Introduction

Approximately 2 billion people present evidence of past or present infection with hepatitis B virus (HBV) over the world. With more than 350 million chronic HBV carriers, this virus stands as one of the most common human pathogens and it causes significant public health problems [1]. Despite the availability of an efficacious and safe hepatitis B vaccine [2], chronic HBV infection remains the major aetiological factor of hepatocellular carcinoma (HCC) worldwide with more than one half of HCC patients being chronic carriers [3]. In recent studies conducted in Asia and Northern America, it was estimated that the lifetime risk of developing HCC is increased by 25–37 times in HBsAg carriers compared to non-infected populations [4,5]. Importantly, the first effects of mass vaccination have been detected in Taiwan with significant reduction of HBV-related HCC in vaccinated children [6]. Although high viral load is known to increase the risk of developing HCC [7], the risk remains elevated in HBsAg-negative HBV infection and occult infection [8–10]. In addition, inconsistent geographical variations in HCC mortality and HBV surface antigen (HBsAg) prevalence have been observed in endemic regions, suggesting that other independent or cooperative factors might be implicated. In highly endemic regions, particularly in South Africa and Mainland China, synergistic effects of aflatoxin B1 (AFB1) and HBV infection have been highlighted in several reports [11].

Hepatocellular carcinoma (HCC) is a common malignancy and a leading cause of cancer death worldwide. Recent epidemiological data have demonstrated that liver cancer incidence is continuously rising and will continue to do so for more than a decade, not only in Asia and Africa but also in North America and Europe [3,12]. HCC generally presents with poor prognosis, and no effective treatment is available for most HCC patients because this tumour remains refractory to current chemotherapeutic regimens [13]. Moreover, HCC is frequently diagnosed when advanced stage of the disease precludes local ablative or surgical interventions that could improve patient outcome. In this context, advances in our understanding of the molecular basis of HCC are urgently needed to develop early tumour markers and novel targeted agents with improved therapeutic efficiency [14,15]. Here we review the molecular mechanisms linking chronic hepatitis B to malignant transformation of liver cells.

The HBV life cycle

HBV is the prototype member of a family of small enveloped DNA virus called hepadnaviruses, which infect a restricted number of mammals and birds. These viruses share a narrow host range and preferential tropism for hepatocytes. The genome of hepadnaviruses is a relaxed circular, partially double-stranded DNA genome that replicates via an RNA intermediate [16] (Fig. 1). The HBV genome is around 3.2 kb in length and presents a highly compact genetic organization with 4 overlapping open reading frames (ORFs) that cover the entire genome. The pre-S/S ORF encodes the three viral surface proteins, the pre-C/C ORF encodes the e antigen (HBeAg) and the core antigen (HBcAg), the P ORF encodes the terminal protein (TP) and the viral polymerase that possesses DNA polymerase, reverse transcriptase and RNaseH activities. The X gene encodes a small protein that is essential for virus replication but whose function remains partially understood. Finally, a viral protein termed HBSP has been shown to be encoded by a spliced viral transcript [17].
Although many steps of hepadnavirus replication have been elucidated (Fig. 2), the initial phases of hepatocyte infection, namely virion attachment, uncoating, and entry remain poorly defined, and a cell-surface receptor has not been identified so far. These limitations may be explained by the low efficiency of HBV infection in few available in vitro systems. In the absence of candidate receptor, a less conventional hypothesis has been proposed, in which scavenging liver sinusoidal endothelial cells (LSEC), rather than hepatocytes themselves, mediate the initial phases of hepatocyte infection.[18] The unit length-strand (minus strand) is covalently linked to the viral polymerase and bears a 9-nucleotide redundant sequence at its extremities. The complementary strand (plus strand) is linked to a capped oligoribonucleotide at its 5' end. The plus strand is less than unit length and terminates at different positions resulting in the presence of a single-stranded region of variable length. Two 11-bp repeats, DR1 and DR2, located at the 5' ends of the minus and plus strands, play a critical role in viral DNA replication. Four open reading frame carried by the minus strand are represented by large arrows.

![Fig. 1. Schematic representation of the circular, partially double-stranded HBV DNA genome. Both DNA strands of the duplex are held together by base-pairing over 250–300 nucleotides at their 5’ extremities. The unit length-strand (minus strand) is covalently linked to the viral polymerase and bears a 9-nucleotide redundant sequence at its extremities. The complementary strand (plus strand) is linked to a capped oligoribonucleotide at its 5’ end. The plus strand is less than unit length and terminates at different positions resulting in the presence of a single-stranded region of variable length. Two 11-bp repeats, DR1 and DR2, located at the 5’ ends of the minus and plus strands, play a critical role in viral DNA replication. Four open reading frame carried by the minus strand are represented by large arrows.](image)

The natural history of chronic HBV infection

The natural history of chronic hepatitis B infection is a dynamic and complex process that can present a variable course, depending on a balance between viral parameters and host immune response [1]. While 90% of infected newborns and 30% of infected children develop chronic hepatitis B, only 5% of individuals infected at the adult age remain chronically infected, illustrating the primary role of the patient’s immune system. Recent consensus statements have schematically classified chronic hepatitis B into five phases: the “immune tolerant phase”, the “immune active phase”, the “inactive HBV carrier state”, the “HBeAg-negative chronic hepatitis B” and finally the “HBsAg-negative phase” [35,36].

Typically, the initial phase of chronic HBV infection is characterized by HBeAg-positivity, high levels of HBV DNA in serum and variable elevation of serum alanine aminotransferase (ALT). However, in a proportion of HBeAg-positive patients, ALT levels are normal and liver damage is minimal despite high levels of HBV replication. This state referred to as the “immune tolerant phase” is frequent after perinatal infections and it can persist for years. The “immune active phase” also referred as “chronic hepatitis B
“Phase” is typified by elevated ALT levels, low levels of HBV DNA in serum, and moderate to severe liver necroinflammation that may progress to fibrosis. While this phase can occur several years after experiencing the “immune tolerant phase” in perinatally-infected patients, it can be detected shortly after infection during adulthood. Large cell dysplasia has been frequently found in advanced chronic hepatitis B with increased activity. It involves hepatocytes with senescent features and no sign of proliferative or apoptotic activity, and develops during periods of frequent neoplastic transformation, thus raising the possibility that it might occur as a safeguard against the development of hepatocellular carcinoma [37]. Senescence in tumourigenesis is regarded as a defense mechanism and evasion of senescence is thought to lead to malignancy [38].

Among patients in the “immune active phase”, a majority eventually progress into the “inactive HBV carrier phase”, characterized by loss of HBeAg, seroconversion to anti-HBe antibodies and decline of serum HBV DNA to barely detectable levels, as well as improvement of fibrosis and inflammation over time [35,39,40]. In prospective studies, this state has been associated with a favorable long-term outcome [41]. However, up to one-third of patients will undergo seroconversion to anti-HBe antibodies and transition to the “HBeAg-negative chronic hepatitis B state”, characterized by periodic reactivation associated with fluctuating levels of ALT and HBV DNA, and active hepatitis with variable degree of fibrosis. This transition has been attributed to the occurrence of nucleotide substitutions in the precore and/or the basal core promoter region that preclude expression of the e antigen [42]. Emergence of these HBV variants is associated with active liver disease, and with a high risk of developing cirrhosis and HCC. By contrast, loss of HBsAg in patients in the

Fig. 2. Schematic representation of the HBV life cycle. HBV infects hepatocytes via an unknown cell-surface receptor. After virion entry and uncoating, nucleocapsids are transported to the cell nucleus where RC-DNA is converted to cccDNA, which is used as template for the transcription of pregenomic and subgenomic RNAs. In the cytoplasm, pgRNA is bound by the viral polymerase and packaged into nucleocapsids, where viral DNA is synthesized. Nucleocapsids can either assemble with envelope proteins in the endoplasmic reticulum, or deliver viral DNA into the nucleus for cccDNA amplification.
“HBsAg-negative phase” has a favorable prognosis, although HCC incidence remains higher than in non-infected populations [43]. This observation may be linked to the finding that persistent traces of HBV DNA are often detectable in the blood for many years after clinical recovery from acute hepatitis, despite the presence of serum antibodies and HBV-specific CTLs [44].

While a majority of liver cancers develop in cirrhotic livers, a significant fraction of HBV-related HCCs occurs in a background of chronic hepatitis B in the absence of liver cirrhosis. The incidence of cirrhosis appears to be about 2-fold higher in HBeAg-negative compared to HBeAg-positive chronic hepatitis [45]. The lower rate of underlying cirrhosis in HBV-related HCCs compared to other aetiologies argues for a more direct role of HBV in the tumoural process. Additionally, distinctive gene expression profiles have been detected in the non-tumoural livers of chronic HBV carriers, such as activated expression of genes implicated in pro-apoptotic, inflammatory and DNA repair responses, suggesting specific pathways triggered by chronic hepatitis B [46,47]. Thus, besides the effects of host immune responses, HBV replication might trigger various signaling cascades in the infected hepatocyte (Fig. 3). Integration of viral, host and environmental parameters with molecular changes might lead to identify novel targeted strategies to improve the management of chronic hepatitis B. Risk scores have been established to estimate the risk of developing HCC in less than 10 years after presentation. Such scores based on age, gender, HBV DNA levels, core promoter mutations and cirrhosis, can be used to identify high-risk patients for treatment and screening of HCC [48].

**Molecular mechanisms of hepatocarcinogenesis**

With the recent availability of integrative systems biology approaches, global insights into genetic alterations and molecular profiles have been reported in large series of HCCs during the last years. These studies have demonstrated a variety of genetic alterations and marked heterogeneity of gene expression profiles among HCC cases, suggesting that HCC might be one of the most complex and heterogeneous solid tumours in humans [49–51]. Such heterogeneity is in agreement with the multiple aetiologies of HCC and the long period of chronic inflammatory disease that fosters the accumulation of genetic and epigenetic defects. Evidence has been provided for deregulation of various signaling pathways in HCC subsets, such as Wnt/β-catenin signaling [52], and the p14ARF/p53 pathway [53], transforming growth factor beta (TGF-β) signaling, Ras/MAPK signaling and the PTEN/Akt and mTOR pathways [54] (reviewed in [50]). Additionally, altered expression of growth factors such as HGF, IGFs and Amphyregulin, as well as genes involved in angiogenesis may participate in the development and progression of HCC (reviewed in [55]).

Aberrant activation of the Wnt/β-catenin pathway through mutations in either β-catenin or Axin genes has been found in 20–40% of HCC cases, predominantly in HCV-associated tumours and in the absence of viral aetiology [56–58]. Interestingly, HCCs carrying mutant β-catenin display distinctive patterns, with highly differentiated morphology comprising microtrabecular and acinar architectures, low proliferative rate, and frequent intrahepatic cholestasis but no steatosis [59]. This tumour type also displays activated expression of liver metabolic enzymes involved in glutamine synthesis, urea cycle and detoxification.
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metabolism, which are normally expressed in perivenous hepatocytes [60,61]. Moreover, β-catenin mutation has been correlated with a low rate of genetic instability [56,62].

TGF-β has a complex role in HCC; it is persistently induced during hepatitis and promotes cirrhosis progression by accelerating the deposition of extracellular matrix (reviewed in [63]). Moreover, persistent upregulation of TGF-β in HCC has been suggested to accelerate neoplastic growth [64]. A bipartite role has thus been ascribed to TGF-β, which has tumour suppressor functions at early stages of liver damage, while at neoplastic stages, TGF-β may switch from being a tumour suppressor into being a tumour promoter that induces invasive and metastatic behaviour. Accordingly, blocking TGF-β using a kinase inhibitor of the TGF-β receptor I has an anti-tumoural effect via the regulation of neo-vascularization [65].

While oncogenic mutations of Ras are rarely seen in HCC, aberrant activation of protein kinases has been implicated in tumour initiation and progression. These kinases include P13K-AKT, Aurora kinase, and receptor tyrosine kinases of VEGF, EGF, PDGF, Herregulin, and IGF-I [66]. Recent studies have suggested a major role of mitogen-activated protein kinases (MAP kinases) such as p38 and the c-Jun N-terminal kinase (JNK) in driving uncontrolled proliferation and invasion of hepatic tumour cells [67–69]. These factors represent interesting targets for therapeutic intervention, as shown recently by the finding that sorafenib, a multikinase inhibitor, provides substantial survival benefits in patients at advanced HCC stages [70]. Moreover, it has been recently suggested that synergistic effects might be obtained in combination therapy using sorafenib and rapamycin for simultaneous targeting of neo-vascularization [65].

Familial cancer genes with high-penetration mutations have not been identified so far in HCC. However, recurrent mutations that compromise p53 function have been evidenced in the TP53 gene (17q13.1) in about 25% of primary HCCs [72]. Loss-of-function mutations of the AXIN1 gene (16p13.3) have been detected in 7–10% of cases, leading to the aberrant activation of Wnt signaling and possibly other pathways [73]. Other mutations affecting the RB, CDKN2A, PTEN or CDH1 genes have been found more rarely, and epigenetic silencing mechanisms such as the methylation of promoter sites may be responsible for the inhibition of tumour suppressor functions, as also recently found for the activation of the Ras and mTOR pathways [71].

The question of whether distinctive molecular mechanisms operate in HBV-related HCCs has been addressed using genetic approaches and microarray technologies for analyzing gene expression profiles. It has been shown that HBV-related HCCs display higher rates of chromosomal alterations than HCCs related to other risk factors [76]. Interestingly, recent studies have evidenced two major routes of hepatocarcinogenesis that differ by the extent of genetic instability, one being predominant in HBV-related tumours [57]. In this work, genetically unstable tumours have been associated with HBV infection, poor differentiation, p53 mutations, and an unfavorable prognosis. Extending these observations, gene expression profiling has revealed that HCC subclasses associated with HBV infection also display an activation of the mitotic cell cycle, deregulated expression of developmental and imprinted genes such as IGF2, as well as activation of the AKT pathway [62]. In different studies, molecular classification of HCC based on gene expression profiles has unveiled the predominant importance of the tumour cell differentiation status for defining molecular subtypes and predicting disease outcome [67,77]. More recently, a meta-analysis integrating transcriptome data from eight independent cohorts has led to the identification of three major molecular subclasses of HCC associated with tumour differentiation and serum alpha-fetoprotein (AFP) levels [78]. Again, in this study, chronic HBV infection was found to be significantly associated with HCC clusters featured by moderate/poor differentiation of tumour cells. Moreover, molecular classification of HBV-related HCCs has been used to predict early recurrence, which is a golden standard to determine the success of curative resection. The group with a high risk of recurrence showed an increased expression of genes involved in cell cycle progression, cell proliferation, migration and motility, and in the Notch signaling pathway [79].

While significant advances can be noted in our understanding of the molecular basis of hepatocarcinogenesis, future studies should be aimed at gaining a comprehensive view of the signaling networks operating in liver cell transformation due to chronic hepatitis B.

Chronic HBV infection and liver oncogenesis

HBV mutants and genotypes

During the progression of chronic hepatitis B from the asymptomatic carrier state to HCC, mutations accumulate in the viral genome in the preS region and in the carboxy-terminal region of the X gene, which overlaps with the enhancer II and the basal core promoter (BCP) [80]. Whether the risk of developing HCC may be influenced by the viral status of the patients, such as HBV genotypes, hepatitis B e antigen (HBeAg) serostatus, and mutations arising during chronic infections are important issues. It has been shown that these parameters play important role, not only in the progression of chronic liver disease, but also in the response to anti-viral therapies [81–83].

While epidemiological studies are usually based on HBsAg positivity, several case-control studies have pointed to HBeAg as a potential predictive marker, with a higher prevalence of HBeAg among patients with HBV-related HCC than among matched HBsAg carriers. In a study of a large cohort of patients, the relative risk of HCC was increased by 6-fold among patients positive for both HBeAg and HBsAg compared with those positive for HBsAg alone [84]. Thus HBeAg may be a useful marker of the risk of cancer, probably because it reflects active HBV DNA replication. The differential oncogenicity of HBV genotypes has been extensively analyzed. Important differences have been observed in the prevalence of serum HBeAg, the levels of HBV DNA, and the occurrence of precore or core promoter mutations between genotypes B and C [85], and between genotypes Aa, Ae, and D [86]. Evaluation of clinical and virological differences between HCC patients infected with genotype B or genotype C in Japan and Taiwan has given rise to divergent data [87,88]. Other studies have concluded that HBV genotype C takes a more aggressive disease course than genotype B in HBeAg-positive patients [89], and HBV genotype C infection (compared to genotype B) has been found as an independent risk factor for HCC development [90]. By contrast, other studies have shown that the prevalence of T1762/A1764 mutation in the basal core promoter increases with the progression of liver disease, and that this mutation is signifi-
HBV DNA integration into human chromosomes

HBV DNA integration into human host chromosomes occurs in the infected liver since early stages of natural acute infections [92,93]. Multiple integrations have been detected in chronic hepatitis tissues [94,95], and integrated HBV sequences have been seen in most (about 80%) HBV-related HCCs [96,97]. In the absence of complete genomes in virtually all HBV inserts, these sequences cannot serve as template for viral replication.

HBV DNA integration sites in human HCCs have been localized on almost all chromosomes, suggesting a random distribution throughout the host genome [93,98]. These events could either induce chromosome changes or act in cis on the expression or function of nearby cellular genes. For a long time it has been thought that HBV insertional mutagenesis is a rare event, and that simple repetitive elements are hotspots for HBV insertion in the human genome. However, evidence for a direct cis-acting promoter insertion mechanism was first provided in two independent HCCs, in which the HBV insertion targeted either the retinoic acid receptor-β (RAR-β) gene or the human cyclin A gene, resulting in tumour-specific chimeric proteins endowed with novel, pro-carcinogenic functions [99–102,103,104]. Thus, the analysis of single HBV insertion sites has allowed identifying new genes that play critical roles in the control of cell growth and differentiation.

In recent studies, the view of random HBV integration has been challenged by large-scale analysis of HBV DNA insertion sites using the Alu-PCR approach. These studies revealed high rates of gene targeting by HBV integration [105,106]. As previously shown for retroviruses [107,108], HBV DNA integration appears to occur frequently in actively transcribed chromosomal regions, within genes or at their immediate vicinity. Recently, sequence analysis of 68 viral–host junctions from 60 HCCs provided evidence for cellular coding regions within several kilobases in 90% of the cases [106]. Moreover, it was shown that HBV integration often targets gene families involved in cell survival, proliferation and immortalization, such as hTERT, PDGF receptor, MLL, calcium signaling-related genes and 60s ribosomal protein genes. Indeed, recurrent viral insertions nearby the hTERT or MLL gene have been reported by different groups [106,109–111]. It can be envisaged that viral insertion induces the first genetic hit in liver tumourigenesis and that different genes targeted by viral integration play important role in hepatocarcinogenesis. While enhanced expression of some cellular target genes upon nearby viral insertion has been documented, the question of whether these genes play a role in tumourigenesis as drivers or represent only bystanders has rarely been addressed. Finally, although integrated viral sequences are defective for replication, they might also contribute “in trans” to tumourigenesis through the production of truncated and mutated HBx or preS2/S proteins. These proteins may act on HCC development by disrupting the control of cellular gene expression or by activating oncogenic signaling pathways.

Besides acting by cis- or trans-activation, HBV insertions have been associated with major genetic alterations within the cell genome, including large deletions, duplications and chromosomal translocations [112–116]. The association of HBV integration with large genomic changes might reflect the abrogation of control mechanisms that safeguard chromosomal integrity [49].

The HBx regulatory protein: a viral oncogene?

The HBx protein encoded by the X gene has been designated “viral oncoprotein” by several authors. This protein has been involved in liver cell transformation because of its pleiotropic activities on cell cycle regulation, signaling pathways and DNA repair (reviewed in [117–119]). However, evidence for a direct transforming activity of HBx is scarce. HBx has been reported to transform two cell lines immortalized by the simian virus 40 large T antigen (SV40TAg) [120,121]. The viral protein has also been shown to cooperate with Ras in the transformation of primary human fibroblasts, either by activating the phosphatidylinositol-3 kinase and Akt pathway or by overcoming Ras-induced senescence [122,123]. A weak tumorigenicity has been attributed to HBx in TG-α immortalized murine hepatocytes, but no cooperation could be evidenced with mutated p53 [124]. Other studies describe opposite effects, in which the apoptotic properties of HBx suppress transformation of primary rodent fibroblasts by different oncoenes [125]. One caveat with these observations is that most were made in cell culture with high levels of X gene expression. Indeed, in the chronically infected liver, HBx expression is kept at low, barely detectable levels [126].

Studies of HBx oncoenicity in transgenic mice have yielded divergent results that may be related to different murine genetic backgrounds and various promoters yielding different expression levels of the viral protein. HCC development has been associated with high-level hepatic expression of HBx in a transgenic mouse line generated in the outbred CD-1 background [127]. In most other transgenic lines in different backgrounds, expression of HBx by itself did not lead to HCC development, although slight histopathologic alterations could be observed in the liver [128]. Transgenic expression of HBx in the murine liver activates both proliferation and apoptosis, and sensitizes hepatocytes to oncogenic transformation [129–133]. Consistent with the ability of HBx to deregulate the cell cycle, premature cell cycle entry or impaired regeneration has been observed after partial hepatectomy of the liver of HBx mice from different transgenic lines [134–136].

Among the different activities of HBx, its trans-activation function may play a crucial role in hepatocarcinogenesis because it is involved in the activation of a large number of signaling pathways and cellular genes that are involved in oncogenesis, proliferation, inflammation and immune responses [137–139]. In particular, we have recently shown that HBx interacts with the acetyltransferases CBP/p300, and that this interaction plays a decisive role in the activation of CREB-dependent transcription [140]. The activation of CREB/ATF trans-activation function by
HBx appears double since HBx has been shown to increase CREB/ATF DNA-binding affinity as well as to enhance the recruitment of CBP/p300 to CREB/ATF bound to cellular DNA [140,141] (Fig. 5). The modulation of CREB/ATF activity by HBx represents an important aspect of HBx activities since the CREB/ATF family members play an essential role in liver metabolism and proliferation, and CREB has been implicated in hepatocarcinogenesis [142]. Moreover, the coactivators CBP/p300 are known to bind and activate a large variety of cellular transcription factors [143]. Some of these factors, such as c-Jun, c-Fos and NF-κB are also activated by HBx, and the interaction between HBx and CBP/p300 could explain, at least partially, the broad activity of HBx on transcription.

Another reported activity of HBx targets centrosome dynamics and mitotic spindle formation through the binding of HBx with different cellular partners implicated in centrosome formation. HBx has been shown to bind and partially inactivate BubR1, an effector of multiple mitotic kinases that specifies microtubule attachments and checkpoint functions [144]. HBx also interacts with HBXIP, a major regulator of centrosome duplication.
required for bipolar spindle formation and cytokinesis [145]. Interestingly, recent data have linked DNA re-replication induced by HBx to partial polyploidy, known to be associated with cancer pathogenesis [146]. Although the precise HBx activities that interfere with the mitotic cell cycle remain to be determined, these data provide a strong link between HBx expression and chromosomal instability in HBV-related carcinogenesis.

Conclusions and perspectives

Consistent with the multifactorial aetiology of HCC and the long latency period of tumour formation, a large variety of oncogenes, growth factors and tumour suppressors have been implicated in human hepatocarcinogenesis [49,51]. The question of whether HBV and other aetiological factors and co-factors may trigger different tumourigenic pathways can now be addressed, owing to the development of novel technologies for genome-wide scans of genetic alterations and gene expression, and to new technologies in proteomic analysis. Increased frequency of p53 mutations in HCC has been associated with AF11 exposure and chronic HBV infection, while HBV-related tumours display high chromosomal instability and low rate of β-catenin mutation. So far, studies have mainly been focused on tumours; it will therefore be important to analyze gene expression and proteomic changes in large series of samples from chronic hepatitis B at different stages in order to identify suitable prognostic markers and therapeutic targets. Besides genomic alterations, epigenetic factors like methylation-associated gene silencing and altered expression of microRNAs may play an important role in the deregulation of cellular functions leading to malignant transformation, and they warrant attention. Better understanding of the complex role of HBV in liver tumourigenesis will undoubtedly contribute to improve the management of liver diseases induced by chronic hepatitis B.

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