have been recognized so far (named with capital letters from A to H) on the basis of a divergence of >8% in the nucleotide sequence of the whole genome (34). HBV genotypes have a different geographic distribution with a predominance of genotype A in North-Western Europe, North America and South Africa, genotypes B and C in highly endemic Asiatic areas and genotype D in the Mediterranean basin and Eastern Europe (35). The remaining genotypes are less widespread in more restricted geographic areas: genotype E is found in West and South Africa, genotypes F and H in Central and South America and genotype G has been detected in France and the USA (35).

Interest in the possible influence of HBV genotypes on the clinical evolution of the infection and particularly on the risk of HCC development has grown in the last few years (35–37). Most of the studies in this field have been conducted in the Far East, thus on patients infected with genotypes B and C, showing that genotype C is associated with a more aggressive disease and a greater progression towards cirrhosis than genotype B (38,39). On the contrary, investigations into the relationship between these two genotypes and HCC have provided conflicting results. Studies in Japan, Taiwan and Hong Kong support the theory of a higher risk of HCC development with genotype C rather than genotype B (40–43), whereas cohort studies in the same geographic areas failed to demonstrate differences in HCC prevalence with respect to these two genotypes (44,45). Furthermore, a case–control study in Taiwan suggests that genotype B is associated with HCC when it develops in young patients, whereas genotype C is associated with HCC in older patients (46). Considering the discrepancies among the available data and the very little information concerning other HBV genotypes and HCC, it appears evident that the problem of the hypothetic influence of genotypes on HCC development is still far from being solved.

Even more intriguing are the studies concerning a possible pro-oncogenic role played by peculiar HBV variants. In fact, the genetic variability of viral isolates from tumour tissues of patients with HCC has been extensively investigated (47–55). Although definitive

Table I. Hepadnaviridae family

Virus	Natural host	Infection/disease
Genus *Ortohepadnavirus*		
Hepatitis B Virus (HBV)	Man, chimpanzee, gibbon, woolly monkey	Inactive infection, hepatitis, chirrosis, HCC
Ground squirrel hepatitis B virus (GSHV)	Californian squirrel, Pennsylvania's woodchuck, chipmunk	Inactive infection, hepatitis, HCC
Woodchuck hepatitis virus (WHV)	Pennsylvania's woodchuck	Inactive infection, hepatitis, HCC
Genus *Avihepadnavirus*		
Duck hepatitis B virus (DHBV)	Pekinese duck, goose	Inactive infection, hepatitis
Heron hepatitis B virus (HHBV)	Heron	Inactive infection, hepatitis

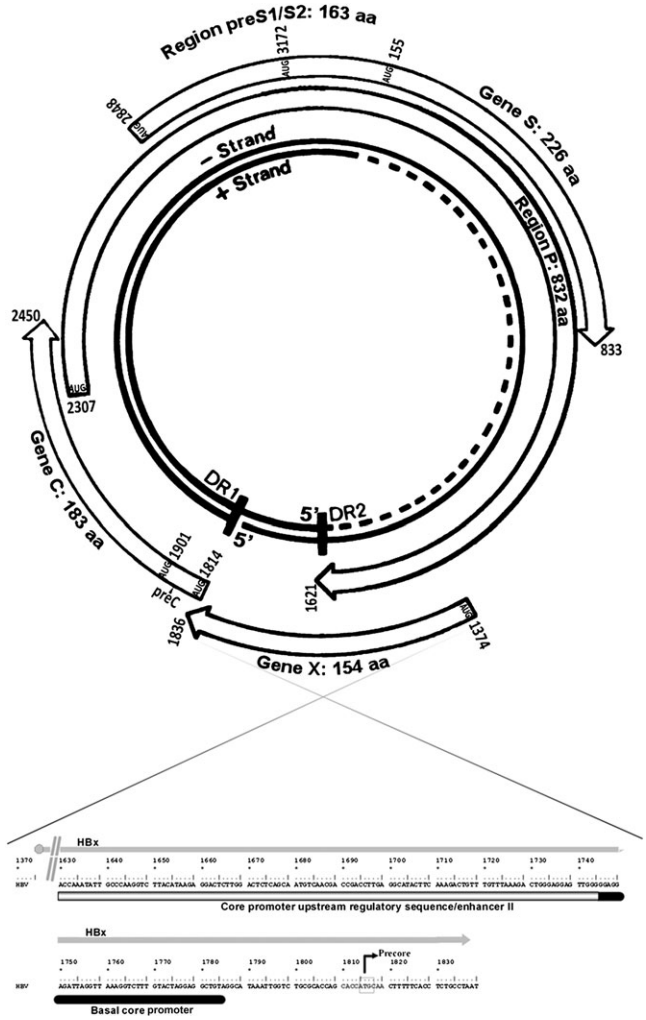

Fig. 1. Schematic representation of the HBV genome structure. In the bottom, overlap between BCP region and X gene is highlighted.

evidence has not been obtained, the available data on the correlation of HBV variants carrying mutations at the level of the basal core promoter (BCP) and/or of the X and preS viral genomic regions and HCC development are of great interest and worthy of discussion.

BCP mutations determining the substitution of a thymine with an adenine at position 1762 (T1762A) and of a guanine with an adenine at nucleotide 1764 (G1764A) appear to be the most frequently detected in HCC patients. These HBV mutants show a more efficient replicative activity than the wild-type viral strain in experimental conditions (56–62). However, clinical investigations failed to find any difference in serum HBV DNA levels between patients infected with HBV carrying BCP mutations and patients infected with the wild-type viruses (63–68). The only effect of BCP mutations confirmed in clinical studies is the 'down-regulation' of HBeAg production, an event that usually anticipates the seroconversion to anti-HBe (69–72). Many reports from different geographic areas reveal a strong association between HCC and infection sustained by BCP-mutated HBV (41,55,73–77). However, a certain number of studies have questioned this association because of the highly frequent occurrence of BCP mutations in HBV chronic carriers with different clinical pictures (54,66). Moreover, no *in vitro* study has yet provided any evidence concerning the possible tumorigenic effects of BCP-mutated HBV strains. However, it must be remembered that BCP completely overlaps the X gene and that the T1762A and G1764A mutations are the cause of the K130M and V131I amino acid substitutions in the X protein (Figure 1), which is strongly suspected to play a key role in the oncogenic property of HBV (see below). Therefore, it is possible that involvement of such mutations in cellular transformation is related to the modification of biological characteristics of the X protein more than to the effect exerted on the core promoter activity. In this context, there is evidence that mutated HBx of viruses isolated from patients with HCC are able to stimulate cell proliferation and determine neoplastic transformation when expressed *in vitro* (78,79). It has been shown that specific HBX mutations are able to abrogate the pro-apoptotic and antiproliferative effect of the 'wild-type' protein, suggesting an important role of these HBX mutants in the first steps of the hepatocarcinogenic process (80). Of particular interest are the data from a recent study showing that the expression of a C-terminal deleted HBX is associated with the up-regulation of Wnt-5a gene expression in Huh7-transfected cells as well as in human HCC tissues but not in the corresponding non-cancerous liver tissues (81).

The first studies on the clinical meaning of HBV genetic variants already provided evidence on the association between infection with preS1- and/or preS2-mutated HBV strains and HCC (78,82–91). These HBV variants are more frequently preS2 defective because of mutations at the level of the preS2 start codon and/or large in frame nucleotide deletions or—less frequently—preS1 defective because of large in frame nucleotide deletions in the preS1 genomic region (Figure 3). There is evidence showing that infections with preS1/preS2 HBV variants lead to the retention of surface viral proteins in the endoplasmic reticulum (ER) of hepatocytes and to the induction of ER stress (92,93), oxidative stress and DNA damage, events that are responsible for severe liver injury and that predispose to hepatocyte transformation, as recently confirmed in experimental studies using a transgenic mice model (94).

The clinical relevance of these HBV variants has been recently confirmed by an observational cohort study showing that patients infected with preS-defective viruses have a significantly higher risk of HCC development than patients infected with the wild-type virus, during a 10-year follow-up (95).

Additional aspects of the pro-oncogenic role of X and preS-defective variants will be further discussed below. Regarding HBV genetic heterogeneity and HCC in general, however, it has to be underlined that very few studies have dealt with the clinical impact of 'complex HBV variants' carrying various combined mutations in different genomic regions (96–99); the functional and biological characteristics, together with the pathogenetic and pro-oncogenic capabilities of these complex HBV variants, remain totally unexplored.

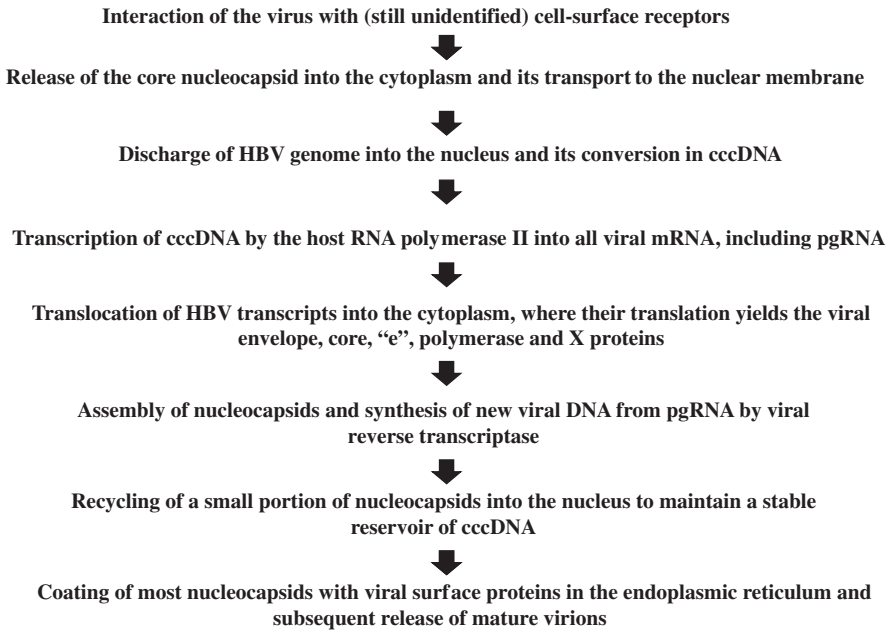

Fig. 2. Schematic representation of the HBV life cycle main steps.

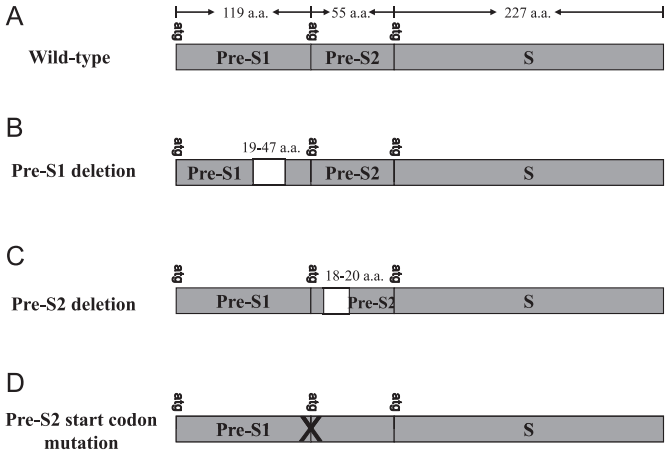

Fig. 3. Schematic representation of the genomic region encoding the preS/S proteins. (**A**) Wild-type sequence. (**B**) Frequent in frame preS1 deletion responsible for the production of a shorter preS1 protein. (**C**) Frequent in frame preS2 deletion responsible for the production of a shorter preS2 protein. (**D**) PreS2 start codon mutation preventing the synthesis of the corresponding protein.

HBV replicative activity and HCC

Chronic HBV infection can show very different—and variable overtime—virological and clinical profiles. Very schematically, we can find conditions of intense viral replication and high serum HBV DNA levels that usually occur in HBeAg-positive individuals, less frequently in subjects positive for the corresponding antibody (anti-HBe). Anti-HBe positive status is more often characterized by suppression of viral replication and low serum HBV DNA levels. This suppression may be persistent overtime ('inactive' infection) or may present periodic viral reactivation with a pattern of fluctuating levels of HBV DNA and aminotransferases and active hepatitis (100). HBV infection may occur also in HBsAg-negative subjects, who generally have low amounts of viral DNA frequently detectable only in the liver, HBV covalently closed circular DNA being a replicative intermediate persisting indefinitely in the nuclei of hepatocytes also in cases with strong suppression of viral replication ('occult' infection, see below) (20). HBV seems to maintain its pro-oncogenic role in all clinical/virological situations, although literature data strongly support the presence of an out-and-out risk 'gradient' correlating HCC development with the replicative activity of the virus (101,102). In fact, several studies on large cohorts of patients demonstrated that HBeAg-positive individuals with higher serum HBV DNA levels have a significantly higher relative risk of HCC development than HBeAg-negative subjects with lower viraemia levels (103). Furthermore, there is evidence showing that serum HBV DNA levels persistently >2000 IU/ml represent an important predictive factor for HCC development also in HBeAg-negative subjects (43,102), whereas carriers of inactive HBV infection do not seem to be at risk of HCC development in the absence of other co-factors of hepatic damage (102,104–106). The pro-oncogenic relevance of the intense replicative activity of HBV raises the hypothesis that antiviral treatment inducing a persistent inhibition of viral activity may determine a significant reduction of HCC risk, especially if such therapy is started in the pre-cirrhotic phase. Although this hypothesis has not yet been confirmed by prospective clinical studies, it must be underlined that prolonged follow-up of patients treated with Interferon-alfa showed that subjects achieving a sustained viral response (persistent suppression of viral replicative activity) had a significant reduction of HCC development risk compared with non-responders (107,108). Analogously, there are data showing that patients successfully treated with Lamivudine have a lower risk of HCC development than non-treated or non-responder patients (109). All these data are strengthened by a certain amount of evidence showing that HBV does not seem able to induce cancer *per se*, either in inactive carriers or in occult HBV infection. The pro-oncogenic activity of HBV seems to be multifactorial and it might act through direct and indirect mechanisms, the latter represented by hepatic necroinflammation that is mild or absent in cases of persistently low viral replication. Probably, in most cases, both these mechanisms are needed to induce cancer. In inactive or occult infection, HBV can probably act as a co-factor of HCC development when a concomitant cause of liver injury is present, such as HCV infection or alcohol abuse (Table II).

Occult HBV infection and HCC

Occult HBV infection deserves particular mention since its potential pro-oncogenic role further emphasizes the strong connection between HBV and HCC worldwide.

Table II. Impact of the different phases of HBV infection on the oncogenic activity of the virus

Phases of infection	Pro-oncogenic role
Active (HBeAg positive)	Primary
Active (anti-HBe positive)	Primary
Inactive	Co-factor
Occult	Co-factor

Molecular epidemiological studies conducted since the early 80s showed almost unanimously that HBV persistence can play a critical role in HCC development also in occult HBV carriers (110–122). These data have been widely confirmed in animal models prone to infection by other hepadnaviruses. In fact, both woodchuck and ground squirrel, when infected with the corresponding Hepadnavirus (WHV and GSHV, respectively), are at high risk of HCC development even after apparent recovery from the infection with seroconversion from HBsAg to anti-HBs (123,124). Occult HBV in humans can probably represent a risk factor for HCC development only in case of concomitant other causes of hepatic diseases (122,125). These data are of great importance if one takes into account that HBV prevalence in the Caucasian American population is one of the lowest in the world. The strong association between occult infection and HCC development was confirmed by an observational cohort study evaluating a large number of HBsAg-negative patients (most of whom HCV infected) with chronic liver disease who had been tested for occult HBV and then followed up for >4 years (126). Occult HBV can persist in the hepatocytes both integrated into the host genome and as free episome, maintaining its transcriptional activity and ability to synthesize proteins, albeit at very low levels (121). In addition, there is evidence suggesting that occult infection may determine a mild but continuous status of chronic necroinflammation (110,127). Thus, it is believed that occult HBV infection can contribute to hepatocellular transformation through the same direct and indirect mechanisms usually attributed to the overt infection.

Pathogenesis

Necroinflammation and cirrhosis

HBV is not directly cytopathic and the induced liver injury essentially has an immune-mediated pathogenesis, related to the cytotoxic T lymphocytes response specifically directed against the viral antigens displayed on infected hepatocytes (26). However, there is evidence that an important role in determining hepatocellular damage is also played by the innate immunity, through the action of the natural killer cells, neutrophils and activated lymphocytes recruited by non-specific chemokines in the inflamed areas. These inflammatory cells release cytokines and chemokines capable of favouring cellular transformation and tumour growth (32,128).

Experiments on HBV-transgenic mice demonstrated that the immune-mediated hepatic damage is sufficient to determine HCC and that its development is avoided by the inhibition of both apoptosis and cytotoxic T lymphocytes-induced chronic inflammation, through Fas ligand (FasL) neutralization (18).

An additional factor that may play a role in HBV-related hepatocarcinogenesis is the peculiar natural history of chronic hepatitis B. In fact, the clinical–virological course of chronic hepatitis B is often characterized by alternation of phases of reduction of viral replication and gene expression with phases of re-exacerbation of viral activities and recurrence of immune-mediated hepatic injury. These flares trigger stimuli able to induce cell death and proliferation which, reiterated overtime, may cause the appearance of genetic alterations predisposing to cellular transformation (129–133). In fact, it is known that a high cellular proliferation index may be a risk factor for HCC development, particularly in cirrhotic livers (32,36) (Figure 4).

Cirrhosis appears to be an essential step for HCC development in patients with HCV or alcoholic liver disease (18). Although it is an important predisposing factor to HCC also in cases with HBV infection, literature data indicate that ∼20% of all HBV-related HCC develop in livers without cirrhosis and, in some cases, even without signs of chronic hepatitis (78,79). Although an uncommon event, the chance of tumour development in a normal or mildly damaged liver is considered further proof of the direct pro-oncogenic properties of HBV (Figure 4).

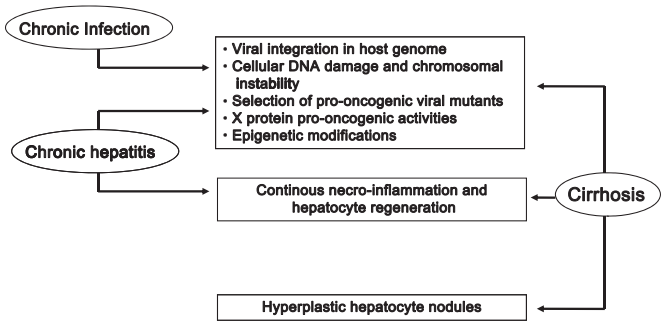

Fig. 4. Schematic representation of the molecular events associated with HBV hepatocarcinogenesis according to infection and liver disease status.

Furthermore, chronic hepatitis B is much more frequent in men than women and males are about three to five times more prone to develop HCC than females (134,135); this trend is even more pronounced in rodent HCC models (136,137). A recent study showed increased HBV transcriptional activity in the liver of male transgenic mice. In these animals, the enhancer I region of HBV is responsive to ligation-stimulated androgen receptor and this binding determines an increased transcriptional activity of HBV that might explain the higher viral DNA levels found in male HBV carriers and the consequent increased risk of HCC development (138). Of note, a recent population-based cohort study on Taiwanese mothers screened for HBV infection at each delivery from 1984 to 2008 demonstrated that the risk of HCC development was significantly higher in women with persistent HBsAg-positive status, but among the HBsAg-negative mothers those who underwent HBsAg sero-clearance during follow-up had a significantly higher risk of HCC development compared with HBV-unexposed women, indirectly confirming once more HBV's maintenance of its pro-oncogenic role also in the occult status (139). Also metabolic changes might contribute to liver damage and favour hepatocarcinogenesis in case of HBV infection. In particular, a deregulation of the insulin-like growth factor (IGF) axis—including the autocrine production of IGFs, IGF-binding proteins, IGF-binding protein proteases and the expression of the IGF receptors—has been described in hepatoma cell lines and during hepatocarcinogenesis due to various oncogenic agents, including Hepadnaviridae in transgenic mice and woodchucks (140–147). These data are consistent with an interplay between IGF axis and HBV infection, but this hypothesis needs further validation by *in vivo* studies.

HBV DNA integration

The ability of HBV to integrate into the genome of the infected host hepatocytes is considered one of the most important confirmations of its direct pro-oncogenic role. Integrated viral DNA has been found in 85–90% of HBV-related HCCs and its presence in tumours developed in non-cirrhotic livers of children or young adults appears to further support the role of viral DNA integration in the hepatocarcinogenesis process (148,149). However, unlike the woodchuck model where the WHV insertional activity in the *myc* family oncogenes is a crucial event for HCC development (150–154), HBV DNA integration occurs randomly in the context of human genomes and may involve multiple sites of different chromosomes (155). Thus, HBV seems to behave like an insertional, non-selective mutagenic agent and the important rearrangements of the host genome associated with viral integration suggest that its main oncogenic effect is the induction of a higher genomic instability (156). In fact, most of the integration events

reported in the literature occur near or within fragile sites or other repetitive regions of the human genome as Alu sequences and microsatellites that are prone to instability in tumour development and progression (157).

Recent studies on duck and woodchuck models infected with the corresponding Hepadnavirus allowed identification of the peculiar biological conditions and molecular factors possibly involved in the viral integration process (158,159). Summarizing, the available data suggest that during HBV infection, long-term chronic inflammation associated with continuous cycles of cell death and proliferation induces an increment in the amounts of DNA ends in host genomic DNA, thus favouring the process of viral integration. In this process, cellular topoisomerase I acting as endonuclease and transferase seems to play an important role in the linearization and integration of viral replicative intermediates (159,160). Furthermore, there is evidence demonstrating that some conditions modifying cellular homoeostasis may increase the frequency of insertional events. In particular, it has been shown that exposure to oxidative stress or mutagens and coinfection with other viruses may favour HBV DNA integration (159).

The integration of HBV genome into the DNA of the infected cells can be responsible, besides cellular genome alterations, also for ruptures and/or rearrangements of the viral DNA (52,79,159,160). Indeed, the integration of complete and structurally unaltered viral genomes have never been found in the genome of infected hepatocytes and the integrated viral sequences, showing deletions of large genomic portions, are replication incompetent and differ from each other in size and structure. However, it must be stressed that integrated HBV DNA might contribute to hepatocellular malignant transformation through the production of mutated viral proteins such as truncated X proteins or preS/S proteins which may activate signalling pathways implicated in tumorigenesis (trans-activation) (161–165). The insertion of viral DNA into cellular genomic regulatory regions or coding regions with consequent modification of gene expression (cis-activation) or structural and functional alteration of the produced cellular proteins is another possible consequence of HBV DNA integration (79,149,156,166–169). One study identified the cyclin A gene as the viral integration site (170). The product of such genomic recombination was the 'HBV-cyclin A' fusion protein, in which 152 amino acids at the N-terminal end of cyclin A were substituted by 156 amino acids of the Middle HBV protein. This fusion protein had strong tumorigenic properties (78,79,159,169,171,172). Another study described an HCC showing the insertion of viral DNA into the gene coding for the β retinoic acid receptor (173). In this tumour, the retinoic acid receptor β gene was mutated because of the integration of an HBV sequence comprising the cohesive region DR2, the core gene and the preS1 genomic region. The fusion protein resulting from this genomic recombination was overexpressed since it was synthesized under the control of the integrated preS1 viral promoter and showed carcinogenic potential (159,174). Among the numerous viral integration sites described one may also mention the tyrosine-protein-kinase domain of the epidermal growth factor receptor gene (175), the mevalonate kinase (176,177), the carboxypeptidase (178), the platelet growth factor receptor genes (31) and the telomerase reverse transcriptase gene (hTERT), which encodes the enzyme responsible for telomeres reconstitution in cellular immortalization process and that represents the first gene where HBV DNA integration was described in more than one case (30,79,159,179–181). In this context, a large scale study showed that the genes involved in signalling and control of cellular death and proliferation are frequent targets of HBV integration (31), although it has to be considered that for many HBV integrations, there are no experimental data able to definitively prove their role in hepatocellular transformation.

HBV proteins with pro-oncogenic activities

X protein. Most HBV-related HCCs show the integration of viral genomic sequences including the HBV X gene (HBx). The integrated forms of HBx are frequently rearranged and may show numerous point mutations, deletions or truncation with fusion to cellular DNA; but despite this integrated HBx might encode functionally active proteins with trans-activating ability (78,79,161). Although characterization of HBx expression in malignant hepatocytes and infected liver tissues has been often hampered by the difficulty in obtaining valid high-affinity anti-HBX antibodies for immunodetection (182), there is strong evidence demonstrating that the expression of HBx is maintained through the multistage process of hepatocarcinogenesis from preneoplastic nodules or foci of transformed hepatocytes to HCC (182–185).

Through the use of hepatic laser microdissection, it has been shown that HBx sequences deleted in the C-terminal portion are frequently and specifically detectable in HCC tumoral cells (54) and *in vitro* analysis has demonstrated that these HBX mutants are able to induce hepatocellular transformation. Other HBX genetic variants frequently isolated from HCCs are those showing the amino acid substitutions at positions 130 and 131 of the protein (54,186,187) and a recent study has indicated that the selection of these mutations precedes HCC development (55). However, the possible HBX functional modifications induced by the 130 and 131 amino acidic substitutions have not yet been investigated.

The potential hepatocarcinogenic effects of the integrated X gene have been largely analysed in HBx transgenic mouse models. Most studies demonstrate that the liver of these animals shows the typical features of the multistep neoplastic transformation process (188,189), although much evidence indicates that HBX must be expressed at high levels or the animals have to be exposed to additional hepatocarcinogenic agents, (i.e. diethylnitrosamine) to obtain the neoplastic transformation of the hepatocytes (158,190–194). Indeed, HBX does not act as a dominant oncogene and several different mechanisms have been implicated in HBX-induced hepatocarcinogenesis.

HBX is mainly detectable in the cell cytoplasm (182) and does not bind directly to DNA but functions by protein–protein interaction causing the transcriptional activation of several viral and cellular promoters and enhancers. It may deregulate the expression of oncogenes (c-Myc and c-Jun), cytokines (tumor necrosis factor-α and transforming growth factor-β) and transcription factors (nuclear factor-kappaB, activator proteins 1 and 2, RPB5 subunit of RNA polymerase II, the TATA-binding protein and activating transcription factor/cyclic adenosine 3′,5′-monophosphate-response element-binding protein) (161–163,195–199) and may modulate cytoplasmic signal transduction pathways (ras-raf-MAP kinase, Src kinase, jun-N-terminal kinase, Jak1/STAT, protein kinase C and Polo-like kinase 1) (200–204) involved in oncogenesis, cell proliferation, senescence and apoptosis, inflammation and immune response (205,206). It has been shown that HBX may interact with p53 protein causing its cytoplasmic delocalization and the inactivation of several important p53-dependent activities including p53-mediated transcriptional activation, p53 sequence-specific DNA-binding activity, cell cycle check point controls and p53-mediated apoptosis (207–214). However, the interaction between HBX and p53 has been questioned by two different studies showing the lack of their coimmunoprecipitation (185,215).

One of more extensively investigated mechanisms by which HBX may contribute to the development of HCC is its role in cell death pathways. To address the effects of HBX on apoptosis, several different experimental systems have been utilized, and contradictory results have been reported, most probably dependant upon the cell setting utilized and the levels of X gene expression induced. Indeed, it has been shown that HBX may inhibit apoptosis in a p53-indipendent manner through multiple mechanisms including the inhibition of caspase-3 and anti-Fas antibody-dependent apoptosis (216,217) or the modulation of activities of the serine protease *hepsin* (218) and up-regulation of *survivin* (219). However, in other experimental conditions, it has been demonstrated that HBX may also induce apoptosis through the regulation of the expression of Fas/FasL (220–223), caspase-8, cFLICE and Bax/Bcl-2 (224,225).

HBX also seems to contribute to carcinogenesis through the modulation of angiogenic pathways. In fact, HBX is able to induce both overexpression of vascular endothelial growth factor (VEGF) gene and stabilization of hypoxia-inducible factor-1, an important angiogenic factor induced in hypoxic conditions (79,226–228). More

recently, it has been shown that HBX may also exert an important role in modulating the epigenetic control of viral (229) and cellular genes, including a number of tumour suppressor genes (230–235).

Surface proteins. The preS1/preS2 sequence, encoding the Large hepatitis B (LHBs) and Middle hepatitis B (MHBs) virus surface proteins, is another region of the viral genome able to produce a transcriptional transactivator with transforming potentials. The *trans*-activating properties are acquired by LHBs or MHB only after C-terminal truncation (78,86). Of particular interest, studies on human tumoral tissues as well as experimental data from transgenic mice or hepatoma cell cultures showed that HBV preS/S genes truncated at the 3′ end and integrated into the host genome encode for C-terminally truncated surface proteins (MHBs^{t}) that progressively accumulate in the ER and display regulatory functions, such as the trans-activation of cellular genes including *c-myc*, *c-fos* and *c-Ha-ras oncogenes* and the specific activation of the c-Raf-1/MEK/Erk2 signal transduction cascade, resulting in the induction of an enhanced hepatocellular proliferative activity (14,32,87,164,236).

The most typical histological picture of HBV infection is the presence of 'ground-glass' hepatocytes (GGH) that is due to the accumulation of viral surface proteins in the cellular cytoplasm and more precisely in the ER, as highlighted since the 70s (237–239). At least two different types of GGH have been recognized and associated with different stages of chronic liver disease: the type I GGH, which is the classic GGH characterized by an 'inclusion-like' accumulation of HBsAg, and the type II GGH—also defined 'marginal type GGH'—that is characterized by the accumulation HBsAg at the cell margin or periphery of hepatocytes and that appears to be highly prevalent in the advanced stages of chronic HBV infection. Of note, it has been shown that the different pattern of HBsAg distribution in type I and type II GGH is linked to the accumulation of different mutated forms of LHBs due to relevant mutations occurring either in the preS1 or in the preS2 genomic region, respectively (95). Moreover, type II GGH associated with preS2-deleted LHBs may cluster in nodules, thus suggesting their higher proliferative activity and potential clonal expansion (95,240). These data are in accordance with the previously mentioned studies showing that infection with preS2-defective HBV mutants significantly correlates with HCC development (96,241). Furthermore, both studies in transgenic mice and cell cultures have provided evidence on the pro-oncogenic role of preS-mutant LHBs. It has been shown that the accumulation of these proteins in the ER determines the activation of stress-signalling pathways with induction of oxidative DNA damage and genomic instability (83,89,242). Moreover, preS-mutant proteins may induce the overexpression of both cyclooxygenase 2 and cyclin A, thus causing cell cycle progression and proliferation of the hepatocytes which in the presence of DNA damage and genomic instability may progress towards transformation and tumour development (95,243).

Genetic and epigenetic alterations

Numerous genetic abnormalities have been described in HCC, including chromosomal deletions and rearrangements, gain and loss of alleles with loss of heterozygosity, gene amplifications and mutations frequently involving oncogenes and tumour suppressor genes, aneuploidy as well as epigenetic alterations. HBV-related HCCs commonly exhibit a higher rate of chromosomal abnormalities than liver tumours linked to other risk factors (244,245) and it has been suggested that HBV might generate genomic instability, either through viral DNA integration or through the activity of its proteins. Moreover, HBV-related HCCs show gain or loss of chromosomal segments at similar sites including 1p, 2q, 4q, 5q, 6q, 8p, 10q, 11p, 16p, 16q, 17p and 22q chromosomal arms (246–253). Of interest, the results of microarray-based gene expression profiles have demonstrated that specific regulatory pathways, such as the ones related to cell death, DNA damage, signal transduction and metastasis are activated in HBV-associated HCC (254,255) and that chronic HBV infection is significantly associated with poor differentiated tumours, showing early recurrence and unfavourable prognosis (256,257). Besides genomic alterations, epigenetic factors like methylation-associated gene silencing and altered expression of microRNAs (miR) have been shown to be frequently involved in the deregulation of cellular functions in HCC. The expression of DNA methyltransferases (DNMTs), which catalyze the methylation of CpG groups, is often increased in livers affected with chronic hepatitis and cirrhosis as well as in HCC (258–260). Considerable evidence indicates that the presence of HBV in HCC significantly associates with aberrant DNA methylation of the host genome (231,261–263). It has been demonstrated that both HBV-infected cells and HCC show elevated expression of DNMT1, DNMT3A and DNMT3B compared with non-infected cells and matched normal tissues, respectively (264). Moreover, it has been shown that overexpression of HBx can induce DNMT1 and DNMT3A (232,234), thus suggesting that this viral protein may be responsible for what has been defined as a 'methylator phenotype' in HBV-related HCC. In accordance with this hypothesis, HBX may repress transcription of E-cadherin, IGF-3, glutathione *S*-transferase P1 and p16^{INK4A} through CpG methylation of the respective regulatory elements (230–233). In all these cases, the repression seems to be a consequence of HBx-mediated up-regulation of DNMT1 and DNMT3A gene expression. In this context, it has very recently been shown that HBx can induce DNMT1 expression by inhibiting miR-152. In fact, miR-152 can target the 3′ untranslated region of DNMT1 RNA, causing a marked reduction of the messenger RNA and protein levels. Of particular interest, the expression of miR-152 appears to be down-regulated in HCC tissues compared with paired non-tumour liver tissues and inversely correlated to DNMT1 in HBV patients (235).

Conclusions and perspectives

Technology progress is providing new and important insights into the comprehension of the pathogenetic mechanisms at the basis of HCC development in which the prominent etiopathogenetic role of HBV infection is widely confirmed. As highlighted by several authors, the prevalence studies based only on HBsAg evaluation determine an underestimation of the real impact of HBV on HCC development and any future study investigating the relationship between HBV and cancer cannot ignore the use of the most sensitive biomolecular techniques for viral DNA research which are able to identify the numerous cases of occult HBV infection. It is established that HBV genome does not encode a dominant oncogene but multifactorial pathogenetic mechanisms subtend HBV-related HCC development. In addition to indirect mechanisms (i.e. necroinflammation and host's immune response processes), HBV may also exert its oncogenic role through direct pathogenetic mechanisms mainly represented by the propensity of its DNA to integrate into the host's genome and by the production of proteins with transforming properties. Important contributions for discerning these direct mechanisms will likely be provided by analysing tumour tissues from patients under specific anti-HBV antiviral treatment in which the necroinflammatory injury is abolished or strongly reduced by the suppression of viral replication. In analogy, the direct oncogenic mechanisms potentially implied in the HCC development should also be more extensively investigated in tumours from patients with occult HBV infection.

The vaccination programs extended to the general population are showing significant efficacy in reducing incidence of HCC, thus demonstrating that the prevention of HBV infection is the best weapon to defeat this dangerous enemy of human health. The recent availability of specific antiviral drugs gives new hopes to patients with chronic infection, hypothesizing that their use in the pre-cirrhotic phase can reduce the risk of cancer development by stopping the necroinflammatory processes and reducing the chance of integration and production of proteins with pro-oncogenic activity. Prospective studies prolonged overtime are obviously needed to confirm the strength of this hypothesis.

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