Review

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Review

Mechanisms of HBV-induced hepatocellular carcinoma

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Summary

Hepatitis B virus (HBV) contributes to hepatocellular carcinoma (HCC) development through direct and indirect mechanisms. HBV DNA integration into the host genome occurs at early steps of clonal tumor expansion and induces both genomic instability and direct insertional mutagenesis of diverse cancer-related genes. Prolonged expression of the viral regulatory protein HBx and/or altered versions of the preS/S envelope proteins dysregulates cell transcription and proliferation control and sensitizes liver cells to carcinogenic factors. Accumulation of preS1 large envelope proteins and/or preS2/S mutant proteins activates the unfold proteins response, that can contribute to hepatocyte transformation. Epigenetic changes targeting the expression of tumor suppressor genes occur early in the development of HCC. A major role is played by the HBV protein, HBx, which is recruited on cellular chromatin and modulates chromatin dynamics at specific gene loci. Compared with tumors associated with other risk factors, HBV-related tumors have a higher rate of chromosomal alterations, p53 inactivation by mutations and overexpression of fetal liver/hepatic progenitor cells genes. The WNT/β-catenin pathway is also often activated but HBV-related tumors display a low rate of activating β-catenin mutations. HBV-related HCCs may arise on non-cirrhotic livers, further supporting the notion that HBV plays a direct role in liver transformation by triggering both common and etiology specific oncogenic pathways in addition to stimulating the host immune response and driving liver chronic necro-inflammation.

Introduction

Hepatocellular carcinoma (HCC) represents, with an estimated 500,000–600,000 deaths/year [1,2], the second cause of cancer death worldwide [3]. HCC development is driven by the interaction of genetic predisposition, environmental factors (metabolic syndrome, alcohol, aflatoxin B1, aristocholic acid) and viruses (hepatitis B virus (HBV), hepatitis C virus (HCV)). Despite the establishment of HBV vaccine programs since the early 90s, the decreased incidence of HBV new infections in many countries and the availability of potent antiviral treatments that lead to long-term inhibition of HBV replication, 240 million people are chronically infected with HBV and remain at risk of developing liver cirrhosis and HCC [2,3].

Driving forces in hepatocyte transformation, HCC development and progression are chronic inflammation, DNA damage, epigenetic modifications, senescence and telomerase reactivation, chromosomal instability and early neoangiogenesis. Although most HCCs develop in the context of liver cirrhosis, which is recognized as a pro-carcinogenic field, HCC can also develop in non-cirrhotic livers. All "etiologic" factors seem to act through similar mechanisms (i.e. point mutations, chromosomal aberrations, epigenetic changes) that converge to affect common pathways. Notably, mutations and chromosomal aberrations have been predominantly found in benign and malignant tumor tissues whereas the dysregulation of signaling pathways and epigenetic changes are also detected earlier in the natural history of HCC development, at the stage of cirrhosis. In the last 10 years, genome-wide technologies and next generation sequencing (NGS) have enabled the identification of molecular signatures to classify subgroups of HCCs and stratify patients according to prognosis, and have highlighted the role of pathways previously underexplored in the HCC field, such as chromatin remodeling and autophagy. The molecular

pathogenesis and classification of HCCs and their impact on the design of new therapeutic approaches has been the object of several recent and comprehensive reviews [4–8]. Here, we focus on the molecular characterization of HBV-related carcinomas, the contribution of HBV genetic variability, HBV integration into the host genome and wild-type and mutated/truncated viral proteins to HCC development.

Epidemiology and co-factors

Recent estimates attribute over 50% of HCC cases worldwide to HBV [1,2], making it the most common carcinogen after tobacco. 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 [9]) and after hepatitis B surface antigen (HBsAg) clearance [10–12]. The fraction of HCC attributable to HBV reflects the distribution of HBV infection and varies significantly in the different geographical areas, representing less than 20% of all cases of HCC in the United States and up to 65% in China and Far East. Europe comprises low risk (18% in west and north Europe) and high risk (51% in east and south Europe) [3].

The lifetime risk of developing HCC is 10- to 25-fold greater for chronic HBV carriers, as compared with non-infected populations [13]. HBV-related HCC patients are a younger age at presentation compared with HCC cases related to alcohol, non-alcoholic steatohepatitis, and HCV. Also, in HBV-related HCC, cirrhosis is absent in up to one-third of patients whereas HCC related to other risk factors are developed in 80% of the cases in a cirrhotic ground [13,14]. Hepatitis severity and co-infection with hepatitis D virus and HCV, or human immunodeficiency virus (HIV) have been found to augment the risk of HCC in chronic HBV infection.

Increasing age, reflecting longer exposure to HBV, and male gender, have long been known to enhance the risk for HCC [14]. Gender disparity in HCC risk, resulting in three males developing HCC for 1 female, may reflect a protective role of estrogen, mediated by complex networks that involve hepatocyte nuclear factor-4a [15] and IL-6 signaling [16] but other explanations remain to be identified. Alcohol consumption plays a synergistic role, with a more than 2-fold increase of the carcinogenic risk of HBV [17]. 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. The combined exposure to aflatoxin B1 (AFB1) and HBV chronic infection leads to the particularly high HCC frequency in some subtropical areas of Asia and Africa [1,18]. Many other known etiological factors of HCC development, including haemochromatosis, non-alcoholic fatty liver diseases and diabetes act as co-factors of overt and occult HBV infection for HCC development [17–19].

HBV life cycle, viral heterogeneity and HCC

HBV genomic variability is attributed to lack of proof-reading by the HBV polymerase and the high copy number of the virus. This leads to the selection of HBV quasi-species containing several mutations; some providing a replicative advantage to the virus while others are detrimental. Circulating infectious HBV particles contain a circular partially double-stranded DNA of about 3200 nucleotides [20]. Soon after infection, the HBV DNA is converted into a covalently closed circular DNA (HBV cccDNA) that accumulates in the nucleus of infected cells as a stable episome organized into nucleosomal structures [21–23]. HBV cccDNA minichromosome is responsible for persistent HBV infection of hepatocytes and is the template for the transcription of all viral mRNAs. The 3.5 kb pre-genomic HBV RNA (pgRNA), is encapsidated into cytoplasmic core particles where it is reverse transcribed by the viral polymerase to produce the first HBV DNA strand and sustain the viral replication within the cell [20]. Chromatin modifying enzymes, cellular transcription factors and the viral proteins HBx and HBc are recruited on the cccDNA minichromosome to regulate its transcription and, ultimately, viral replication [21–26]. The goal of therapy in chronic hepatitis B (CHB) is the persistent suppression of HBV replication [27]. Due to the lack of direct effect on the cccDNA in the nucleus, a sustained suppression of HBV replication by nucleos(t)ide analogs (NUCs) does not lead to cccDNA eradication [22]. Long-term inhibition of HBV replication by NUCs reduces the risk of HCC in non-cirrhotic patients by preventing progression to cirrhosis whereas in cirrhotic patients the reduced rates of liver mortality due to strong impact on clinical decompensation have not been paralleled by a similar impact on HCC development and HCC related mortality [27,28].

The integration of viral DNA into the host genome, that occurs randomly in regenerating infected hepatocytes [20], does not contribute to HBV replication but is an important factor in 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

Key point

HBV is responsible for over 50% of HCCs; the second cause of cancer death worldwide.

transforming properties [6]. A clonal expansion of hepatocytes containing unique virus-cell DNA junctions formed by the integration of HBV DNA can be detected in patients at various stages of chronic infection [29]. Although the size of clones with integrated HBV DNA tended to increase with age, inflammatory activity, progression of fibrosis and sero-conversion to anti-HBe, they contained mostly normal-appearing hepatocytes and their size or frequency did not differ between patients with and without a coexisting HCC [29]. The nature of the selective advantages that sustain the expansion of hepatocyte clones containing integrated HBV DNA and whether they represent a true pre-neoplastic condition as well as their relation with early HCCs are still unclear.

The risk of developing HCC also correlates with HBV replication, HBV genotype and HBV genomic mutations [11,30]. High serum HBV DNA levels correlate in the clinical setting with liver damage accumulation, evolution to cirrhosis and HCC development [11]. At least 8 different HBV genotypes with a nucleotide sequence variation >8% have been identified (A-H). HBV genotype C has been associated with a higher risk of HCC development [31]. 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 [32]. 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 [32].

Key point

HBV contributes to HCC development through direct and indirect mechanisms.

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 [33] 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 [33].

Point mutations, deletions, or insertions in the preS1 and preS2 sequences are often found in patients with long-lasting CHB and HCC (reviewed in [34]). 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 [35] and the predictive value of preS deletion mutants has been recently confirmed in a prospective study [36]. 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 [34]. Both preS1 and preS2 mutations may lead to unbalanced production of the different envelope proteins and the accumulation of mutated L protein in the endoplasmic reticulum (ER) of hepatocytes, resulting in the activation of the ER stress signaling pathway [34,37–40]. ER stress can generate reactive oxygen species (ROS) and cause oxidative DNA damage, genomic instability, and ultimately favor HCC development [34,40–44].

HBV mutants with premature stop codon at position 172 or 182 of the S gene have also been frequently found in patients with cirrhosis and HCC [45–48]. 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 [49]. Importantly, the emergence of the rtA181T/sW172* mutant is associated with an increased risk of developing HCC in patients [50].

The genetic/epigenetic landscape of HBV-related HCC

Extensive evidence indicates that HCC is an extremely heterogeneous tumor at the genetic and molecular level, with a complex mutational landscape and multiple transcription and signaling pathways involved [8] (Fig. 1).

Genetic predisposition

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 [8]. Genome-wide association studies have identified SNPs associated with HCC development in the *STAT4* (a key protein of the inflammatory pathway) [51], *TPTE2* (a homolog of PTEN) [52], *DCL1* (a tumor suppressor gene implicated in HCC pathogenesis) [53] genes and in the region containing the *UBEB4*, *KIF1B* and *PGD* [54] genes. Additional SNPs associated with HCC development in patients with chronic hepatitis B have been described in the *CTL-4* (cytotoxic T-lymphocyte antigen 4) [55], *TGF-β1* (transforming growth factor beta1) [56], *XRCC3* (X-ray repair complementing defective repair in Chinese hamster cells 3) [57], *MDM2* (mouse double minute 2 homolog) [58], *MTHFR* (methylenetetrahydrofolate reductase) and *TS* (thymidylate synthase) [59]. SNPs of *GSTM1* (Glutathione-S-Transferase Mu 1) and *GSTT1* (Glutathione S-transferase theta-1) genes

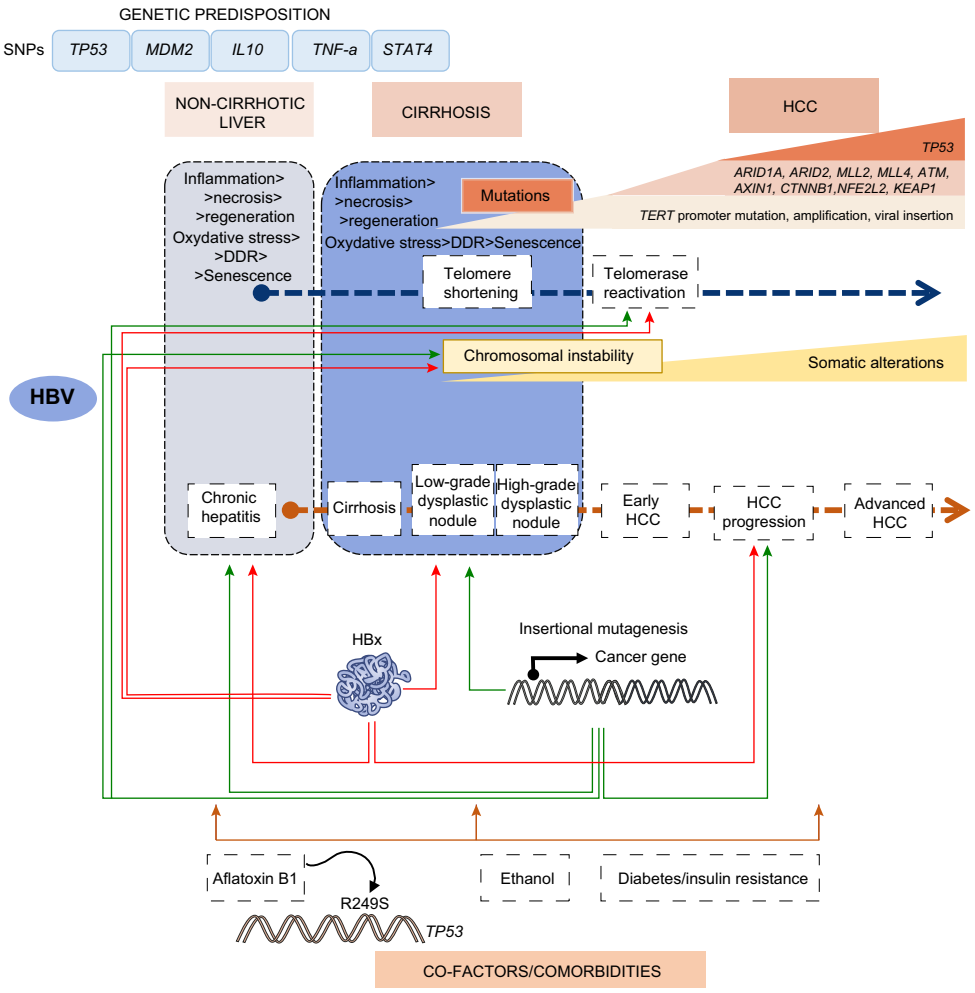

Fig. 1. Interactions between virus, host genetic predisposition and environmental factors in HBV-related HCC development and progression. HBV contributes to HCC by: a) insertional mutagenesis due to integration of the viral DNA into host chromosomes; b) increased genomic instability caused by both viral integration and the activity of the viral protein HBx; c) reactivation of telomerase activity, d) modifications of the epigenome promoted by HBx and HBc; e) 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).

confer a high risk of HCC development to chronic HBV patients exposed to AFB1, suggesting a functional interplay between genetic predisposition, exposure to genotoxic contaminants and HBV persistence [60]. Overall, the predictive power of all these polymorphisms seems to be low and need to be validated in larger cohorts of multiple ethnicity and in at different stages of the liver diseases.

Chromosomal aberrations and gene mutations

The combined analysis of whole-exome sequencing data from over 1150 HCCs has unveiled a complex repertoire of somatic genetic alterations with 35 to 80 mutations in coding regions per tumor and has allowed the identification of six major pathways that are recurrently mutated in HCCs [61–67].

Telomere maintenance

In HCCs, telomerase is reactivated in more than 90% of cases [61], due to somatic *TERT* promoter mutations (54–60%) [61,68], *TERT* amplification (5–6%) [61], or HBV insertion in the *TERT* promoter (10–15%) [69,70]. In HBV-related HCCs, *TERT* re-expression can also be the result of a direct transcriptional activation of the *TERT* promoter by the wild-type HBx protein as well as truncated HBx and MHBst proteins [71,72]. *TERT* promoter mutations are more frequent in HCV-related HCCs in association with *CTNNB1* mutations, suggesting functional cooperation between telomerase maintenance and β-catenin pathway in liver tumorigenesis [61,62,68].

WNT/β-catenin pathway

The WNT/β-catenin pathway is frequently activated in HCC by activating mutations of *CTNNB1*

Key point

HBV-related HCCs may arise on non-cirrhotic livers and display a high rate of chromosomal alterations, p53 inactivation by mutations and overexpression of fetal liver/hepatic progenitor cells genes.

(11–37%) [73], inactivating mutations of *AXIN1* (5–15%) [74] or *APC* (1–2%). *CTNNB1* mutations include substitutions or in-frame deletions in the amino-terminal domain targeted by the APC/AXIN1/GSK3B inhibitory complex [73]. Mutated β-catenin HCC accumulates and migrates into the nucleus to activate transcription from β-catenin target genes like *GLUL* and *LGR5* [75]. When compared to HCC related to alcohol, HCV or non-alcoholic steatohepatitis (NASH), HBV-related HCC show less frequent β-catenin activating mutations and more frequent Axin1 inactivation suggesting that the mechanism that activate the WNT/β-catenin signaling can be different in HCCs according to etiology [62,63,75,76].

P53 and cell cycle pathway

Mutations of the *TP53* tumor suppressor gene are found in 12–48% of cases [62,63,77–79], with no specific recurrent *TP53* hotspot mutations [63,80] outside the R249S mutation related to AFB1 exposure that is usually identified in HBV-related HCC. The retinoblastoma pathway, that controls G1/S cell cycle progression, is inactivated by homozygous deletion of *CDKN2A* (2–12%) [61,63], *RB1* mutations (3–8%) [66], amplification of the *CCND1*/*FGF19* locus (5–14%) [81,82] and recurrent HBV insertions in *CCNE1* (cyclin E1, 5%) [69]. The inactivation of P53 and cell cycle pathway activation are particularly frequent in HBV-related HCC and they are associated with tumor aggressiveness and poor prognosis [62,66,76].

Oxidative stress pathway

Mutations activating NRF2 (coded by the *NFE2L2* gene) or inactivating KEAP1, that prevent NRF2 ubiquitinylation and its physiological degradation [63,65,83], can be found in 5–15% of the HCCs. *In vitro* studies have shown that NRF2 activation protect tumor cells from ROS and death [84].

Epigenome modifiers

Epigenome modifiers are frequently altered in HCC [62] with mutations of *ARID1A* (4–17%) [63] and *ARID2* (3–18%) [85] (part of SWI/SNF chromatin remodeling complexes (BAF and PBAF)) and mutations of H3K4 histone methyltransferases *MLL* (3–4%), *MLL2* (2–3%), *MLL3* (3–6%), and *MLL4* (2–3%) as well HBV insertions in *MLL4* (10%) [62,65,69].

RAS/RAF/mitogen-activated protein kinase and the PI3K/AKT/mTOR pathways

Activating mutations of the RAS genes are rarely observed in HCC (<2%) whereas inactivation of the *RP6SKA3* gene, coding for the RAS inhibitor RSK2, is found in 2–9% of tumors [62,64]. A persistent RAS activation has been implicated in HCC resistance to sorafenib [86]. Homozygous deletion of *PTEN*, an inhibitor of the PI3K kinase, has been identified in 1–3% of the HCC [62]. The amplification of the *FGF19*/*CCND1* locus in around 5–10% of HCCs leads to the activation of the PI3K/AKT/mTOR and RAS/RAF/mitogen-activated protein kinase [81,87], whereas activating mutations of *PIK3CA* (0–2%) and inactivating mutations of *TSC1* or *TSC2* (3–8%) lead to activation of the AKT/mTOR signaling [61,62,67].

HBV-related tumors generally harbor a higher rate of chromosomal abnormalities than tumors linked to other risk factors [8,62,63], 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 specific genetic alterations including viral DNA integration and *IRF2* inactivation [63]. However, all the pathways that are dysregulated downstream of the genomic defects, are common to tumors related to other risk factors. The mechanisms at the origin of this pathways dysregulation, for example higher frequencies of *TP53* or *AXIN1* mutations, could differ and their consequences remain to be more precisely analyzed.

Transcriptomic analysis

Genome-wide transcriptomic analysis of well annotated HCCs have identified subgroups of HCC associated with specific clinical and genetic characteristics [63,75,88–91]. These studies along with a meta-analysis [92] showed that, regardless of the specific nomenclature used for each classification, HCC can be roughly divided into 2 major molecular subtypes; one characterized by an enrichment of signals related to cell proliferation and progression in the cell cycle (proliferation class), and the second class retaining more molecular features similar to normal hepatocytes (non-proliferation class).

Proliferation subclass patients display a remarkable heterogeneity at the genomic and phenotypic levels with higher rates of chromosomal instability, the enrichment in aberrant epigenetic changes and the activation of multiple pathways (AKT/mTOR [93], MET [94], TGFB [95], IGF [96], RAS/mitogen-activated protein kinase [97]). Other characteristics include: a) the expression of Notch [98], progenitor cells and hepatoblastoma-like markers [89,90,99]; b) the enrichment of gene signatures derived from hepatoblastomas and cholangiocarcinomas [100–102]; c) a progenitor cells-like DNA methylation-based signature [103]; d) a prominent dysregulation of miRNAs [104]. Proliferation class tumors are more aggressive, with higher α-fetoprotein (AFP) levels, moderately/poor cell differentiation, frequent vascular invasion, higher risk of recurrence after resection and lower survival rates [87,91,105].

Non-proliferation subclass tumors show less aggressive phenotype, better histologic differentiation, lower AFP, lack of enrichment in poor prognosis signatures. Nuclear β-catenin accumulation, upregulation of liver-specific Wnt-targets and enrichment of *CTNNB1*-mutations are present in up to 25% of cases [75,106]. Subgroups of patients within this class show an enrichment in inflammation-related signatures or show broad gains in chromosome 7, associated with overexpression of epidermal growth factor (EGF) receptor [107].

HBV-related HCCs are predominantly tumors belonging to the proliferation class whereas HCV and alcohol-related HCCs are more prevalent in the non-proliferation class [62,63,75]. In a study focused on the molecular characterization of HBV-related HCCs, Amaddeo and colleagues [76] confirmed that *TP53* 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, which controls p53 protein activation, was exclusively identified in HBV-HCC (7%). However, HBV-related HCCs display a wide genomic diversity and can belong to all transcriptomic subgroups. HBV-related HCCs with non-proliferation class profiles were found in older patients with other co-factors such as HCV, alcohol consumption or NASH [76].

Non-coding RNAs

Non-coding RNAs, including miRNAs (small non-coding single-stranded RNAs that regulate gene expression, primarily at the post transcriptional level) and lncRNAs (non-coding RNAs varying in length from 200 nt to 100 kb that: a) regulate the expression of protein-coding genes; b) function as scaffold molecules for chromatin remodeling complexes c) bind one or more miRNAs acting like miRNA sponges) are increasingly recognized as key players in the regulation of liver functions and in hepatocarcinogenesis [108–110]. 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, miR155, miR-221, miR-222, miR-224, miR-301, miR331-3P, miR-373, miR-485, miR-495, miR-664), or downregulated (miR-26a, miR-29, miR-122, miR-125, miR-130a, miR-150, miR-193b, miR-199a-3p, miR-200, miR-223 and let-7 family members) in HCC [108,109]. Differences between HCV- and HBV-related HCC associated miRNAs have been reported. miR-143, miR-34 and miR-19 are upregulated in HBV-related HCC and promote the more aggressive biological phenotype of HBV-related HCCs [111,112]. Downregulation of LET7a by HBx is associated with upregulation of STAT3-induced cell proliferation [113]. HBx suppression of miR-152 leads to upregulation of DNMT1, which methylates the promoters of many tumor suppressors [114]. Finally, miR-26 expression is low in HBV-related HCCs, lower in men than in women and is associated with a poor survival and lower response to adjuvant therapy with interferon-α [115,116].

lncRNAs that are dysregulated in HBV-related HCCs include high expression in HCC (HEIH), highly upregulated in liver cancer (HULC), HBx-interspersed nuclear element 1 (HBx-LINE1), H19, maternally expressed imprinted gene 3 (MEG3), HOTAIR, low expression in tumor (LET), downregulated expression by HBx (DREH) and microvascular invasion in HCC (MVIH) [109,110]. Other lncRNAs deregulated in HCCs include metastatic lung adenocarcinoma transcript 1 (MALAT-1), HOXA transcript at the distal tip (HOTTIP), RERT, Long intergenic ncRNA regulator of reprograming (lincRNA-RoR), lincRNA–UFC1, downregulated in liver cancer stem cells (lncRNA.DILC) [110,117].

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 remodeling complexes.

Global hypo-methylation of DNA with selective hyper-methylation and silencing of a number of tumor suppressor genes promoters containing CpG islands, including *RASSF1A*, *p16/INK4A*, *APC*, E-cadherin, *SOCS-1*, IGF-binding protein 3 (*IGFBP3*) and glutathione *S*-transferase P1 (*GSTP1*), have been shown to start at the pre-neoplastic/cirrhotic stage [118]. 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 [118,119]. Genome-wide methylation profiling confirmed many genes known to be dysregulated by aberrant methylation in HCC (e.g., *RSSFA1*, *APC*, *NEFH*, *IGF2*, *RAFF5*, *NKX6.2*, *SFRP5*) as well as in other tumors (e.g., *NOTCH3*; *NSD1*; *ZIC1*) and identified new epigenetic drivers in HCCs (e.g., *SEPT9*, ephrin-B2 ligand (*EFNB2*), homeobox A9 (*HOXA9*), forkhead box G1 (*FOXG1*) and runt-related transcription factor 3 (*RUNX3*), *FGF8* and *FGF6*) [103]. A 36-probe methylation signature correlated with poor outcome in both HBV and HCV-related HCCs [103].

Histone deacetylases, *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 [120]. 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 [121]. Notably, combining the pan-HDAC inhibitor panobinostat and sorafenib strongly potentiated treatment efficacy and improved survival in HCC xenograft models [121]. An increased expression of the EZH2 and G9a histone methyl-transferases has also been reported in HCCs [122–124]. EZH2 overexpression helps to discriminate between pre-neoplastic/dysplastic lesions [124,125] and, in overt HCCs, correlates with tumor aggressiveness and poor prognosis of HCCs [123,124]. 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 [126]. The HCC-specific lncRNA HEIH associates with EZH2 to repress EZH2 target genes and facilitate HCC tumor growth in HBV-related HCCs [127] and, in particular, EZH2-mediated repression of WNT antagonists has been found to promote β-catenin-dependent hepatocarcinogenesis [128].

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 [63,69,80,85]. ARID1A and ARID1B are crucial and mutually exclusive subunits of the SWI/SNF ATPase-powered nucleosome remodeling 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.

Direct oncogenic roles of HBV

HBV can promote carcinogenesis by three different mechanisms: a) a classic retrovirus-like insertional mutagenesis with the integration of viral DNA into host cancer genes like *TERT*, *CCNE1*, and *MLL4*; b) the promotion of genomic instability as the result of both the integration of viral DNA into the host genome and the activity of viral proteins; c) the ability of wild-type and mutated/truncated viral proteins (HBx, HBc and preS) to affect cell functions, activate oncogenic pathways and sensitize liver cells to mutagens.

Key point

HBV DNA integration into the host genome is an early event that induces genomic instability and eventually direct insertional mutagenesis.

Insertional mutagenesis

HBV DNA integration in host chromosomes, although dispensable for viral replication, is an early event in HBV infection and it is detected in about 80% of HCCs [129]. HBV integration at specific genomic sites is thought to provide a growth advantage to a clonal cell population that eventually accumulates additional mutations. HBV integrations within the retinoic acid receptor β (*RARβ*) [130] and the cyclin A [131] as target genes 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 [71,132–135]. The analysis of 399 HBV integration breakpoints from 81 HBV-positive HCCs by NGS showed 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 were located near the viral enhancer and the X gene and core ORFs [69]. Most HBV breakpoints were 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) and *ROCK1* (Rho-associated coiled-coil containing protein kinase 1) genes, whose expression was upregulated in tumors *vs.* the normal tissue [69]. Additional recurrent integrations were found in the *FN1* (fibronectin 1) [69,136] and the *ARHGEF12* (Rho guanine nucleotide exchange factor (GEF) 12), *CYP2C8* (cytochrome P450, family 2, subfamily C, polypeptide 8), *PHACTR4* (Phosphatase and actin regulator 4), *PLXNA4* (Plexin A4), *RBFOX1* (RNA binding protein, fox-1 homolog (C. elegans) 1) and *SMAD5* (SMAD family member 5) genes [136] but their overexpression in the HCC tissues was not uniform [69,136].

Recurrent HBV insertion sites also occur within or near repetitive, non-coding sequences, such as long interspersed nuclear elements (LINEs), Alu (named after the restriction enzyme specifically cutting those sequences), other short interspersed nuclear elements (SINEs) and the long terminal repeats (LTR) of endogenous retroviruses (ERVs) [69,134,136]. HBV integration with the generation of an HBx-LINE1 chimeric transcript was reported in 21 out of 90 HBV-related HCC patient tumors (23%) and was significantly associated with poor patient outcome [137]. These results were confirmed in a second study from China (17 out of 40 cases, 42.5%) [138] whereas the HBx-LINE1 fusion transcripts were not detected in 50 HBV-related HCCs from Europe [139]. The high frequency of this oncogenic transcript might be restricted HBV genotype C infection that is predominant Chinese patients and needs to be validated in other independent series of HCC. Mechanistically, the HBx-LINE1 transcript was shown to act as a lncRNA and its oncogenic properties are independent from its protein product [137]. HBx-LINE1 expression drives migration and invasion of tumor cell lines through the induction of

epithelial mesenchymal transition (EMT) and the nuclear localization of β-catenin [137,138] and increases the incidence of HCCs in diethylnitrosamine treated mice [137]. Recent evidence indicate that HBx-LINE1 functions, as previously shown for other lncRNAs, by sequestering miRNAs, namely miR-122 [138]. These results highlight an opposite function of miR-122 in HCV infection, where Ago bound miR-122 promotes HCV replication via binding the viral 5′UTR, and HBV infection, where miR-122 inhibits HBV replication directly by targeting HBV transcripts [140] and indirectly by modifying the cellular environment [141]. HBx-LINE1 expression in HBV-related HCCs reduces the levels of functional miR-122 potentially leading to the derepression of hundreds of miR-122 targeted genes and the disruption of liver homeostasis [138]. The negative correlation between HBx-LINE1 levels and miR-122 abundance in HBV-related HCCs [138] and the role of miR-122 as a potent anti-inflammatory tumor suppressor in the liver [142,143] support this model.

Genomic instability

Loss of heterozygosity rate is higher in HBV-related HCCs [144] and HBV promotes genomic instability as the result of both the integration of viral DNA into the host genome and the activity of viral proteins. Whole genome sequencing of HBV-related HCCs detected an increased in copy number variations at HBV breakpoint locations indicating that HBV integration likely induces chromosomal instability [69], thus indirectly supporting the old observation that a 61-bp subgenomic HBV DNA sequence (designated as 15AB, nt 1855–1915) can act as a hot spot for genomic recombination by binding a cellular recombinogenic protein [145]. HBx directly induces chromosomal instability by affecting the mitotic checkpoints [146], binding and inactivating p53 and by interacting with the DNA repair protein DDB1 [129]. Aberrant accumulation of cleaved cyclin A in preS2 mutant-transgenic mice has been involved in centrosome over-duplication and chromosome instability [43].

Wild-type and mutated/truncated viral proteins

The third direct mechanism of HBV carcinogenesis is based on the ability of viral proteins (HBx, HBc and preS) to affect cell functions, including cell proliferation and cell viability and to sensitize liver cells to mutagens. In transgenic mouse models, the unregulated expression of the HBx and the large S proteins are associated, through different mechanisms, with hepatocarcinogenesis [39,147].

HBx protein

HBx regulatory protein is both required for HBV cccDNA transcription/viral replication [24,148], and thought to contribute to HBV oncogenicity (Fig. 2). Regulation of transcription, through a direct impact on chromatin function and transcription in the nucleus and/or indirectly mediated by the modulation of cell signaling in the cytoplasm, is thought to play an important role.

HBx, chromatin and transcriptional control. HBx is recruited to the cccDNA minichromosome in HBV replicating cells to increase transcription of the nuclear cccDNA minichromosome [24,148]. In the absence of HBx, cccDNA-bound histones are hypo-acetylated, and the cccDNA transcribes significantly less pgRNA [24]. HBx also binds and blocks the inhibitory activity on HBV transcription exerted by the PRMT1 methyltransferase [25], the Tudor-domain protein Spindlin-1 [149] and the SETDB1 histone methyltransferase [150]. Additional mechanisms by which HBx can potentiate HBV replication include: a) the downregulation of *DNMT3A* expression through the induction of miR-101 [151]; b) a direct transcriptional activation of genes and miRNAs that potentiate endocytosis (RAB family) and autophagy (ATGs, beclin-1, miR-33a) [152], both required for viral replication [153–155]; c) binding to the UV-DDB1 protein [156] and inactivation of the Smc5/Smc6-mediated restriction of cccDNA transcription (Strubin M *et al.* presented at the HBV Molecular Biology Meeting 2015); d) the elevation of cytosolic calcium levels [157] and e) the direct transcriptional repression of miRNAs (miR-138, miR-224, miR-596) that inhibit HBV replication by directly targeting the HBV pgRNA [152].

The genome-wide analysis of HBx chromatin recruitment in HBV replicating cells [152] revealed a specific binding of HBx to a large number target sequences, including protein-coding genes and non-coding RNAs (16 lncRNA promoters and 32 lncRNA intragenic regions, 44 snoRNA, 3 snRNA and 75 miRNA promoter regions) [152]. Pathway analysis showed an enrichment in genes/non-coding RNAs involved in cell metabolism, chromatin dynamics and cancer as well as genes/non-coding RNAs known to modulate HBV replication (RAS, calcium transport, endocytosis, MAPK/WNT pathways, SRC and the EGF/HGF family) [152].

Mechanistically, the activity of HBx on transcription of both cellular genes and the viral genome rely on the modulation of epigenetic modifications and the interaction with multiple transcription factors (ATF/CREB, ATF3, c/EBP, NF-IL-6, ETS, EGR, SMAD4, OCT1, RXR receptor and p53), chromatin modifying enzymes (CBP, p300 and PCAF) and component of the basal tran-

Key point

Wild type and mutated/truncated viral proteins (HBx, HBc and preS) deregulate cell transcription and proliferation control and sensitize liver cells to carcinogenic factors.

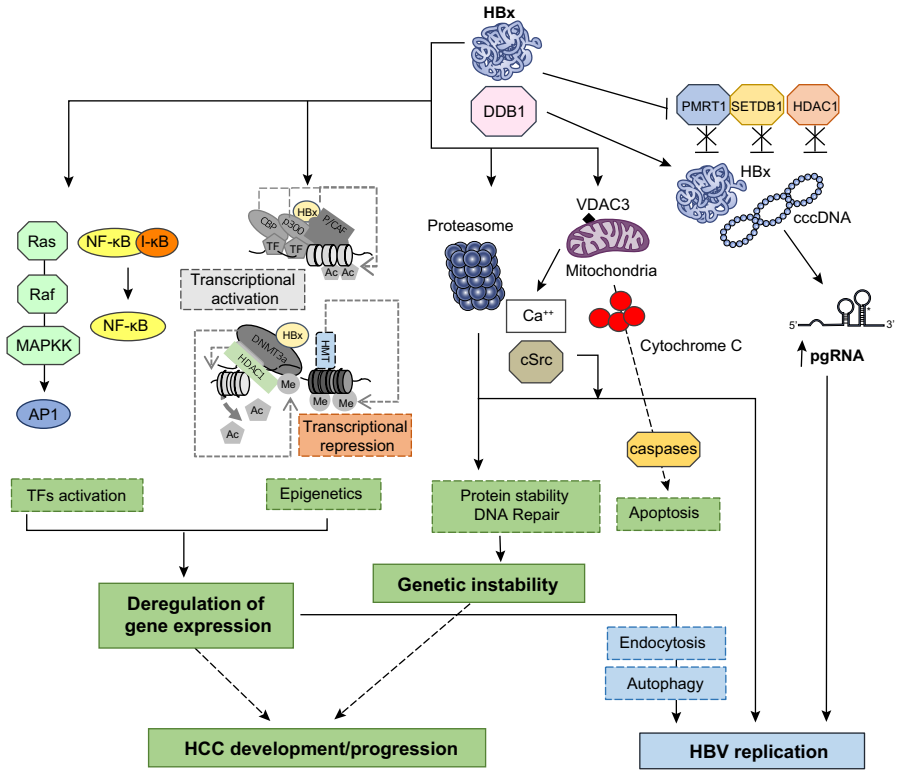

Fig. 2. HBx Networks. The regulatory protein HBx, in addition to being required for viral replication, contributes to hepatocytes transformation by multiple mechanisms, mediated by its interaction with a large number of cellular targets. HBx physically interacts with several cellular proteins that modulate cell proliferation, cell death, transcription and DNA repair.

scriptional machinery (RPB5, TFIIB, TBP and TFIIH). The functional outcome of these interactions can be either activation or repression of transcription and involves different mechanisms (Fig. 3):

a) HBx binding *in vivo* to a subset of CREB-regulated promoters, promotes their expression by increasing CBP/p300 recruitment [158] and by preventing CREB inactivation by a PP1 (Protein Phosphatase 1)/HDAC1 complex [26].
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) and ZNF198 (that stabilizes the LSD-Co-RESR-HDAC1 repressor complex), leading to the overexpression, both in animal models and human HBV-related HCCs, of SU12/PRC2 direct target genes, including the hepatic cancer stem cell markers BMP And Activin Membrane-Bound Inhibitor (BAMBI) and epithelial cell adhesion molecule (EpCAM) [159]. SUZ12 and ZNF198 degradation is enhanced by the lncRNA HOTAIR that serves as a bridge between the PRC2 and the LSD-Co-RESR-HDAC1 repressor complexes [160].
c) EpCAM and DLK1 overexpression in hepatic cells that have lost SUZ12 is mediated by HBx and involves a regional active demethylation of the CpG dinucleotides flanking NF-κB-half-sites that is mediated by the formation of a multi-protein complex containing the transcription factor RelA, the methyltransferase EZH2, the catalytically inactive DNA methyltransferase DNMT3L and the TET2 enzyme, catalyzing the conversion of 5-methylcytosine to 5-hydroxymethylcytosine [161].
d) HBx downregulation of gene and non-coding RNA transcription by: i) increasing total DNA methyltransferase (DNMT) activity through the upregulation of DNMT1, DNMT3A1 and DNMT3A2 [162]; ii) promoting the relocation of the DNMT3a to facilitate the regional hypermethylation of the promoters of certain tumor suppressor genes, such as *p16/INK4A* [163] and iii) by converting positive transcription factors complexes into negative (formation of a HBx-NF-κB repressor complex on the miR-224 promoter) [152].

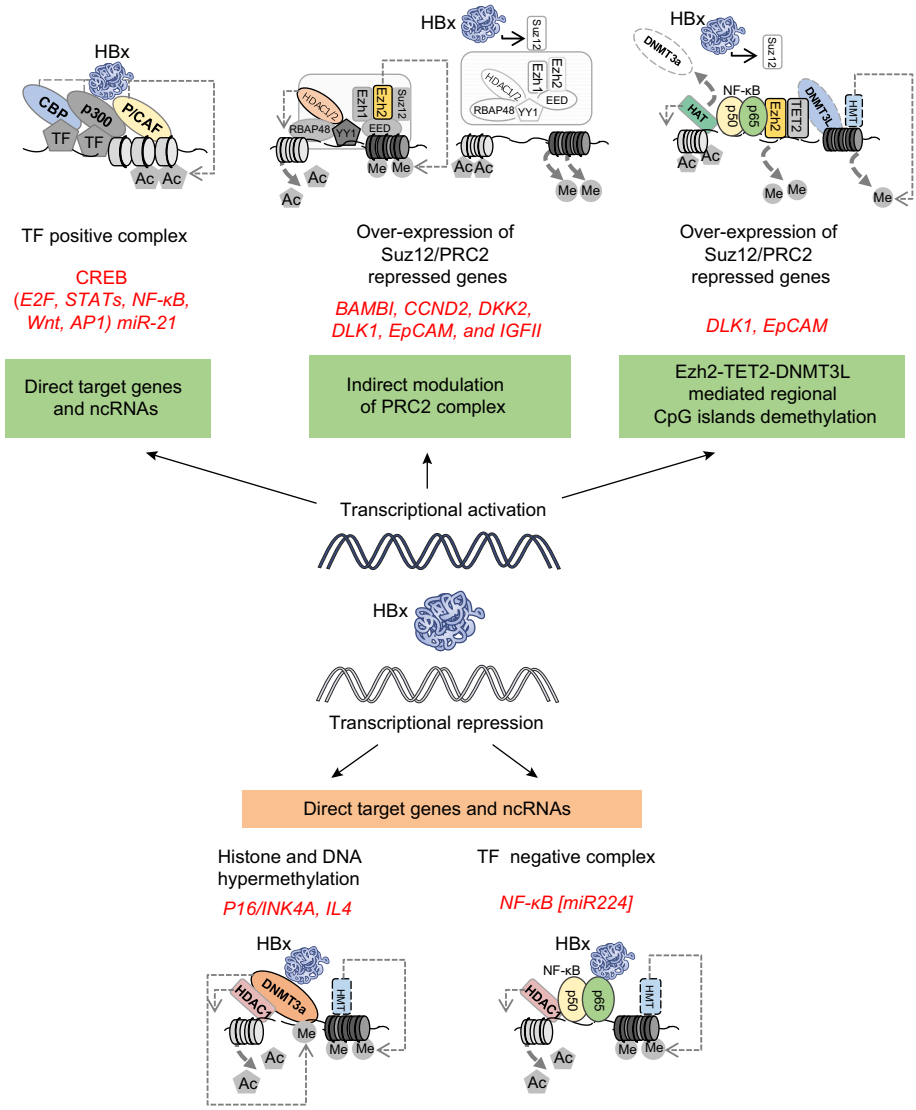

Fig. 3. HBx and chromatin dynamics. HBx binds several nuclear proteins involved in chromatin dynamics and the regulation of transcription leading to: a) an increased recruitment of CBP/p300 to the promoters of CREB-regulated genes (upper left panel); b) PLK1- and proteasomal-dependent degradation of 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 (upper mid panels); c) DNA de-methylation of Suz12/PRC2 repressed genes mediated by a complex containing EZH2, TET2 and DNMT3L (upper right panel); d) repression of gene and ncRNAs transcription by: i) promoting the re-location of the DNMT3a DNA methyltransferase to facilitate the regional hypermethylation of the promoters of certain tumor suppressor genes, such as *p16*/*INK4A* (lower right panel) and ii) by converting positive into negative transcription factors complexes (lower right panel).

HBx, senescence and telomeres. Inflammation and oxidative and oncogenic stress accelerate cellular senescence in chronic HBV (and HCV) infections [1]. Telomeres progressively shorten from normal liver to cirrhosis and reach the shortest levels in HCC [164,165]. Senescence limits the proliferation of damaged cells and reduces the risk of malignancy by triggering the expression of tumor suppressors [166]. Many studies have showed that 80-90% of HCCs display a high telomerase activity [167] to bypass senescence. *TERT* promoter mutations activating telomerase expression represent the single most frequent genetic alteration in HCC [68,168] but are less represented in HBV-related HCCs that reactivate TERT by other mechanisms including the integration of HBV DNA sequences into the *TERT* gene [70,80,133,134] and the upregulation of *TERT* expression by HBx and PreS2 proteins [169]. HBx also contributes to overcome senescence by: a) inhibiting the p53 nucleotide excision repair and transcription-coupled repair functions [170]; b) decreasing the expression of the p53 activators *ASPP1* and *ASPP2* [119]; sup-

pressing the cyclin-dependent kinase (CDK) inhibitors *INK4A* and *p21* via promoter methylation, resulting in the inactivation of the RB tumor suppressor [171].

HBx, cell proliferation and cell death. 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 pre-neoplastic 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 Ca^{2+} accumulation in mitochondria and increased levels of ROS [157], ER stress and activation of the unfolded protein response (UPR) [172]. HBx also binds to mitochondrial voltage-dependent anion-selective channel protein 3 (VDAC3) [173], leading to membrane depolarization, ROS production [173] and eventually apoptosis [174]. 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 NF-κB [175], suggesting that infected hepatocytes may survive immune-mediated damage whereas uninfected hepatocytes undergo apoptosis. HBx interaction with the peptidyl-prolyl *cis*/*trans* isomerase Pin1 leads to HBx stabilization, enhanced transactivation of HBx target genes and increased cellular proliferation [176]. Cirrhotic nodules have a "relative" defect of vasculature that may generate local reductions in oxygen tension and hypoxia that upregulate HIF1α expression and promote angiogenesis. HBx binds to and stabilizes HIF1α and stimulates HIF1α transcription [177], thus promoting angiogenesis and tumor growth. HBx also promotes angiogenesis by upregulating the pro-angiogenic growth factor angiopoietin 2 (ANG2) [178]. HBx, similar to HCV core [179,180], also seem to convert TGFβ1 signaling from negative to positive growth regulation and shift TGFβ responses from tumor suppression to epithelial–mesenchymal transition and tumor growth. HBx also directly upregulates TGFβ1 [181] and TGFβ1 signaling by SMAD-dependent (via stabilization of the SMAD4 complex) and non-SMAD-dependent pathways (via activation of RAS–ERK and PI3K–AKT) [182]. HBx also suppresses E-cadherin by promoter DNA methylation and by upregulating SNAIL [183].

HBx and "stemness". HBx promotes the expression of NANOG, KLF4, OCT4 and MYC as well as EpCAM and β-catenin [184]. Stabilization of β-catenin transcriptionally upregulates *EpCAM* [185]. EpCAM+ cells display CSC-like properties and generate invasive tumors in HCC xenograft experiments [186]. HBx also promotes the expression of miR-181 family members, which upregulate EpCAM and are highly expressed in embryonic livers, in HSC, and in patients with AFP-positive tumors [187]. HCCs with progenitor cell features have a worse prognosis and higher recurrence after treatment compared to HCCs, which are negative for these markers analysis [89]. In animal models, liver cancers can originate from hepatocytes as well as from immature progenitor cells [188].

A unifying picture of HBx role in liver carcinogenesis, that could reconcile all HBx reported activities, is still missing. Wild-type HBx and truncated HBx proteins both have oncogenic functions and promote tumorigenesis [189–191]. 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 would suggest that HBx inactivation could have a role in liver carcinogenesis or tumor progression [76]. The presence of HBx inactivating mutations was correlated with *TP53* mutations, a G1–G3 transcriptomic profile, a strong expression of onco-fetal genes (*EpCAM*, *AFP* and *KRT19*) and poorer prognosis [76], adding further complexity to the understanding of HBx contribution to HCC development. It is important to emphasize that the scientific literature reporting on HBx activities over almost two decades is exceedingly vast but many findings have not been generated in the context of HBV infection systems or confirmed *in vivo* in animal models or *ex vivo* in CHB and HBV-related HCC patients samples.

HBV core

We and others have shown that the HBV capsid protein HBc not only binds the HBV minichromosome, i.e. the cccDNA nuclear replicative intermediate [21,23] but also a subset of cellular genes involved in innate immunity, inflammatory responses and the control of cell proliferation [192–194].

PreS/S proteins

The inappropriate expression and accumulation of wild-type large envelope protein in the ER membranes can be directly cytotoxic to the hepatocyte and initiate, in transgenic mice, a cascade of events that ultimately progress to malignant transformation [39] (Fig. 4).

Many studies in both transgenic mice and cell culture have confirmed the pro-oncogenic potential of PreS2 mutated proteins [40,72,195,196]. The interaction with the JUN activation

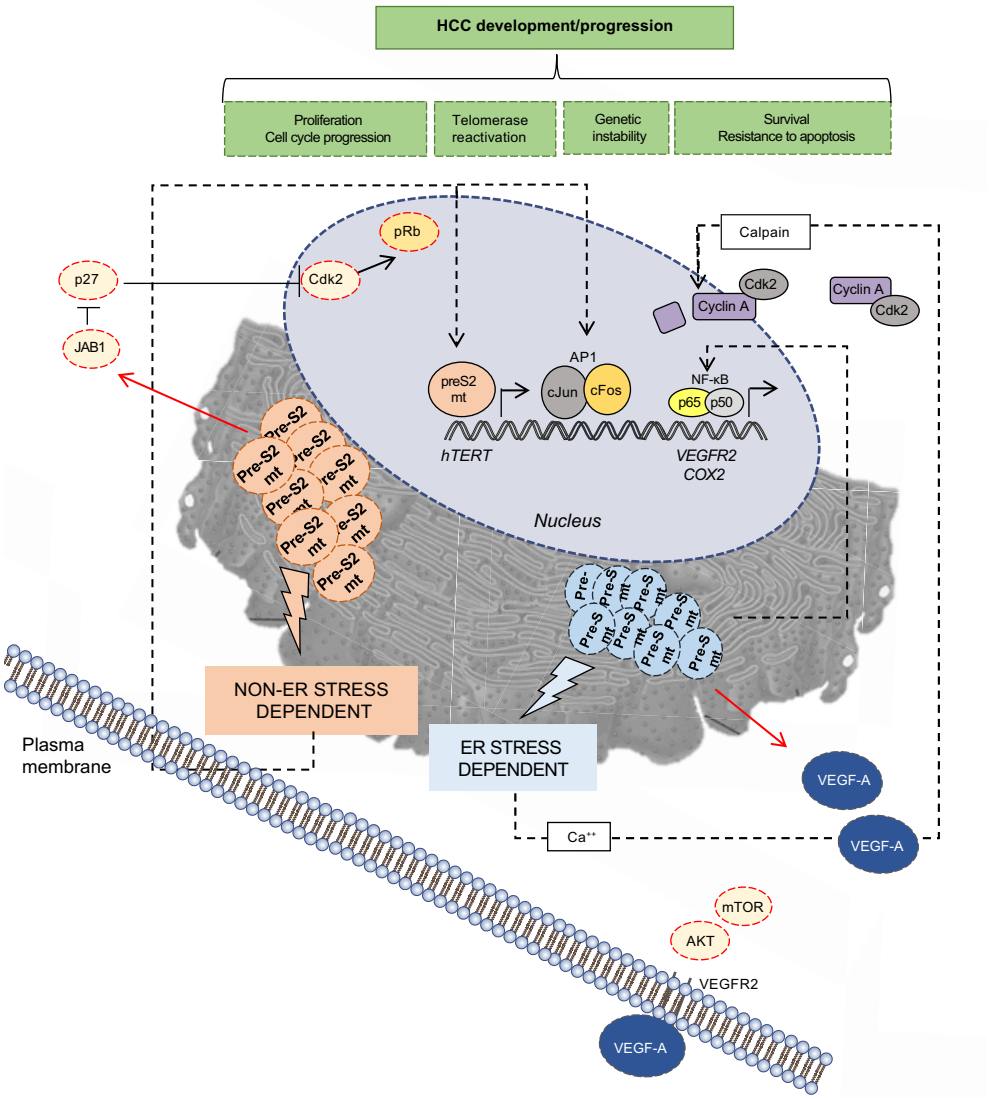

Fig. 4. Mechanisms of preS/S hepatocarcinogenesis. The preS mutants activate both ER stress-dependent and ER stress-independent signals. The accumulation of mutated envelope proteins in the ER leads to ER stress, DNA damage and genomic instability. The preS mutants-induced ER stress: a) activates the calcium-dependent protease calpain that cleaves cyclin A and generates a N-terminus-truncated product that translocates into the cytoplasm, causes centrosome over-duplication and activates sell signals; b) upregulates NF-κB and AKT/mammalian target of rapamycin (mTOR) signaling; c) protects the hepatocytes from apoptosis. PreS2 mutants can additionally promote hepatocyte proliferation by inducing an ER stress-independent activation of a signal transduction pathway that involves the JUN activation domain-binding protein 1 (JAB1), the CDK inhibitor p27, and the retinoblastoma tumor suppressor.

domain-binding protein 1 (JAB1) triggers the degradation of the CDK inhibitor p27, the hyper-phosphorylation of pRb and cell cycle progression [197]. Cyclin A and cyclooxygenase-2 overexpression driven by preS2 mutants induces both cell proliferation and anchorage-independent growth [42,43]. PreS/S sequences deleted at the 3′-end and producing functionally active MHBst are found in many viral integrants from HBV-associated HCCs [198–202]. MHBst proteins retained in the ER have been shown to trigger a protein kinase C dependent activation of c-Raf-1/MEK/Erk2 signal transduction cascade, the induction of AP-1 and NF-κB transcription factors and to enhanced hepatocytes proliferation [203]. MHBst also directly interacts with a preS2-responsive DNA region in the *hTERT* promoter, resulting in the upregulation of telomerase activity and in the promotion of HCC development [73].

Conclusions

HBV is a major risk factor worldwide for developing HCC and contributes to HCC development

through direct and indirect mechanisms. Productive HBV infections trigger inflammation and continuous necrosis mediated by the immune response against infected hepatocytes. Compensatory proliferation of adult hepatocytes as well as of the bipotential hepatobilliary progenitors acting as facultative stem cells and residing in bile canaliculi (hepatic progenitor cells (HPCs) in humans, oval cells in rodents) [188] favors the accumulation of genetic and epigenetic lesions. Indeed, the cell of origin of HCC, as for most cancer types, remains unknown and it is still debated whether HCCs arise from transient-amplifying HPCs/oval cells or terminally differentiated hepatocytes that de-differentiate. The lifetime risk of developing HCC and other cancers has been correlated with the accumulation of genetic errors during the division of adult stem cells [204]. In view of their high proliferative potential, HPCs/oval cells have been proposed as the cell of origin of HCC [188]. However, recent observations in animal models of HCC have challenged this view. The HCC progenitor cells (HcPC) identified in diethylnitrosamine (DEN)-induced HCCs share transcriptomic features with oval cells but, since DEN is metabolically activated by CYP2E1 only in fully differentiated zone 3 hepatocytes, they are not likely derived from oval cells but rather from hepatocytes [205]. Moreover, hybrid hepatocytes (HybHP), a subpopulation of periportal hepatocytes found in healthy livers and capable of extensive proliferation in response to chronic hepatocytes depleting damages, do not originate tumors in any of the HCC models investigated [206]. These results have given new support to the role of adult hepatocytes de-differentiation in HCC development, a notion that is particularly relevant in the context of virus-related HCCs and the direct role of HBV in hepatocytes transformation.

HBV DNA integration into the host genome is an early event that may precede clonal tumor expansion and induces genomic instability and eventually direct insertional mutagenesis. Prolonged expression of the viral proteins (wild-type and mutant HBx; wild-type and mutant large envelope proteins) dysregulates cellular transcription, alters proliferation control and sensitizes liver cells to carcinogenic factors. Epigenetic changes targeting the expression of tumor suppressor genes also occur early in the development of HCC and, again, a major role is likely played by HBx, which is recruited on cellular chromatin and modulates chromatin dynamics at specific gene loci.

Despite the global progress of our understanding of the molecular pathogenesis and, in particular, of the genetic landscape and the driver mutations in HCC, there are a number of areas in which our knowledge is still insufficient. There is a disconnect between the flourishing literature on the impact of HBV (and HCV) proteins on host cells, with the compilation of a kind of repertoire of all possible cellular targets and oncogenic activities, and the evaluation of their true contribution to transformation. Future efforts should aim to address the following open issues: a) what is the relative contribution of virus-induced inflammation, viral integration and the impact of viral proteins? b) Is HBV replicating efficiently in tumor cells or, in other words, is viral replication directly linked with infected hepatocytes clonal expansion, cells transformation and tumor progression? c) Can we measure the burden of viral integration in a given patient and is there a relation with clonal expansion of infected hepatocytes and HCC development? d) Are HBV-related HCCs a homogenous entity or distinct subgroups with different molecular pathogenesis, prognosis and response to therapy can be identified? Finally, the characterization of early events related to HBV-induced tumorigenesis may eventually lead to the development of biomarkers apt to identify subgroups of patients at increased risk of HCC among NUC suppressed patients, to implement more personalized approaches to HCC screening and define new preventive strategies in these patients.

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Conflict of interest

These authors disclose the following: JZR is consultant for IntraGen. ML received consulting honoraria from Gilead, BMS, Assembly, Arbutus, Janssen, Medimmune, Galapagos.

Author contributions

ML and JZR participated in all stages of manuscript production, design, figures, tables, writing, and review of final version.

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