The direct and indirect roles of HBV in liver cancer: prospective markers for HCC screening and potential therapeutic targets

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Abstract

Chronic hepatitis B virus (HBV) infection remains the number one risk factor for hepatocellular carcinoma (HCC), accounting for more than 600 000 deaths/year. Despite highly effective antiviral treatment options, chronic hepatitis B (CHB), subsequent end-stage liver disease and HCC development remain a major challenge worldwide. In CHB, liver damage is mainly caused by the influx of immune cells and destruction of infected hepatocytes, causing necro-inflammation. Treatment with nucleoside/nucleotide analogues can effectively suppress HBV replication in patients with CHB and thus decrease the risk for HCC development. Nevertheless, the risk of HCC in treated patients showing sufficient suppression of HBV DNA replication is significantly higher than in patients with inactive CHB, regardless of the presence of baseline liver cirrhosis, suggesting direct, long-lasting, predisposing effects of HBV. Direct oncogenic effects of HBV include integration in the host genome, leading to deletions, cis/trans-activation, translocations, the production of fusion transcripts and generalized genomic instability, as well as pleiotropic effects of viral transcripts (HBsAg and HBx). Analysis of these viral factors in active surveillance may allow early identification of high-risk patients, and their integration into a molecular classification of HCC subtypes might help in the development of novel therapeutic approaches.

Keywords: HBV; chronic hepatitis B; HBs antigen; HBx; X-protein; liver cancer; HCC; direct role of viral proteins; hepatocarcinogenesis

Received 22 August 2014; Revised 1 September 2014; Accepted 2 September 2014

No conflicts of interest were declared.

Introduction

Chronic hepatitis B virus (HBV) infection accounts for up to 54% of hepatocellular carcinoma (HCC) cases worldwide [1]. This percentage is lower in developed (23%) than in developing countries (59%), reflecting the variable burden of chronic HBV infection in different areas [2,3]. Worldwide, more than 240–350 million individuals are estimated to be chronically infected with HBV, facing a lifetime risk of 15–40% of developing end-stage liver disease, including cirrhosis, liver failure and HCC [4–7], accounting for more than 600 000 deaths/year [8]. Synergistic effects increasing the risk for hepatocarcinogenesis in CHB are demographic factors, such as Asian or African ancestry, male sex and age, as well as environmental factors, such as alcohol abuse, aflatoxin exposure or non-alcoholic fatty liver disease. Furthermore, viral co-infections (hepatitis C, hepatitis D or HIV co-infections), HBV genotype, viral DNA integration into the host genome or other direct effects of viral proteins might increase the risk for HCC development in CHB [9,10]. Patients chronically infected with HBV risk developing HCC in the absence of inflammation, severe liver damage and cirrhosis, due to direct oncogenic viral factors of HBV [3,9–13]. However, the direct role of HBV in HCC development remains to be elucidated.

HBV virology

HBV is a human blood-borne virus, infecting hepatocytes of humans or humanoid primates due to specific binding of the preS1 domain of the large envelope viral protein to hepatocytes via the bile salt transporter sodium taurocholate co-transporting polypeptide (NTCP) [14,15]. HBV can be transmitted by direct blood contact or sexual contact (horizontal transmission) as well as before or during birth from mother to child (vertical transmission) [16]. A very low number of HBV particles (<10) was shown to be sufficient to establish hepatocyte infection in vivo [17,18], demonstrating extremely high infectivity. The individual age at time of infection, as well as the implementation of vaccination programmes, vary greatly between different geographic regions, which at least partially accounts...
for the different prevalence of CHB and HCC worldwide [1,3,19]. A 90% risk of developing chronic HBV infection is found after vertical transmission, commonly seen in Oceania, South and South East Asia [20–22]. In cases of horizontal transmission, age at time of infection remains the highest risk factor for CHB development. In immune-competent adults the risk for CHB drops below 1%, while young children face a risk of 20–30% of chronic infection [23–25].

HBV, a member of the Hepadnaviridae family, is an enveloped DNA virus replicating via an RNA intermediate with a partially double-stranded relaxed circular DNA (rcDNA) molecule of 3.2 kb [26,27] (see Figure 1). Upon translocation to the nucleus, the rcDNA genome is converted into a covalently closed circular DNA (cccDNA), which serves as a template for viral transcription and allows effective HBV persistence [16,28,29]. The four overlapping open reading frames (ORFs) of the HBV genome encode for preS/S, preCore/Core, Pol and X (see Figure 1). The regulatory elements (enhancer II/basal core promoter, preS1 promoter, preS2/S promoter and enhancer I/X promoter) are also coding sequences for viral proteins within the ORFs [30]. Following transcription, all viral RNAs are translated in the cytosol. The non-structural X-protein (HBx) translated from a small 0.7 kb RNA is essential for viral replication and undergoes multiple interactions with the non-structural protein HBx. Besides serving as template for HBeAg, HBeAg and Pol translation, the pgRNA serves as a matrix for viral replication after being incorporated into nucleocapsids and being reverse-transcribed by the viral polymerase into new viral rcDNA [33]. rcDNA is either redelivered into the nucleus for a cccDNA pool or secreted with envelope proteins as infectious virions [32].

CHB and necro-inflammation: key drivers for liver fibrosis and HCC

HBV is a prototype, non-cytopathic virus [34] and in CHB the main liver damage-mediating cells driving HCC are CD8+ T cells [35–37]. The correlation between the strength of HBV-specific T cell responses and virus clearance after HBV infection has been well established. Adaptive immune responses ultimately mediate viral clearance and protective immunity, if the acute HBV infection can be cleared. For efficient control of HBV infection, an interplay between the innate and the adaptive immune response is required. Viral clearance is mediated by an effective adaptive CD4+ and CD8+ T cell response, whereas protective assembly and these proteins are secreted as spherical or subviral non-infectious particles [26,31,32]. The core protein, which forms the nucleocapsid (HBc) and its secreted counterpart HBe, arise from alternative translation initiation sites in the pregenomic RNA (pgRNA, ~3.5 kb). The viral polymerase, which is also translated from the pgRNA, has multiple functions, such as serving as reverse-transcriptase, DNA-dependent DNA polymerase and RNase H. The pgRNA furthermore serves as matrix for viral replication after being incorporated into nucleocapsids and being reverse-transcribed by the viral polymerase into new viral rcDNA [33]. rcDNA is either redelivered into the nucleus for a cccDNA pool or secreted with envelope proteins as infectious virions [32].
immunity is mediated by a B cell response, generating neutralizing antibodies [29,38]. Impaired virus control during chronic infection is correlated with functionally exhausted antiviral T cells. Nevertheless, they may still amplify liver injury [35–37,39]. Factors responsible for shifting the balance from immune tolerance to immune clearance are poorly understood. The liver has a unique immunoregulatory function to prevent inadvertent organ damage, which renders the liver an attractive target site for pathogens such as HBV [16]. The innate immune response, early adaptive B and T cell responses, regulatory T cells, hepatocyte-intrinsic factors and the liver microenvironment all seem to play a role in mediating liver damage [39]. For example, modulation of the cytokine response (up-regulation of IL-10 and TGFβ) and a putative role of regulatory T cells (Treg) have been proposed [40–43]. Tregs, for example, mitigate immune-mediated liver damage by down-regulating the activity of effector T cells and limiting cytokine production and cytotoxicity in acute HBV infection [44], yet their actual role in CHB remains elusive. In addition, viral factors of HBV, such as induction of regulatory cytokines, hepatocyte-restricted viral gene expression and high levels of circulating viral antigens, also allow escape from the immune response [16]. After acute damage, the liver has a unique capacity to regenerate via proliferation of normally quiescent hepatocytes, which ultimately restores liver function and size [45]. However, in CHB, persistent viral infection and ineffective T cell responses lead to chronic inflammatory liver damage and repeated compensatory proliferation of hepatocytes (necro-inflammation), which may subsequently lead to liver fibrosis or cirrhosis and HCC [35–37,46–49] (see Figure 2). This sequence was partially dissected in several mouse models, underlining the high importance of compensatory proliferation and deregulated hepatocyte apoptosis [50], chronic inflammation [37] and altered cytokine networks, including NF-κB and other signalling cascades [51,52], as tumour-promoters in the pathophysiology of HCC development (reviewed in [53]). Large-scale longitudinal epidemiological studies have revealed the strong correlation of high viral loads and risk of progression to HCC, pointing to the importance of subsequent inflammation in correlated phases of high viral replication. In CHB, baseline HBV DNA levels were even shown to be an independent predictive factor for the development of HCC, even after adjustment for epidemiological (sex, age, alcohol consumption) and other known risk factors, such as severity of liver damage (serum ALT level) [5,54,55]. Nucleoside/nucleotide analogues (NA) controlling HBV replication represent a successful treatment option to overcome this underlying process of chronic inflammatory liver damage and compensatory proliferation.

Figure 2. Different sequences from CHB to HCC. After HBV infection, a strong efficient immune response can clear the infection. However, due to pleiotropic effects with an inefficient T cell response, a chronic HBV infection (CHB) might develop. The risk for CHB depends on the route of infection (vertical versus horizontal transmission) and age at time of infection (adults <1%; young children 20–30%). An inefficient immune response might cause ‘active’ chronic hepatitis, with severe necro-inflammation, increased fibrosis and the risk of cirrhosis and subsequent HCC development. HBV DNA levels (>2000 IU/ml), ALT levels and fibrosis are typical findings in ‘active’ CHB. In chronic HBV infection, HCC development also occurs in the absence of cirrhosis and severe prolonged liver damage. Such cases might be ‘inactive’, asymptomatic carriers and even cases of ‘occult’ CHB; this is due to direct oncogenic viral factors of HBV, such as HBV DNA integration, the viral surface proteins and the non-structural protein HBx.

Treatment options for CHB: primary prophylaxis for HCC?

Because cure can hardly be achieved, the current goal of antiviral therapy for hepatitis B is to improve quality of life and survival by preventing the progression of chronic liver disease (CLD) to end-stage liver disease (cirrhosis ± decompensation) and HCC by sustained suppression of HBV replication. Indications for treatment vary between different countries, since therapy is

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expensive, usually long in duration and not widely available, particularly in many developing countries with the highest prevalence [56]. Algorithms were proposed for treatment stratification by, for example, the American Association for the Study of Liver Diseases (AASLD) [57], the European Association for the Study of the Liver (EASL) [58,59], the Asian Pacific Association for the Study of the Liver (APASL) [60] and several others. According to EASL guidelines, the decision should be based on serum HBV DNA levels (>2000 IU/ml/10 000 copies/ml) and/or serum ALT levels (>upper limit of normal) and histology (active necro-inflammation and/or fibrosis; > A1 F1 by META VIR) [58,59]. During interferon-α (IFNα) treatment, HBV DNA levels and HBsAg become undetectable in 30–40% of patients and 10% of patients seroconvert to anti-HBs. However, 5–10% of patients relapse, with full-blown disease after IFN therapy has ended [61,62]. While only a few patients seroconvert to anti-HBs during IFNα therapy, seroconversion rates increase to about 15–20% after the end of treatment [7,27].

NAs block reverse transcription and thus effectively suppress HBV replication. This seems sufficient to decrease necro-inflammation and lower the risk of cirrhosis and HCC development [58,63–65]. As cccDNA remains in the nucleus of infected hepatocytes, however, HBV infection is not eliminated by NA [27]. Treatment benefit with regard to HCC incidence depends on baseline HCC risk, stage of fibrosis/cirrhosis and the type of NA used (high genetic barrier) [57,58,63]. However, antiviral therapy reduces, but does not eliminate, the risk of HCC in chronic hepatitis B patients, regardless of the presence of baseline liver cirrhosis [63,65–67]. The ultimate goal is to achieve a sustained virological response and clearance of cccDNA in CHB. Just recently, a distinct antiviral mechanism that interferes with cccDNA stability driven by antiviral cytokines (IFNα and lymphotixin-β receptor activation) by up-regulation of APOBEC3A and APOBEC3B cytidine deaminases in hepatocytes was shown to effectively result in cccDNA degradation [27,68]. This might serve as a starting point for new therapeutic approaches that hopefully will eventually be able to cure hepatitis B.

Direct oncogenic effects of HBV in hepatocarcinogenesis

In addition to causing necro-inflammation, HBV has direct oncogenic potential that contributes to the development of HCC, independent of inflammatory liver damage [3,9,11,12]. This is reflected best by the fact that patients chronically infected with HBV face possible HCC development in the absence of inflammation, severe liver damage and cirrhosis, due to direct oncogenic viral factors [13]. The precise role of these direct viral factors in HCC development, however, remains controversial. The association between occult HBV infection (persistence of viral DNA after integration, or as cccDNA in patients negative for HBsAg) and HCC strongly supports the view of direct oncogenic factors of HBV [13]. The current debate on the significance of occult HBV infection in HCC development is ongoing.

There is compelling evidence for subgenotypes of HBV being risk factors for early HCC development in genotypes B2–5 [11], and patients bearing genotypes A1, C, Ba and F1 more frequently progress to HBV-related liver disease than genotypes A2 and Bj [3,69–71]. However, further studies focusing on HBV genotypes are needed to dissect direct effects from epidemiological risk factors. Another important risk factor is HBV variants, selected for during the natural course of chronic HBV infection or during treatment. The double substitution A1762T and G1764A in the basal core promoter (BCP) region of HBV and pre-core mutants seems to be an independent risk factor for HCC development associated with high viral titres and increased liver damage, even in the presence of anti-HBe seroconversion [6,9].

The role of HBV integration in HCC development

HBV integration into the host hepatocyte genome is a frequent event in HCC (86.4%) and is also found in adjacent liver tissues (30.7%) [72], which might lead to deletions, cis/trans-activation, translocations, the production of fusion transcripts and generalized genomic instability [12]. Integration is detrimental for HBV replication, yet it is a dynamic process and was reported over 30 years ago to precede HCC development [73]. However, it is unclear whether HBV integration initiates, or rather is a consequence of, transformation [49]. Hence, the distinct role of HBV DNA integration in hepatocarcinogenesis and the attributed risk of occult HBV-infection in HCC development remain controversial. Factors provoking liver cell death and proliferation increase dynamic rates of HBV DNA integration [74,75]. HBV integration was initially proposed to be a random event [12,76,77].

More recent high-throughput, next-generation, sequencing approaches identified recurrent sites for integration. Genes reported to be frequently altered are TERT, MLL4, CCNE1, NTRK2, IRAK2 and p62MAPK1 [72,78,79]. Secondly, viral promoter-driven human transcription and viral–human transcript fusion have been reported [80]. A common effect, increasing with rising numbers of integrations, seems to be increased genomic instability, and a correlation was observed between the number of chromosomal aberrations and the status of tumour suppressor genes (TP53, RB1, CDKN2A and TP73) [81]. Lately, the identification of insertion sites within or close to repetitive, non-coding sequences, such as long interspersed nuclear elements (LINEs) or short interspersed nuclear elements (SINEs) has garnered notice [82,83].

Using mathematic algorithms (ViralFusionSeq), the detection of viral–human fusion transcripts was enabled in an unbiased fashion [84] and helped to identify a
novel fusion sequence of the human LINE(1) and HBV-encoded HBx [HBx-LINE(1)] after insertion in chr. 8p11. Interestingly, this non-coding fusion RNA (HBx–LINE1) was detected in 23.3% of HBV-associated human HCCs, was correlated with poor survival and showed tumour-promoting properties affecting β-catenin activity and epithelial–mesenchymal transition (EMT) [85]. However, integration and low frequencies of fusion transcripts do not suffice to explain the multitude of direct effects of HBV in HCC development. Comparing HBV-associated HCC to other aetiologies shows a significantly more frequently altered p53 pathway (47%), up-regulation of other genes involved in cell-cycle regulation and progenitor phenotypes, as well as specific gene mutations (HBx, TP53, IRF2, AXIN1 and CTNNB1) [86].

HBs antigen and pre-S/S mutants in HCC development: link to UPR

A direct factor driving hepatocarcinogenesis could be the expression level of HBsAg (see Figure 3). In a small cohort of NA-treated patients, hepatocarcinogenesis was observed in patients with high HBsAg levels [≥2000 IU/ml], despite efficient suppression of HBV viraemia as a result of long-term NA therapy [65]. Also, patients from a big Taiwanese cohort, who maintained a high HBsAg load (>1000 IU/ml) showed an elevated risk for HCC development [66]. Furthermore, a subgroup of patients of the REVEAL HBV cohort with HBsAg > 1000 IU/ml, despite spontaneous HBV DNA clearance, still showed an HCC risk ratio of 3.86 above baseline [67].

One possible direct carcinogenic effect is the accumulation of HBV surface proteins in the endoplasmic reticulum (ER), leading to the typical histological finding of hepatocytes overloaded with virus protein in HBV-infected livers, referred to as ‘ground glass’ hepatocytes (GGHs), first described by Hadziyannis and Popper in 1973 [87]. Many studies have shown that GGHs correlate with the expression pattern of HBV antigens and activity of CHB and appear in preneoplastic lesions, harbouring HBsAg with specific pre-S mutants [88]. Type I GGHs harbour mutants with deletions within the pre-S1 region (mutated L protein), whereas type II GGHs contain pre-S2 mutants and are distributed in clusters, showing patterns of proliferative nodules [89]. Over two decades ago, a mouse model over-expressing the HBV large envelope polypeptide (L), PreS1 and parts of HBx [90] was shown to have liver cell injury, inflammation and compensatory proliferation, finally progressing to HCC due to apoptotic dysregulation [91]. This was also found in HBV transgenic mouse models expressing mutated preS2 [92]. Linking different types of GGHs to preS/S mutants identified a direct oncogenic effect of HBV. The emergence of HBV variants carrying mutations in the preS/S genomic region is reportedly a quite frequent event, especially as a consequence of antiviral therapy. Selected changes in the polymerase ORF of HBV in the course of therapy with NA may also cause changes in the structural proteins due to their overlapping ORFs. Some major drug-resistant mutations against NA in the HBV polymerase cause a premature stop codon in the overlapping HBsAg (ie rtA181T→SW172stop, rt184M→SL176stop). These premature stop codons at positions 172 or 181 of the S gene lead to secretory defects and are significantly associated with cirrhosis and HCC [93,94]. However, only alterations that do not compromise viral fitness will be selected. For example, the M surface protein (consisting of preS2 and S), which overlaps a spacer domain of the Pol ORF, is not essential for formation and secretion of virions and tolerates mutations and even large deletions without causing loss of polymerase activity [31,32,95,96]. Of note, viral mutants with antigenically modified surface antigen (HBsAg ‘a’ escape variants) are potentially infectious to vaccinated patients and may be overlooked by common HBsAg assays in the clinic, thus possibly accounting for cases of ‘occult’ hepatitis B infection [31].

Accumulation and imbalance of unfolded or misfolded proteins activates intracellular signalling pathways associated with ER stress. This is buffered by the activation of the so-called unfolded protein response (UPR), a homeostatic signalling network recovering ER function, or, if this fails, triggering apoptosis [97]. Additional functions of the UPR described even include a role in innate immunity, metabolism and cell differentiation [98]. UPR has been shown to be an important source of intracellular ROS [99], and oxidative stress caused by increased ROS is known to have a high oncogenic potential by inducing DNA damage and changes in signalling cascades that control proliferation, cell survival and cell death [99,100]. The role of such events in HCC development has been described in many experimental studies [101–103].

Both experimental and human data support an important role of preS/S mutants in the induction of ER stress, with consequent oxidative DNA damage and genomic instability. Pre-S mutants hence may promote hepatocyte proliferation, up-regulate vascular endothelial growth factor-A (VEGF-A), cyclin A and activate Akt–mammalian target of rapamycin (Akt–mTOR) signalling by the induction of UPR [31,35,104–106]. Furthermore, PreS2 mutants can exert oncogenic functions directly by interacting with intracellular signalling pathways [31,104]. A frequent mutant is the C-terminally truncated M protein (see above), also referred to as MHBS(t) [96,104], which can frequently be found integrated in HCC [107]. MHBS(t) may induce TRAIL-induced, caspase-3-dependent apoptosis [108], increase hepatocyte proliferation, trigger PKC-dependent activation of c-Raf-1→Erk2→Ap-1 and NF-κB activation and show trans-activation potential [104]. In a meta-analysis of 11 582 patients, the presence of preS mutants was found to correlate with a 3.77-fold higher risk for HCC development [109], underlining the significance of these findings.
HBx/HBV X-protein and frequent truncations in the course of HCC development

The HBx protein has been implicated as a direct onco-genic protein [110–112]. Studies using HBx transgenic mouse models have shown spontaneous HCC development [113,114]; however, other groups merely observed an increased sensitivity to other known carcinogens [115,116]. HBx, a small polypeptide (154 amino acids), is usually expressed at low levels during HBV infections and is frequently detected at high levels in HBV-related HCCs [117]. HBx can be found in the cytoplasm of infected hepatocytes and at low levels in the nucleus, and has been identified as a trans-activating protein [118], playing a crucial role in virus gene expression, viraemia and infectivity [119]. An X-protein-negative clone of woodchuck hepatitis virus failed to establish chronic infection [120]. Severity of liver damage correlates with levels of HBV due to increased integration of HBx in active chronic liver disease and increased hepatocyte proliferation [121,122]. It has been reported that HBx is involved in hepatocarcinogenesis, due to pleiotropic effects on several signalling pathways regulating cell death, proliferation, differentiation (including EMT), oxidative stress and DNA repair, as possible oncogenic factors in hepatocarcinogenesis (see Figure 3) (reviewed in [123–125]). For example, HBx has been reported to drive pro-proliferation [126], induce cell-cycle arrest [127] and prevent [128] or even induce apoptosis [129].

One proposed possible direct carcinogenic effect of HBx is the induction of regional hypermethylation or

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**Figure 3.** Examples of proposed, direct HBV effects driving hepatocarcinogenesis. The key players of HBV integration, HBx, c-terminally truncated HBx and Pre/S/S mutants, as well as their proposed signalling pathways in HBV-associated hepatocellular carcinoma (HCC), are shown. These factors directly alter the expression and activity of selected signalling pathways and drive malignant transformation. Secondly, these factors can drive metastatic disease as well as worsen overall survival by making HCC more aggressive: CHB, chronic hepatitis B; TFIH, transcription factor II H; miR, microRNA; DNMT1, DNA methyltransferase 1; ERK, extracellular signal-regulated kinases; INK4a, also known as p16/cyclin-dependent kinase inhibitor 2A; RB, retinoblastoma protein; RAS, protein family class of small GTPases; RAF, family of three serine/threonine-specific protein kinases; MAPK, mitogen-activated protein kinases; EGFR, epidermal growth factor receptor (also known as ErbB-1/HER1); ErbB-2, also known as CD340/proto-oncogene Neu/human epidermal growth factor receptor 2 HER2/neu; SRC, c-Src, a non-receptor protein tyrosine kinase; URG11, up-regulated gene 11; PTEN, phosphatase and tensin homologue; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; AKT, also known as protein kinase B (PKB); mTOR, mammalian target of rapamycin; MMP10, matrix metalloproteinase 10; ER, endoplasmic reticulum; AP1, activator protein 1; NF-κB, nuclear factor-κ light chain enhancer of activated B cells; VEGF, vascular endothelial growth factor; JAB1, JAK–binding protein; p27/Cip1, cyclin–dependent kinase inhibitor 1B; TRAIL, TNF-related apoptosis-inducing ligand; FADD, Fas-associated protein with death domain; EMT, epithelial–mesenchymal transition; UPR, unfolded protein response)
global hypomethylation, eg by inducing DNA methyltransferase 1 (DNMT1) expression, silencing tumour suppressor genes and inducing chromosomal instability [130]. Accordingly, HBx was reported to complex with wild-type p53 protein, limiting p53-specific tumour suppressor functions (for review, see [131]) by inhibiting sequence-specific DNA binding and transcriptional activation, as well as suppressing nucleotide excision repair [132,133]. In the same direction, HBx was shown to activate the RAS–RAF–MAPK [134] and ERK–INK4a–RB pathways [135]. This abrogates expression of tumour suppressors and overcomes senescence, which is usually triggered due to chronic inflammation and oxidative stress to protect from carcinogenesis [136]. Next, HBx was reported to overcome senescence by suppression of cyclin-dependent kinase inhibitors INK4a (p16), as well as p21 Cip1/CDKN1A) [137] and to deregulate the G1–M checkpoint, resulting in a rescue from HBx-mediated apoptosis [138]. Another proposed key player in suppressing DNA repair and tumour suppressors is directly by HBx-up-regulated URG11, altering expression of multiple microRNAs. Resulting up-regulation of miR-148a has been shown to drive cell cycle progression and cell migration by suppressing PTEN, thus increasing Akt–mTOR signalling [139]. Furthermore, other microRNAs are possibly also directly targeted by HBx-like miR-122a, which targets cyclin G1 [140] and inhibits HBV replication, as well as inhibiting expression of p27 [121].

Other effects attributed to HBx are enhanced oxidative stress, increased levels of ROS and calcium signalling by interaction with the ER [141] and mitochondria [123,142,143]. Various studies point to sustained higher cytosolic calcium levels and a stimulating effect on HBV replication, in addition to other possible oncogenic effects, such as activating src and ras signalling [144] (reviewed in [143]). HBx was shown to activate FOXO4, which enhances resistances to ROS-induced cell death [145], activating autophagy as a pro-survival factor facilitating virus persistence and hepatocarcinogenesis [100,146]. Additionally, hypoxia induced by necro-inflammation induces binding and stabilizing hypoxia-induced factor 1a (HIF1a) by HBx, promoting angiogenesis and cell migration by up-regulation of VEGF and matrix metalloproteinases [147–149].

Increased EGFR signalling and up-regulation of ErbB2 (Her2/Neu) and other members of the epidermal growth factor receptor tyrosine kinases, as well as loss of inhibitor proteins, have been reported in a subset of HCCs, creating autocrine/paracrine activation loops [150–153]. ErbB2 up-regulation in CHB and in some HCCs correlated with HBx expression [154,155]. Targeting ERBB2 mRNA by a specific siRNA not only reduced ErbB2 expression but also reduced the expression of β-catenin [154]. This links the ErbB family to the multitude of effects reported by activation of β-catenin signalling in HCC, including proliferation, migration, EMT and loss of adhesion. β-Catenin activation occurs in many cancers, mostly driven by activating mutations in β-catenin itself or by inherited and sporadic mutations in regulatory proteins such as the tumour suppressor APC and AXIN, which normally complex and inactivate β-catenin in the OFF-state of canonical WNT signalling, together with E-cadherin and other factors [156–158]. In human HCC, mutations in WNT/β-catenin signalling are common (~15–33% CTNNB1, ~15% AXIN1 and 1–2% APC) [159–161]. However, these mutations were mostly reported in HBV-negative HCCs and frequent β-catenin activation in CHB-driven HCC must rely on other modes of activation. Consistent with this idea, up-regulation of URG11 through HBx leads to up-regulation of β-catenin [138]; inhibitory binding of β-catenin by E-cadherin is facilitated by HBx through DNA methylation of the E-cadherin promoter [162] or inhibition of miR-373 and up-regulation of SNAIL [163] – all driving increased WNT-independent activation of β-catenin. Furthermore, HBx was reported to activate the proto-oncogene Src, which can also activate β-catenin signalling [164–166].

The analysis of HBx mutants naturally induced by integration in the host genome has helped to define the functional relevance of protein domains of HBx. For example, HBx amino acids 116–140 were shown to be required for mitogenic pathway activation [167]. HBx variants were reported to play an important role in the course of hepatocarcinogenesis [148,167–170] and were reported to be independent predictors for survival after HCC resection [171]. C-terminally truncated HBx, in particular, is frequently found in HCC [81,86,168,169]. A recent study reported detection of C-terminally truncated HBx in 46% of HBV-HCC [168], which was significantly correlated with venous invasion, and in vitro data showed increased cell invasiveness, C-Jun activity and expression of MMP10 [168].

At the same time, multiple functions of HBx, as well as changes in host gene expression mediated by HBx, seem to be essential for virus replication and hepatocyte survival or protection from the immune response, consequently benefiting the virus and hence promoting HCC development [121]. Some of the proposed oncogenic effects of HBx mentioned above were reported from in vitro experiments or mouse models with very high expression levels of the HBx protein. This points to a potent function of HBx also in already established HCC, since the highest accumulation of viral HBx RNA was found in HCC tissue [110,172]. Secondly, the anti-apoptotic and pro-apoptotic effects of the HBx protein seem to be dependent on the status of hepatocyte differentiation [173].

**Future perspective**

**Identification of new therapeutic targets in HCC**

Potentially curative treatment options for HCC, such as liver transplantation and surgical interventions, are limited to early stages [174]. However, many patients face a recurrence of HCC after resection, or are only eligible for palliative treatment due to advanced
tumour stage. Sorafenib, a pan-kinase inhibitor, remains the only approved systemic, standard-of-care drug, improving overall survival by only 2–3 months and with significant side-effects [175,176]. A recent study described that sorafenib not only directly inhibits tumour cell growth but also modifies the crosstalk between tumour-associated macrophages and natural killer cells, thus slowing down HCC growth [177]. The poor prognostic outcome of late-stage HCC results from human HCCs showing a diverse spectrum of cancer subtypes, without common oncogene addiction loops [174,178]. Establishing a comprehensive molecular classification of HCC remains a challenge, due to inter- and intratumor heterogeneity [159,179–181]. The different aetiologies of HCC, and especially the direct carcinogenic effects by HBV, can contribute synergistically or even alone as carcinogens. In this 'non-Vogelstein-type' carcinogenesis, a molecular classification consisting of six HCC subgroups was proposed [182], pointing to a specific role of low or high copy number of HBV (termed ‘G1/G2’ tumours) with specific oncogene patterns [160]. Even though the role of certain viral proteins, as well as HBV integration, seems overly complex, compelling evidence exists for their direct role in hepatocarcinogenesis and they should be further evaluated in carcinogenic-risk stratification and therapeutic approaches. Thus, the integration of a molecular classification, intratumor diversity and aetiological backgrounds, including direct oncogenic factors of HBV, are missing. This might explain the failure of some promising Phase II and Phase III trials using kinase inhibitors, eg sunitinib, brivanib, erlotinib and linifanib and the mTor inhibitor, everolimus [178]. In order to understand which patients might profit most from certain therapeutics, a personalized fingerprinting of HCC is likely to be needed in future clinical trials.

Active surveillance and preventing HCC development in CHB

Hepatocarcinogenesis remains a major risk for chronically HBV-infected patients, despite the existence of potent antiviral NAs with high genetic barriers. Active surveillance programmes can significantly reduce morbidity and mortality from HCC [1,62] because early HCC detection enables potentially curative resection. HBV DNA levels have been confirmed as the most important predictor of disease progression and HCC development [5]. However, HBsAg levels and increased baseline HCC risk should be taken into account for active surveillance, even in HBV DNA-negative patients undergoing NA treatment, since over-expression and truncation of HBsAg increase the risk for HCC [31,62,65,110,172]. Thus, non-invasive markers for risk stratification are necessary to identify patients at high risk for HCC, allowing early diagnosis and treatment. Certain viral proteins (ie HBs, HBx and their mutants), as well as integration of HBV DNA, should be evaluated for inclusion in a screening of a personal risk prediction for HCC.

Acknowledgements

MR received a clinical leave stipend funded by the German Centre for Infection Research (DZIF); MH was supported by the Foundation for Experimental Biomedicine Zürich, Switzerland.

Contributions

MR, UP, TO and MH wrote the text.

Abbreviations

AKT, protein kinase B (PKB); anti-HBc, antibodies against HBeAg; anti-HBe, antibodies against HBcAg; anti-HBs, antibodies against HBsAg; AP1, activator protein 1; BCP, basal core promoter; cccDNA, covalently closed circular DNA; CHB, chronic hepatitis B; CLD, chronic liver disease; DNMT1, DNA methyltransferase 1; EGFR, epidermal growth factor receptor (also known as ErbB-1/HER1); EMT, epithelial–mesenchymal transition; ER, endoplasmic reticulum; ErbB-2, CD340/proto-oncogene Neu/human epidermal growth factor receptor 2; ERK, extracellular signal-regulated kinases; FADD, Fas-associated protein with death domain; GGHs, ‘ground glass’ hepatocytes; HBc, hepatitis B core protein; HBsAg, hepatitis B virus envelope antigen; HBsAg, hepatitis B virus surface antigen; HBV, hepatitis B virus; HBx, hepatitis B virus X protein; HCC, hepatocellular carcinoma; INK4, p16/cyclin-dependent kinase inhibitor 2A; JAK1, JAK-binding protein; LINE, long interspersed nuclear elements; MAPK, mitogen-activated protein kinases; miR, microRNA; MMP10, matrix metalloproteinase 10; mTOR, mammalian target of rapamycin; NA, nucleoside/nucleotide analogues; NF-xB, nuclear factor-xB light chain enhancer of activated B cells; NTCP, sodium taurocholate co-transporting polypeptide; p27/Cip1, cyclin-dependent kinase inhibitor 1B; pgRNA, pregenomic RNA; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; PTEN, phosphatase and tensin homologue; RAF, family of three serine/threonine-specific protein kinases; RAS, protein family class of small GTPases; RB, retinoblastoma protein; rcDNA, relaxed circular DNA; ROS, reactive oxygen species; SINE, short interspersed nuclear elements; SRC, c-Src, a non-receptor protein tyrosine kinase; TFIH, transcription factor II H; TRAIL, TNF-related apoptosis-inducing ligand; Treg, regulatory T cells; UPR, unfolded protein response; URG11, up-regulated gene 11; VEGF, vascular endothelial growth factor.

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