Chapter 5
Pathobiology of Hepatitis B Virus-Induced Carcinogenesis

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Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent solid tumors worldwide, with more than 250,000 new HCC cases annually and an estimated 500,000–600,000 deaths/year [1, 2], and because of its very poor prognosis is the second cause of cancer death worldwide [3].

HCC development is driven by multiple viruses (HBV, HCV) and chronic metabolic alterations that lead to chronic inflammation, DNA damage, and epigenetic and genetic changes that affect both "common" and "etiology specific" oncogenic pathways. The clinical and molecular heterogeneity of HCC translates into "molecular signatures" that identify discrete molecular subgroups of HCC and stratify patients according to prognosis.

Numerous signaling modules are deregulated in HCC, including growth factor signaling (e.g., IGF, EGF, PDGF, FGF, HGF), cell differentiation (WNT, Hedgehog, Notch), and angiogenesis (VEGF). Intracellular mediators such as RAS and AKT/mTOR also play a role in HCC development and progression. The use of novel molecular technologies such as next-generation sequencing (NGS) has enabled the identification of pathways previously underexplored in the HCC field, such as chromatin remodeling and autophagy.

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Different molecular mechanisms are involved in aberrant pathway activation, including point mutations, chromosomal aberrations and epigenetically driven downregulation. Importantly, whereas mutations and chromosomal aberrations have been predominantly found in tumor tissues, with the notable exception of the recently reported TERT promoter mutations, deregulation of signaling pathways and epigenetic changes are also detected early in the natural history of HCC development, at the stage of cirrhosis or dysplastic nodules.

Chronic inflammation, double-strand breaks (DSBs) accumulation, epigenetic modifications, chromosomal instability, and early neo-angiogenesis are the major driving forces in hepatocytes transformation, HCC development and progression. All "etiologic" factors (i.e., chronic HBV and HCV infections, chronic metabolic alterations) seem to act through overlapping and non-overlapping mechanisms that finally converge on these pathways.

Recent views on the molecular pathogenesis and classification of HCCs and their impact on the design of new therapeutic approaches can be found in Refs. 4–10. In this chapter, we focus on the molecular characterization of HBV-related HCCs and the role of HBV genetic variability, HBV integration into the host genome and wild-type and mutated/truncated viral proteins to HBV carcinogenesis.

Epidemiology and Risk Factors

Chronic hepatitis B infection remains a major public health problem worldwide despite the availability of the HBV vaccine since the early 1990s and the decreased incidence of HBV new infections in most countries [2]. Over 400 million people chronically infected with HBV are at high risk of developing liver cirrhosis and hepatocellular carcinoma (HCC) [11], making HBV the most common carcinogen after tobacco. Recent estimates attribute to HBV over 50 % of HCC cases worldwide [1, 2]. Because of geographic variations in the incidence of hepatitis B, the fraction of HCC attributable to HBV varies significantly, representing less than 20 % of all cases of HCC in the USA and up to 65 % in China and Far East; Europe is divided into a low-risk (18 %) area (west and north Europe) and a high-risk (51 %) area (east and south Europe) [3]. The role of HBV in HCC may be greater than that depicted by sero-epidemiologic studies, as suggested by the increased risk of developing HCC in patients with occult HBV infection [defined as persistence of free and/or integrated forms of HBV-DNA in the liver in the absence of the viral marker HBsAg in the serum [12]] and after hepatitis B surface antigen (HBsAg) clearance [13–17].

The lifetime risk of developing HCC is 10- to 25-fold greater for chronic HBV carriers, as compared with non-infected populations [18]. Important epidemiologic features of HBV-related HCC include younger age at presentation compared with HCC cases related to alcohol, non-alcoholic steato-hepatitis, and HCV and the absence of cirrhosis in one-third of patients with HCC [11, 18].

Several virus-related, host-related, dietary, and lifestyle factors are associated with an increased risk of HCC in patients who are chronically infected by

HBV. Increasing age, reflecting longer exposure to HBV, and male gender have long been known to enhance the risk for HCC [18]. More recently, evidence has emerged that gender disparity in HCC risk may also reflect protection against this tumor by estrogen via complex networks involving hepatocyte nuclear factor-4a [19] and IL6 signaling [20]. Hepatitis severity and coinfection with hepatitis D virus and hepatitis C virus (HCV), or human immunodeficiency virus (HIV) have been also found to augment the risk of HCC in chronic HBV infection. Alcohol consumption, a well established independent risk factor for HCC, also plays a synergistic role with a more than twofold increase of the carcinogenic risk of HBV [21]. Tobacco smoking is also associated with an increased risk of HCC in patients with HBV-related cirrhosis, with evidence of a quantitative relationship between smoking and cancer risk. HCC frequency is particularly high in Asia and Africa due to the high frequency of viral hepatitis infections and to Aflatoxin B1 (AFB1) exposure [1, 22]. Other known etiological factors of HCC development, including hemochromatosis, steatosis, nonalcoholic fatty liver diseases, and diabetes, often act as co-factors of overt and occult HBV infection for HCC development [19, 21–23].

HBV Viral heterogeneity and HCC

As discussed in the previous chapters, the HBV genome is, in plasma circulating infectious HBV particles, a circularized linear partially double-stranded DNA of about 3200 nucleotides [24]. Once entered the cell, HBV DNA is converted into a covalently closed circular DNA (HBV cccDNA) that accumulates in the nucleus of infected cells as a stable episome and is organized into nucleosomal structures [25–27]. HBV cccDNA is responsible for persistent HBV infection of hepatocytes and is the template for the transcription of all viral mRNAs, including the pregenomic HBV RNA (pgRNA), the obligatory replicative intermediate, which is reverse transcribed by the viral polymerase to produce the first HBV DNA strand and sustain the viral replication in cytoplasmic core particles [28]. Chromatin-modifying enzymes, cellular transcription factors, and the viral proteins HBx and HBc are recruited on the cccDNA mini-chromosome to regulate its transcription and, ultimately, viral replication [25–27, 29–31]. The integration of viral DNA into the host genome, that occurs randomly in regenerating infected hepatocytes [24], contributes to viral pathogenesis both by cis-acting mechanisms and by the continuous expression of *trans*-acting wild type and truncated HBx or truncated preS/S polypeptides bearing enhanced transforming properties [32]. The goal of therapy in chronic hepatitis B (CHB) is the persistent suppression of HBV replication [33]. Due to the lack of direct effect on the cccDNA in the nucleus, a sustained suppression of HBV replication by NUCs does not lead to cccDNA elimination (eradication) [26]. Long-term inhibition of HBV replication by NUCs has resulted into a reduction of the risk of HCC in non-cirrhotic patients by preventing progression to cirrhosis whereas in cirrhotic patients the reduced rates of anticipated liver mortality due to clinical decompensation, has often translated into increased rates

of HCC-related mortality or, at best, a marginal effect on HCC development in long-term follow-ups [33, 34].

The risk of developing HCC also correlates with HBV genotype, HBV genomic mutations, and HBV replication [14, 15]. At least eight different HBV genotypes have been identified (A-H) where the nucleotide sequence varies by at least 8 %. HBV genotype C has been associated with a higher risk of HCC development [35]. However, the findings are not univocal and no firm conclusion can be drawn on whether HBV genotypes harbor different oncogenic potential. HBV replication drives both disease severity and progression and the persistence of high-serum HBV-DNA levels correlate in the clinical setting with liver damage accumulation, evolution to cirrhosis, and HCC development [14].

Variability of HBV genome is basically attributed to lack of proofreading by the HBV polymerase and the high copy number of the virus that lead to the selection of HBV quasi-species containing several mutations within their viral genome. Some of these mutations may provide a replicative advantage to the virus while others are detrimental. The accumulation of mutations reflects the duration of active HBV infection, the strength of the immune response and the selection pressure exerted by exogenous factors such as antiviral therapies and vaccinations [36]. HBV mutations are not distributed randomly over the entire genome but cluster in particular regions such as the basal core promoter (BCP)/preCore region and the preS/S region [37].

The double-mutation T1762/A1764 in the basal core promoter is significantly associated (OR: 6.72) with the development of HCC in both genotypes B and C [38] and can be detected in plasma up to 8 years before HCC diagnosis suggesting a possible use of this mutation as a strong predictive biomarker, at least in some geographical areas [38].

Several HBV variants with point mutations, deletions, or insertions in the preS1 and preS2 sequences are often found in patients with long-lasting chronic hepatitis B (CHB) [37] and variants defective for the M envelope protein are the most frequently selected [39–44]. A role of HBV preS mutants in the pathogenesis of HCC is supported by a large number of experimental and clinical evidence [45–50]. A meta-analysis of 43 studies and ~11,500 HBV-infected patients has shown that infection with preS mutants is associated with a 3.77-fold increased risk of HCC [51] and the predictive value of preS deletion mutants has been recently confirmed in a prospective study [45]. The HBV variants commonly associated with HCC include (a) mutations of the preS2 start codon and/or deletions in the 5′-terminal half of the preS2 region and (b) deletions in the 30-terminal half of the preS1 region [52, 53]. These preS deletion deletions correspond to viral regions containing B or T cells epitopes and are thought to represent HBV immune escape variants [54]. Both preS1 and preS2 mutations may lead to unbalanced production of the different envelope proteins and the accumulation of mutated L protein in the ER of hepatocytes, resulting in the activation of the ER stress signaling pathway [48, 55–61]. ER stress can generate reactive oxygen species and cause oxidative DNA damage, genomic instability, and ultimately favor HCC development [48, 62–66].

HBV mutants with premature stop codon at position 172 or 182 of the S gene have been frequently found in patients with cirrhosis and HCC [67–70] and

nucleos(t)ide analogues (NUCs) can select mutants in the B, C, and D domain of the reverse transcriptase/DNA polymerase (Pol) associated with pharmacological resistance [71]. Since the HBV surface gene overlaps completely, on a different open reading frame (ORF), with the Pol gene, some changes in Pol ORF selected by NUCs can affect the overlapping surface gene. The rtA181T/ sW172* mutation, selected by lamivudine or adefovir, results in the generation of a stop codon in the S ORF and the production of a truncated S protein with a dominant negative secretion defect that accumulates within the hepatocyte leading to ER stress and activation of oncogenic cellular pathways to be cited [71]. Importantly, the emergence of the rtA181T/sW172* mutant associated with an increased risk of developing HCC in patients [72].

Genetic Alterations in HBV-Related HCC

Extensive evidence indicates that HCC is an extremely heterogeneous tumor at the genetic and molecular and genetic level with a complex mutational landscape and multiple transcription and signaling pathways involved [73–75].

Genetic host factors are thought to play an important role in the development of HCC during HBV infection and several studies of family clusters, mostly performed in the Far East, have identified single-nucleotide polymorphisms (SNPs) associated with an increased HCC risk as compared to the control populations [76]. Risk-associated SNPs in chromosome 8p12 have been associated with DLC1 locus (Deleted in Liver Cancer 1) deletion or chromosomal loss in patients with HBV-related HCC [77]. Additional SNPs associated with HCC development in patients with chronic hepatitis B were identified in the CTL-4 (cytotoxic T-lymphocyte antigen 4) gene [78] and the KIF1B locus in chromosome 1p36.22 [79] but, overall, their predictive power seems to be low and need to be validated in larger cohorts of multiple ethnicity.

HBV-related tumors generally harbor a higher rate of chromosomal abnormalities than tumors linked to other risk factors [80], likely due to the ability of HBV to generate genomic instability through both viral DNA integration and the activity of the X protein (see below). HBV-related HCCs are characterized by higher frequencies of TP53 mutations at, as well as outside, the aflatoxin B1-related codon 249 hotspot mutation [74], and AXIN1 [80], whereas activating β-catenin mutations are more frequent in non HBV-related HCCs [80]. IRF2 inactivation, that leads to impaired TP53 function, was found exclusively in HBV-related tumors [81]. A recent whole-exome sequencing analysis of 243 HCCs [82] identified, by integrating mutations, focal amplifications and homozygous deletions, 161 putative driver genes associated with 11 pathways altered in >5 % of the tumors [TERT promoter mutations activating telomerase expression (60 %); WNT/β-catenin (54 %); phosphoinositide 3-kinase (PI3K)-AKT-mTOR (51 %); TP53/cell cycle (49 %); mitogen-activated protein kinase (MAPK) (43 %); hepatic differentiation (34 %); epigenetic regulation (32 %); chromatin remodeling (28 %); oxidative stress (12 %);

interleukin (IL)-6/JAK-STAT (9 %); transforming growth factor (TGF)-β (5 %)]. New genes found to be recurrently mutated in HCC included β-catenin inhibitors (ZNRF3, USP34 and MACF1), hepatocyte-secreted proteins (APOB and FGA), and the TGF-β receptor ACVR2A. TERT promoter mutation were usually an early event, whereas FGF3, FGF4, FGF19, or CCND1 amplification and TP53 and CDKN2A alterations appeared at more advanced stages in aggressive tumors. HCV infection, metabolic syndrome, and hemochromatosis did not show significant associations. Alcohol-related HCCs were significantly enriched in CTNNB1, TERT, CDKN2A, SMARCA2 and HGF alterations. IL6ST mutations were found in HCCs with no known etiology. HBV-related HCCs were more frequently mutated in TP53 [82].

Genome-wide transcriptomic analysis of well-annotated HCCs identifies subgroups of HCC associated with specific clinical and genetic characteristics [73, 83]. In the study from Boyault et al. [83] the G1–G2 subgroups demonstrated overexpression of fetal stage-associated genes and were controlled by parental imprinting; G3 tumors were characterised by TP53 mutations and demonstrated adverse clinical outcome; G4 was a heterogeneous subgroup of tumours; G5–G6 subgroups were strongly related to β-catenin mutations, leading to Wnt pathway activation. G1 and G2 tumors were both related to HBV infection and displayed frequent activation of the PI3K/AKT pathway, but differed for the overexpression of genes expressed in fetal liver and controlled by parental imprinting (G1) and the frequent mutation of the PIK3CA and TP53 genes (G2) [83]. In a more extended study focused on the molecular characterization of HBV-related HCCs Amaddeo and colleagues [84] confirmed that the TP53 gene was the most frequently mutated gene in HBV-related HCC (41 % vs 16 %, in non-HBV tumors) and that inactivation of the IRF2 tumor-suppressor gene, that controls p53 protein activation, was exclusively identified in HBV-HCC (7 %) but also showed that HBV-related HCCs display a wide genomic diversity and were distributed in all G1–G6 transcriptomic subgroups. In particular G2 and G3 profiles were enriched and genes associated with progenitor features (EpCAM, AFP, KRT19, and CCNB1) were significantly overexpressed in HBV-related HCCs compared to HCCs related to other etiologies [84]. G4–G6 profiles characterized a small subset of HBV-related HCCs in older patients with other cofactors such as HCV, alcohol consumption, or NASH.

microRNAs [small noncoding single-stranded RNAs that regulate gene expression, primarily at the posttranscriptional level] are increasingly recognized as key players in the regulation of liver functions and in hepato-carcinogenesis [85]. Using global miRNA profiling of HCC cell lines or liver tissue, the expression of several miRNAs has been found to be either upregulated (miR-18, miR-21, miR-221, miR-222, miR-224, miR- 373, and miR-301), or downregulated (miR- 122, miR-223, miR-125, miR-130a, miR-150, miR-199, miR-200, and let-7 family members) in HCC [85]. Differences between HCV- and HBV-related HCC associated miRNAs are emerging. miR143, miR34, and miR-19 are upregulated in HBV-related HCC and promote the more aggressive biological phenotype of HBV-related HCCs [86, 87]. Downregulation of Let 7a by HBx is associated with upregulation of STAT3-induced cell proliferation [88]. HBx suppression of miR-152 leads to upregulation

of DNMT1, which methylates the promoters of many tumor suppressors [89]. Finally, miR26 expression is low in HBV-related HCCs and lower in man than in women and associate with a poor survival and lower response to adjuvant therapy with interferon-α [90].

Epigenetic Mechanisms in HBV-Related HCC

The principal mechanisms involved in chromatin remodeling and the epigenetic control of gene expression are DNA methylation, enzymatic covalent *histone modifications*(e.g., acetylation, methylation, and phosphorylation) and nucleosomal re-structuring by ATP-dependent*chromatin re-modeling complexes*.

Global hypo-methylation of DNA with selective hyper-methylation of tumor-suppressor genes promoters containing CpG islands, have been shown to modify gene expression patterns in the liver before HCC appearance. A number of tumor-suppressor genes, including RASSF1A, p16/INK4A, APC, E-cadherin, SOCS-1, IGF-binding protein 3 (IGFBP3), and glutathione *S*-transferase P1 (GSTP1), have been shown to be silenced by DNA methylation in a large proportion of liver tumors, and this process often starts at pre-neoplastic (cirrhotic) stages [91]. A higher rate of promoter methylation for specific genes such as pl6INK4A, E-cadherin, ASPP1, and ASPP1 has been observed in HBV-related tumors compared to non-viral tumors [91, 92]. Using genome-wide methylation profiling, Villaneueva and collegues [93] have identified and validated a 36-probes methylation signature able to accurately predict survival in HCC patients. This signature correlated with known predictors of poor outcome and identified patients with the mRNA signatures of proliferation and progenitor cell features. The study confirmed a high prevalence of genes known to be deregulated by aberrant methylation in HCC (e.g., RSSFA1, APC, NEFH, IGF2, RAFF5, NKX6.2, SFRP5) and other solid tumors (e.g., NOTCH3 in acute leukemias; NSD1 in glioblastoma; ZIC1 in colorectal cancer) and described new potential candidate epidrivers in HCCs (e.g., SEPT9, a tumor suppressor described in colon and ovarian cancer; ephrin-B2 ligand EFNB2, reported hyper-methylated in patients with acute leukemia; homeobox A9, forkhead box G1 and runt-related transcription factor 3, involved in TGF-b receptor-signaling; FGF8 and FGF6) [93].

HDAC1, 2, and 3 are overexpressed in 30–50 % of HBV-related HCCs and HDAC3 is an independent predictor of tumor recurrence following liver transplantation [94]. A significant upregulation of several HDACs (namely, HDAC1, 2, 3, 4, 5, and 11) was also described in HCV-related HCCs where DNA copy gains in *HDAC3 and HDAC5* correlated with their mRNA upregulation [95]. Importantly, combining the pan-HDAC inhibitor panobinostat and sorafenib strongly potentiated treatment efficacy and improved survival in HCC xenograft models [95]. Pathologic activation of *Ezh2 and PRC2*, either through Ezh2 overexpression or Ezh2-activating mutations, is among the most common alterations observed in a wide variety of cancerous tissue types, including non-Hodgkin's lymphoma, prostate, breast and HCCs [96–103]. Increased expression of EzH2 is frequently

detected in HCC tissues, correlate with the aggressiveness and/or poor prognosis of HCCs and may help to discriminate between pre-neoplastic/dysplastic lesions and cancer [104, 105]. Similarly, an increased expression of the G9a histone methyltransferase has also been reported in HCC [105]. Knockdown of EzH2 expression in HCC cells is sufficient to reverse tumorigenesis in a nude mouse model, thus suggesting a potential therapeutic value of EzH2 inhibition in HCC [106]. An HCC-specific long noncoding RNA (lcn) [lncRNA-HEIH] associates with EzH2 to repress EzH2 target genes and facilitate HCC tumor growth in HBV-related HCCs [107] and, in particular, EzH2-mediated repression of Wnt antagonists has been found to promote β-catenin-dependent hepato-carcinogenesis [108]. On the other hand, animal models of HBx- and HBV-mediated tumorigenesis downregulate the chromatin modifying proteins Suz12 [another PRC2 component] and ZnF198 [part of the LSD-Co-RESR-HDAC1 repressor complex] in liver tumors [109]. Suz12/Znf198 downregulation is accompanied, both in animal models and human HBV-related HCCs, by the overexpression of a number of Suz12/PRC2 direct target genes, including the hepatic cancer stem cell markers BAMBI and EpCAM [110].

Several studies also identified mutations in a group of chromatin regulators (*ARID1A*, *ARID1B*, *ARID2*, *MLL*, and *MLL3*) in approximately 20 % of all tumors, including virus- and alcohol-related HCCs [81, 111–113]. ARID1A and ARID1B are crucial and mutually exclusive subunits of the SWI/SNF ATPase-powered nucleosome re-modeling complex. ARID2 is a subunit of the poly-bromo- and BRG1-associated (PBAF) remodeling complex, which is implicated in the control of ligand-dependent transcription by nuclear receptors. Inactivating mutations in *ARID1A*, and its role as a tumor suppressor have been reported in several malignancies, including ovarian, colorectal, and gastric cancer [114–117].

Direct Oncogenic Roles of HBV

The long latency period between HBV infection and HCC and the strong relation between HCC incidence and age has often been used to support an indirect role of HBV in hepatocytes transformation. Increasing evidence suggests, however, that HBV contributes to HCC by directly promoting growth factor-independent proliferation, resistance to growth inhibition, tissue invasion and metastasis, angiogenesis, reprogramming of energy metabolism, and resistance to apoptosis, i.e., the host gene expression pathways and cellular phenotypes that are recognized as hallmarks of cancer [4, 118]. The ability of HBV-encoded proteins to blunt both the innate and adaptive immune responses favors the persistence virus replication and contributes to HCC by sustaining chronic inflammation without viral clearance. Notably, most virus-induced changes in host gene expression that promote HCC also support virus replication and/or protect virus-infected hepatocytes from cytokine- and cell-mediated damage or destruction.

HBV can promote carcinogenesis by three different mechanisms (Fig. 5.1). First, the integration of viral DNA into the host genome contributes to chromosome instability

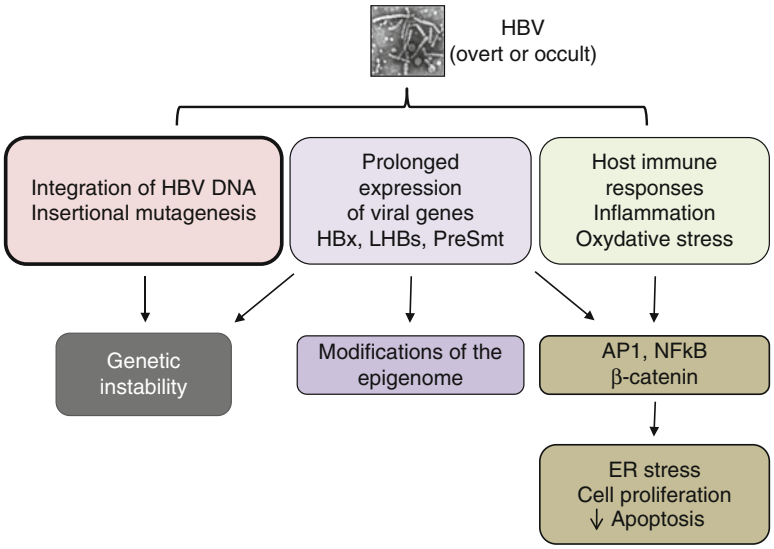

Fig. 5.1 HBV carcinogenesis. HBV contributes to HCC by (**a**) insertional mutagenesis due to integration of the viral DNA into host chromosomes; (**b**) increased genomic instability caused by both viral integration and the activity of the viral protein HBx; (**c**) modifications of the epigenome promoted by HBx and HBc; (**d**) modulation of cell death and proliferation pathways by the prolonged expression of viral proteins (wild-type and mutant HBx, LHB envelope proteins, truncated MHB proteins, HBc)

[119]. HBV DNA integration in host chromosomes, although dispensable for viral replication, is detected in about 80 % of HCCs [32]. Second, classic retrovirus-like insertional mutagenesis can occur. HBV integration at specific genomic sites provides a growth advantage to a clonal cell population that eventually accumulates additional mutations. HBV integrations within the retinoic acid receptor ß (RARß) and the cyclin A as target genes [32] provided the first evidence and additional genes were later found to be targeted by HBV integration in tumors, including recurrent HBV DNA integration into the hTERT gene encoding the catalytic subunit of telomerase [120–123]. More recently, next-generation sequencing (NGS) studies of ~400 HBV integration breakpoints from over 100 HBV-positive HCCs confirmed that HBV integration is more frequent in the tumors (86.4 %) than in adjacent liver tissues (30.7 %). Approximately 40 % of HBV breakpoints within the HBV genome are located near the viral enhancer and the X gene and core open reading frames and copy-number variations (CNVs) are increased at HBV breakpoint locations indicating that HBV integration likely induces chromosomal instability [113]. Multicentric tumor pairs develop from independent mutations [111]. Most HBV breakpoints fall near coding genes, mainly into exons or regulatory regions, including the TERT, MLL4 (mixed-lineage leukemia protein 4), CCNE1 (Cyclin 1), SENP5 (Sentrin-specific protease 5), ROCK1 (Rho-associated coiled-coil containing protein kinase 1) genes, whose expression was upregulated in tumors

versus the normal tissue [111, 113]. More recently, Lau and coworkers [124] have reported the integration of HBV sequences into the host long interspersed nuclear element (LINE) and the generation of a HBx-LINE1 chimeric transcript in 21 out of 90 HBV-related HCC patient tumors that is significantly associated with poor patient outcome [124]. Mechanistically, the HBx-LINE1 transcript acts as a long noncoding RNA by increasing the nuclear localization of β-catenin and by activating Wnt signaling and its oncogenic properties are independent from its protein product [124]. Notably, HBx-LINE1 fusion transcripts were not detected in 50 HBV-related HCCs from Europe [125]. The high frequency of this oncogenic transcript might be restricted to the Hong Kong population where HBV genotype C is predominant and remains to be validated in other independent series of HCC. The third direct mechanism of HBV carcinogenesis is based on the ability of viral proteins (HBx, HBc, and preS) to affect many cell functions, including cell proliferation and cell viability and to sensitize liver cells to mutagens. In transgenic mouse models, unregulated expression of the HBV X and large S proteins are associated with hepatocarcinogenesis [59, 126].

HBx Protein

HBx regulatory protein is both required for HBV cccDNA transcription/viral replication [29, 127], and thought to contribute to HBV oncogenicity. Although the mechanisms underlying these pleiotropic activities of HBx have not been fully elucidated, regulation of transcription, through direct nuclear (transcription and chromatin) and/or indirect cytoplasmic (cell signaling) mechanisms, is thought to play an important role (Fig. 5.2). In the following subsections we provide a rather comprehensive description of the vast scientific literature reporting on HBx activities over almost two decades. We have underlined wherever possible the relevance of those results that have been generated in the context of HBV replication/infection systems and/or conformed in either in vivo animal models or through the ex vivo evaluation of CHB- and HBV-related HCC patients samples.

HBx, Chromatin and Viral/Cellular Transcriptional Control

HBx is recruited to the cccDNA minichromosome in HBV-replicating cells to increase transcription of the nuclear cccDNA minichromosome [29, 127]. In the absence of HBx, cccDNA-bound histones are hypoacetylated, and the cccDNA transcribes significantly less pgRNA [29]. HBx also binds and blocks the inhibitory activity on HBV transcription exterted by the PRMT1 methyl-transferase [30] and the Tudor-domain protein Spindlin-1 [128]. Additional mechanisms by which HBx can potentiate HBV replication include the down-regulation of DNMT3A

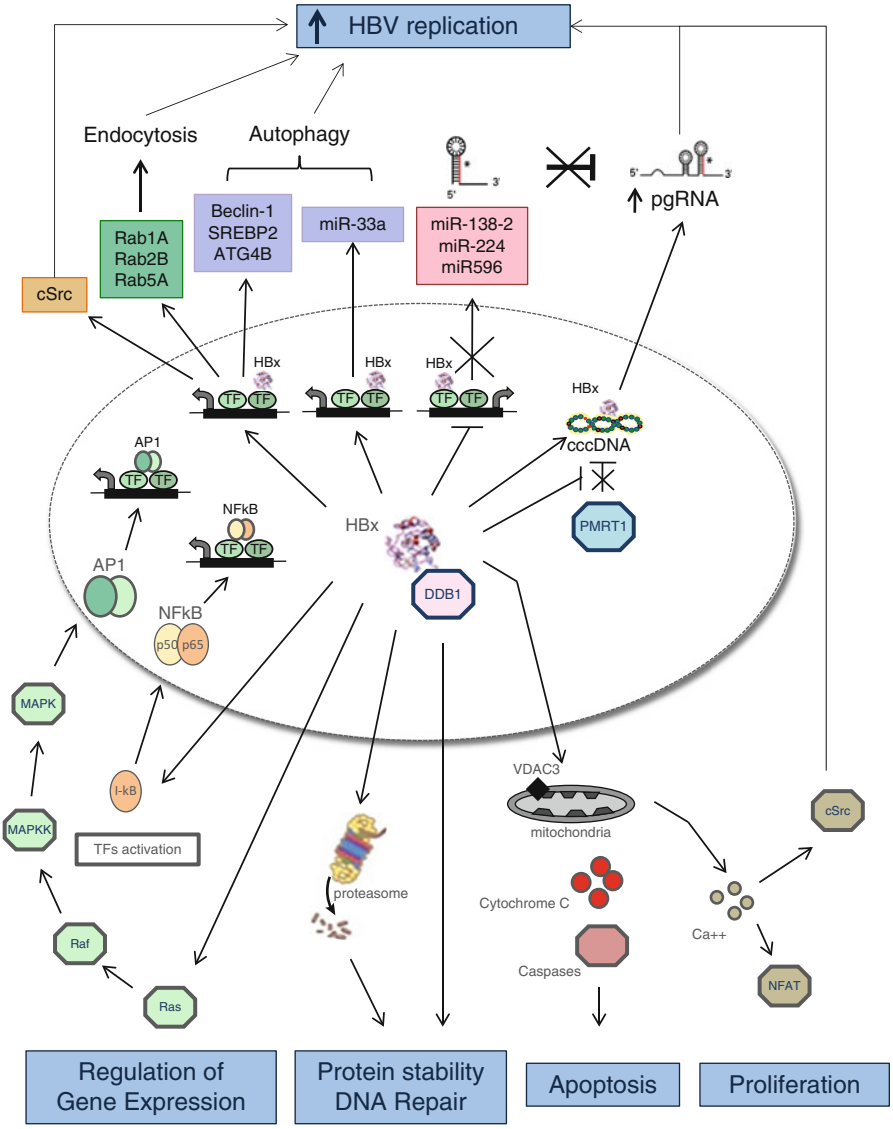

Fig. 5.2 Multiple cellular targets of the regulatory protein HBx. The regulatory protein HBx, in addition to be required for viral replication, contributes to hepatocytes transformation by multiple mechanisms, mediated by its interaction with a large number of cellular targets. HBx physically interacts with several cellular proteins that modulate cell proliferation, cell death, transcription, and DNA repair

expression through the induction of miR-101 [129], induction of endocytosis and autophagy that are required for viral replication [130–132], binding to the UV-DDB1 protein [133], and elevation of cytosolic calcium levels [134].

ChIP-Seq genome-wide analysis of HBx chromatin recruitment in HBV-replicating cells revealed a specific binding of HBx to a large number of new and known HBx target sequences [135], including protein-coding genes and ncRNAs [16 lncRNA promoters and 32 lncRNA intragenic regions, 44 snoRNA, 3 snRNA, and 75 microRNA promoter regions]. 39 out of the 75 HBx-targeted miRNAs are classified as intragenic and 15 of them display HBx peaks in the promoter region of their target genes. Multiple transcription factors seem to mediate the recruitment of HBx on the target chromatin (i.e., E2F1, CREB, β-catenin, NFkB) [135]. Pathway analysis of the protein-coding genes and miRNAs potentially regulated by HBx showed an enrichment in genes/ncRNAs involved in cell metabolism, chromatin dynamics and cancer but also in genes/ncRNAs that modulate HBV replication. (Ras, calcium transport, endocytosis, MAPK/WNT pathways, Src, the EGF/HGF family). Functional experiments identified new mechanisms by which HBx, in addition to its activity on the viral cccDNA, boosts HBV replication, mediated by direct transcriptional activation of genes and miRNAs that potentiate endocytosis (RAB family) and autophagy (ATGs, beclin-1, miR-33a) and the transcriptional repression of miRNAs (miR-138, miR-224, miR-596) that directly target the HBV pgRNA and would inhibit HBV replication [135].

Mechanistically, the activity of HBx on transcription of both cellular genes and the viral genome rely on the interaction with transcription factors and chromatin modifying enzymes and the modulation gene expression through epigenetic modifications (Fig. 5.3). Indeed, HBx binds several nuclear proteins involved in the regulation of transcription including component of the basal transcriptional machinery (RPB5, TFIIB, TBP, TFIIH), coactivators (CBP, p300, and PCAF) and transcription factors (ATF/CREB, ATF3, c/EBP, NF-IL-6, Ets, Egr, SMAD4, Oct1, RXR receptor, p53) [4]. HBx binds in vivo to the promoters of a number of CREB-regulated genes and increases the recruitment of CBP/p300 to these promoters leading to increased gene expression [136]. More recently, the same group has also shown that HBx prevents the inactivation of CREB by a PP1 (protein phosphatase 1)/HDAC1 complex [31].

HBx also increases total DNA methyltransferase (DNMT) activity by the upregulation of DNMT1, DNMT3A1, and DNMT3A2 [137] and, by relocating DNMT3a [138], selectively facilitates the regional hypermethylation of the promoters of certain tumor-suppressor genes, such as p16/INK4A (Fig. 5.3).

Animal models of HBx- and HBV-mediated tumorigenesis downregulate the chromatin-modifying proteins Suz12 [a component of the polycomb repressive complex 2—PRC2, that directs the (tri)methylation of lysine 27 on histone 3 9H3K27Me3) and gene silencing] and ZnF198 [that stabilizes the LSD-Co-RESR-HDAC1 repressor complex] in liver tumors [110]. SUZ12 and ZNF198 are targeted to poly-ubiquitibnation and proteasomal degradation by a Plk1-dependent phosphorylation that is enhanced by the long noncoding RNA HOTAIR that serves

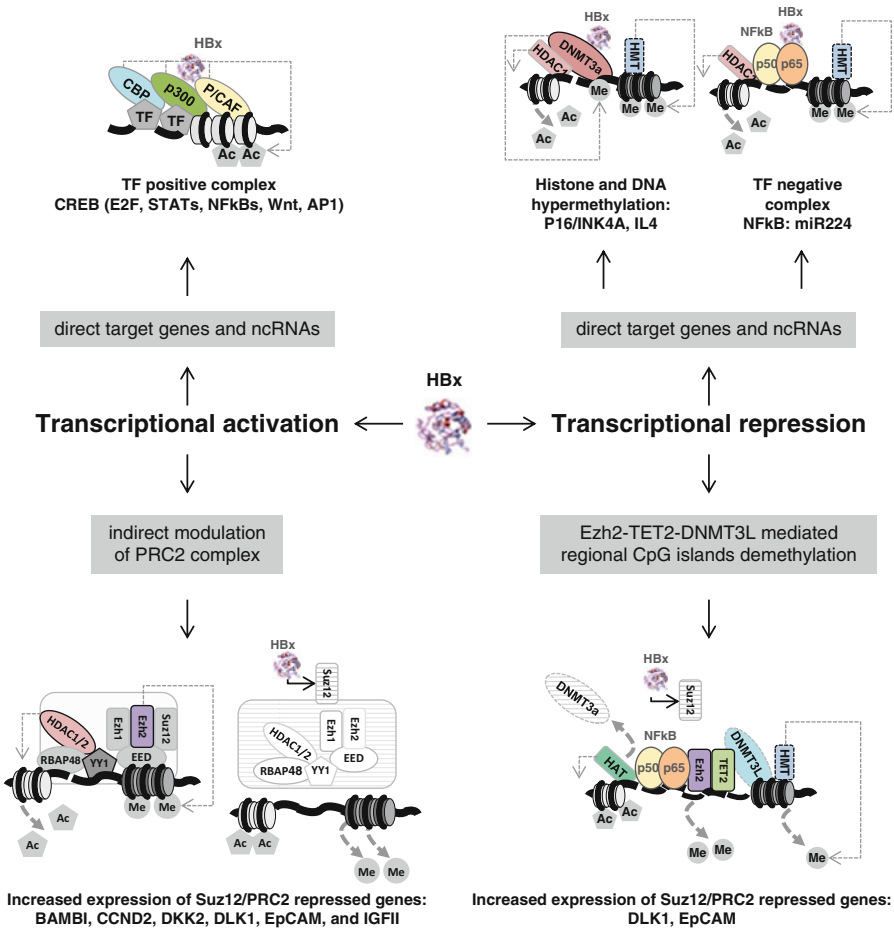

Fig. 5.3 HBx and chromatin dynamics. HBx binds several nuclear proteins involved in chromatin dynamics and the regulation of transcription: (**a**) HBx binds in vivo and increases the recruitment of CBP/p300 to the promoters of CREB-regulated genes (*upper left panel*); (**b**) HBx induces the PLK1- and proteasomal-dependent degradation of Suz12 [a component of the polycomb repressive complex 2—PRC2, that directs the (tri)methylation of lysine 27 on histone 3 (H3K27Me3) and gene silencing], leading to the overexpression of Suz12/PRC2 direct target genes, including the hepatic cancer stem cell markers BAMBI and EpCAM (*lower left panels*); (**c**) in Suz12-depleted cells expressing HBx a regional DNA de-methylation mediated by a complex containing Ezh2, TET2 and DNMT3L around half-NFkB sites releases the expression of EpCam and DLK1 expression (*lower right panel*) (**d**) HBx downregulates gene and ncRNAs transcription by (1) promoting the re-location of the DNMT3a DNA methyl-transferase to facilitate the regional hypermethylation of the promoters of certain tumor-suppressor genes, such as p16/INK4A and (2) by converting positive into negative transcription factors complexes (*upper right panels*)

as a bridge between the PRC2 and the LSD-Co-RESR-HDAC1 repressor complexes [139]. Suz12/Znf198 downregulation is accompanied, both in animal models and human HBV-related HCCs, by the overexpression of a number of Suz12/PRC2 direct target genes, including the hepatic cancer stem cell markers BAMBI and EpCAM [110] (Fig. 5.3). EpCAM over-expression in hepatic cells that have lost Suz12 is mediated by HBx and involves an active demethylation of CpG dinucleotides flanking NF-κB-binding sequences and the formation of a multiprotein complex containing the transcription factor RelA, the methyltransferase EZH2, the TET2 enzyme catalyzing the conversion of 5-methylcytosine to 5-hydroxymethylcytosine, and the catalytically inactive DNMT3L [140] (Fig. 5.3).

HBx, Oxidative Stress, and Apoptosis

HBx has been shown to have both pro-apoptotic and anti-apoptotic properties, depending on its levels, the cell context (i.e., quiescent hepatocytes, neoplastic or preneoplastic liver cells with defective growth control, liver progenitor cells) and the experimental system used. In HBV replicating cells HBx promotes cytosolic calcium signaling, resulting in Ca2+ accumulation in mitochondria, increased levels of ROS [134] and the activation of the PYK2 and SRC kinases, which also promote HBV replication [32]. HBx also binds to mitochondrial voltage-dependent anion-selective channel protein 3 (VDAC3) [141], leading to membrane depolarization, reactive oxygen species (ROS) production [141] and eventually apoptosis [142]. Ca2+ signaling and increase ROS levels trigger ER stress and the unfolded protein response (UPR) [143]. On the other hand, high levels of HBx have been reported to block tumor necrosis factor-α (TNFα)- and FAS-mediated apoptosis by activation of NFκB [144], suggesting that infected hepatocytes may survive immune-mediated damage whereas uninfected hepatocytes undergo apoptosis in CLD.

HBx, Epithelial–Mesenchymal Transition, and Fibrogenesis

Epithelial–mesenchymal transition (EMT) plays multiple roles in the pathogenesis of CLD by promoting fibrogenesis, tumor progression, and metastasis. TGFβ is central to EMT by inducing collagen synthesis and promoting transcription factors that suppress epithelial markers [145–147]. HBx upregulates TGFβ1 [148] by SMAD-dependent (via stabilization of the SMAD4 complex) [149] and non-SMAD-dependent pathways (via activation of RAS–ERK and PI3K–AKT) [150]. HBx, similar to HCV core [151, 152], also seems to convert TGFβ1 signaling from negative to positive growth regulation and shift TGFβ responses from tumor suppression to EMT. Liver fibrogenesis (type 2 EMT) and HCC metastasis (type 3 EMT) are also mediated by miR 21, which is upregulated by NFκB in HBV-associated HCC [153]. As miR 21 activation usually occurs early during HBV CLD

progression, when ROS and HBx stimulates NF-κB and AP1, these observations link HBx, chronic inflammation, and hepatocytes transformation. HBx-stimulated SRC signaling promotes EMT [154] by destabilization of adherens junctions [155]. HBx also suppresses E cadherin by promoter DNA methylation and by upregulating SNAIL [156].

HBx, Hypoxia, and Angiogenesis

Cirrhotic nodules have a "relative" defect of vasculature that may generate local reductions in oxygen tension and hypoxia, upregulate HIF1α expression, and promote angiogenesis. HBx binds to and stabilizes HIF1α and stimulates HIF1α transcription [157], thus promoting angiogenesis and cell "stemness." HBx also promotes angiogenesis by upregulating the pro-angiogenic growth factor angiopoietin 2 (ANG2) [158]. HIF1α is also stabilized by increased insulin-like growth factor receptor 1 (IGFR1), epidermal growth factor receptor (EGFR) and PI3K–AKT signaling that are all activated by HBx [159].

HBx and Hepatic Stem/Progenitor cells (HSCs/HPCs)

Stemness markers [such as NANOG, OCT4, SOX2, and Krüppel-like factor 4 (KLF 4)] are reactivated and expressed in HCC [160]. About 20–40 % of HCCs display phenotypic markers of hepatic progenitor cells (HPCs) [161, 162] and share a common transcriptional signature with normal HPCs in cDNA microarray-based analysis [163]. HCCs expressing progenitor cell features have a worse prognosis and higher recurrence after treatment compared to HCCs, which are negative for these markers analysis [163]. Although a clinicopathological analysis of surgically resected HCC specimens suggested that $EpCAM^+$ CSCs were more frequently detected in HBV-related HCCs than in HCV-related HCCs [162] a validation on larger independent cohorts including HCCs from multiple etiologies is still lacking. HPCs (also called oval cells in rodent models of carcinogenesis) are small epithelial cells that can differentiate towards both hepatocytes and cholangiocytes and are located in the smallest branches of the biliary tree (canal of Herring and/or the ductular compartment). In animal models, liver cancers can originate from hepatocytes as well as from immature progenitor cells [164]. HBx promotes the expression of NANOG, KLF4, OCT4, and MYC as well as EpCAM (epithelial cell adhesion molecule) and β-catenin [160]. Stabilization of β-catenin transcriptionally upregulates EpCAM [160] and promotes the transcription of stemness genes in association with TCF/LEF1, OCT4, and NANOG. EpCAM+ cells display CSC-like properties and generate invasive tumours in HCC xenograft experiments [162]. HBx also promotes the expression of miR 181 family members, which upregulate EpCAM [165] and are highly expressed in embryonic livers, in HSC, and in patients with α-fetoprotein (AFP)-positive tumours [165].

HBx, Senescence, and Telomeres

Inflammation, oxidative, and oncogenic stress accelerate cellular senescence in chronic HBV (and HCV) infections. In cirrhotic livers, hepatocytes display decreased proliferation rates with a dominant replicative senescence phenotype, critically shortened telomeres and reduced regenerative potential [1]. Indeed, the length of telomeres progressively shortens from normal liver to chronic liver disease, and reaches the shortest levels in HCC [166, 167]. Senescence limits the proliferation of damaged cells and reduces the risk of malignancy by triggering the expression of tumor suppressors [168]. Transformed hepatocytes must bypass senescence and can survive despite critically shortened telomeres. Many studies have indeed showed that 80–90 % of HCCs display a high telomerase activity [169]. TERT promoter mutations activating telomerase expression represent the single most frequent genetic alteration in HCC [170, 171] but are less represented in HBV-related HCCs that re-activate TERT by other mechanisms including the integration of HBV DNA sequences into the TERT gene [111, 113, 121, 122] and the upregulation of TERT expression by HBx and PreS2 proteins [172]. Despite TERT activation telomers remain very short in HCC cells, predisposing to occasional telomere instability, chromosomal instability and polyploidy [172]. Indeed, the majority of HCC cells display a high incidence of chromosome instability that, similar to the accumulation of senescent cells [1], is already evident in cirrhotic liver tissues and increases during the hepato-carcinogenesis process [173]. LOH rate is higher in HBV-related HCCs [173] and HBx directly induces chromosomal instability by affecting the mitotic checkpoints [174]. HBx also binds and inactivates p53 and interacts with the DNA repair protein DDB1, which in turn affect repair functions and allow the accumulation of genetic changes [32]. RAS signaling and the AKT–ARF–p53–p21 and RAS–MEK–ERK–INK4A/p16–RB pathways, linked to oncogene-induced senescence (OIS) [175], are both active in HCC and are activated by HBx [176–178]. At the same time, HBx contributes to overcoming senescence by: a) upregulating DNA methyltransferases (DNMTs) [89]; b) inhibiting the p53 nucleotide excision repair and transcription-coupled repair functions [179]; and c) decreasing the expression of the p53 activators ASPP1 and ASPP2 [92]. HBx also suppresses the cyclin-dependent kinase (CDK) inhibitors INK4A and p21 via promoter methylation, resulting in the inactivation of the RB tumor suppressor [180]. miR 221, which is upregulated in HBV- (and HCV)-related HCCs, blocks the expression of the CDK inhibitor p27 and promotes tumor growth and progression by activation of the PI3K–AKT–mTOR pathway [181]. HBx also interacts with the peptidyl-prolyl cis/trans isomerase Pin1 and this interaction leads to HBx stabilization, enhanced HBx-mediated transactivation of target genes, and increased cellular proliferation [182].

HBx, Tumor Promotion, and Tumor Progression

Despite the large number of published studies, we still lack a unifying picture of HBx role in liver carcinogenesis that reconcile all HBx reported activities. Both wild-type HBx and truncated HBx proteins could demonstrate oncogenic functions and promote tumorigenesis [183–186]. However, it is not yet clear whether mutated HBx proteins "gain" oncogenic functions or rather "lose" activities that would restrain the oncogenic potential of wild-type HBx or that would not be no longer required for tumor progression. The recent demonstration in a large series of HBV-related HCCs that premature stop codon and large deletions leading to a complete inactivation of the HBx gene are selected and accumulate in the tumors in contrast to the surrounding non-tumor liver tissues in more than 70 % of the tumors suggests that HBx inactivation could have a role in liver carcinogenesis or tumor progression. The reported correlation between HBx inactivating mutations, the presence of TP53 mutations, a G1–G3 transcriptomic profile [83], an abnormal expression of onco-fetal genes (EPCAM, AFP and KRT19), and poorer prognosis [84] adds a further layer of complexity to the understanding of HBx contribution to HCC development.

HBc Protein

We and others have shown that the HBV capsid protein HBc not only binds the HBV minichromosome, i.e., the cccDNA nuclear replicative intermediate [25, 27] but also a subset of cellular genes involved in innate immunity, inflammatory responses, and the control of cell proliferation [187–189].

PreS/S Proteins

The potential pro-oncogenic role of mutated envelope proteins has been confirmed in many studies in transgenic mice and cell cultures [48, 61, 190–193]. PreS2 mutants may induce cyclin A and cyclooxygenase-2 overexpression leading to cell proliferation and anchorage-independent growth [65, 66]. PreS2 mutated proteins also directly interact with the Jun activation domain-binding protein 1 (JAB1), thus triggering cyclin-dependent kinase (Cdk) inhibitor p27 degradation, Retinoblastoma hyper-phosphorylation and cell cycle progression [64]. Cyclin A is located in the cytoplasm rather than in the nucleus in preS2 mutant-transgenic mice where favors centrosome over-duplication and consequently chromosome instability [61, 66]. Finally, the ER stress response induced by preS-mutated proteins increases vascular endothelial growth factor-A (VEGF-A) expression [193]. PreS/S sequences deleted at the 3′-end and producing functionally active MHBst are found in many viral

integrates from HBV-associated HCCs [50, 190, 194–196]. MHBst proteins retained in the ER trigger a PKC dependent activation of c-Raf-1/MEK/Erk2 signal transduction cascade, induction of AP-1 and NF-kB transcription factors, and an enhanced proliferative activity of hepatocytes [191, 197]. MHBst directly interact with a preS2-responsive DNA region in the hTERT promoter, resulting in the upregulation of telomerase activity and in the promotion of HCC development [192]. On the other hand, the inappropriate expression and accumulation of wild-type large envelope protein in ER membranes can be directly cytotoxic to the hepatocyte and initiate a cascade of events that ultimately progress to malignant transformation [59, 198].

Conclusions

HBV is a major risk factor worldwide for developing HCC. HBV contributes to hepatocellular carcinoma (HCC) development through direct and indirect mechanisms. Productive HBV infections triggers inflammation, continuous necrosis mediated by the immune response against infected hepatocytes, and cell regeneration favoring the accumulation of genetic and epigenetic lesions. HBV DNA integration into the host genome occurs at early steps of clonal tumor expansion and induces genomic instability and eventually direct insertional mutagenesis. Prolonged expression of the viral regulatory protein HBx and the large envelope protein deregulate the cellular transcription program and proliferation control and sensitize liver cells to carcinogenic factors. Epigenetic changes targeting the expression of tumor suppressor genes occur early in the development of HCC. A major role is played by HBx that is recruited on cellular chromatin and modulates chromatin dynamics at specific gene loci. Genome wide approaches begin to identify homogeneous subgroups of HBV-related tumors with defined genotypes and signaling pathways alterations.

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