

Mini-Review

Hepatitis B Virus-Related Hepatocellular Carcinoma: Pathogenic Mechanisms and Novel Therapeutic Interventions

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Chronic hepatitis B · Hepatitis B virus · Hepatocellular carcinoma · Pathogenic mechanisms · Therapeutic interventions

Abstract

Background: Infection with the hepatitis B virus (HBV) is one of most important risk factors for hepatocellular carcinoma (HCC). Indeed, HBV is considered a group 1 human carcinogen and is a highly oncogenic agent. HBV cannot be effectively controlled or completely eliminated, so chronic HBV infection is a public health challenge worldwide. **Summary:** It is now believed that HBV-induced HCC involves a complex interaction between multiple viral and host factors. Many factors contribute to HBV-associated HCC, including products of HBV, viral integration and mutation, and host susceptibility. This review outlines the main pathogenic mechanisms with a focus on those that suggest novel targets for the prevention and treatment of HCC. **Key Message:** HBV infection is an important risk factor for HCC. Understanding the interaction between viral and host factors in HBV-induced HCC will reveal potential targets for future therapies. **Practical Implications:** The two main therapeutic strategies consist of antiviral agents and immunotherapy-based approaches. Dendritic cell-based immunotherapy is promising for restoring the T cell-mediated antiviral immune response. Another approach is the specific expansion of the host's pool of HBV-specific T cells. Stimulation of the Toll-like receptors (TLRs), particularly TLR9, provides another means of boosting the antiviral response. Combination therapy with cytokines (interferon gamma and tumor necrosis factor alpha) plus lamivudine is more effective than these agents used alone. Therapeutic vaccines are being developed as an alternative to long-term antiviral treatment or as an adjunct.

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Introduction

Around 2 billion people are infected with hepatitis B virus (HBV), and more than 350 million have become chronic carriers [1] who have persistent virus and subvirus particles in their blood for more than 6 months. HBV infection is endemic in China. The hepatitis B surface antigen carrier rate is 7.2%, and about 93 million people are chronically infected by HBV [2, 3]. Progressive chronic liver disease appears as hepatitis, fibrosis, cirrhosis and finally hepatocellular carcinoma (HCC) [4]. Chronic HBV infection is a major etiological factor of HCC in HBV endemic areas worldwide [5]. HCC is a common solid tumor worldwide and represents the third leading cause of cancer mortality [6, 7]. Individuals with chronic HBV infection are at increased risk of developing HCC, especially those with chronic liver disease and cirrhosis [8, 9]. As a result, HBV was categorized as a group 1 human carcinogen and one of the most important oncogenic agents by the World Health Organization [10].

There are three reported mechanisms by which HBV promotes carcinogenesis: (1) HBV proteins are involved in many signaling pathways in hepatocytes, thereby affecting the expression and functions of specific genes and contributing to liver disorders. Most of these changes are associated with HCC. (2) Integration of HBV DNA into the host genome alters the function of endogenous genes or induces chromosomal instability. (3) Inflammation-mediated alteration of specific signaling pathways contributes to tumorigenesis. Chronic inflammation plays a vital role in the development of HCC. Repeated cycles of inflammation-induced apoptosis and hepatocyte regeneration increase the risk of hepatocarcinogenesis.

HBV Integration and Mutation in HBV-Related HCC (table 1)

HBV Gene Integration

Integration of the viral gene into the host genome is an important mechanism responsible for HCC development among HBV-infected individuals. 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. The presence of integrated HBV DNA sequences in cellular DNA from human HCCs was first reported in the early 1980s [11–14]. Afterwards, many studies were carried out to further investigate HBV integration. In particular, tumors related to HBV are mostly found in subclasses characterized by high genetic instability, notably with aberrations at chromosomes 4q, 13q, 16p, 16q and 17p [15, 16].

The first reported recurrent HBV integration event was found to be located at the human TERT gene in two liver tumor samples. TERT, a gene encoding telomerase reverse transcriptase, plays an essential role in overriding cellular senescence. This gene is frequently overexpressed in cancer cells and its dysregulation in somatic cells was found to be linked to carcinogenesis [17]. Subsequently, Sung et al. [18] focused on the events of HBV integration and their effects on the HCC genome using whole genome sequencing and integrated expression profiling analyses. They found that HBV integration is observed more frequently in the tumors (86.4%) than in adjacent liver tissues (30.7%). Copy number variations were significantly increased at HBV breakpoint locations where chromosomal instability was likely induced. Approximately 40% of HBV breakpoints within the HBV genome were located within a 1,800-bp region where the viral enhancer, X gene and core gene are located. They also identified recurrent HBV integration events (in ≥ 4 HCCs) that were validated by RNA sequencing and Sanger sequencing at the known and putative cancer-related TERT, MLL4 and CCNE1 genes, which showed upregulated gene expression in tumor versus normal tissue [18–20].

Table 1. HBV mutation and integration sites

HBV mutation sites	HBV integration sites
rtA181T/sW172 3' end of the HBx gene X/preC regions: G1613A, C1653T, T1753V, A1762T, G1764A, A1846T, G1896A and G1899A YMDD mutation (M204V/I)	chromosome 2 (FN1 gene) chromosome 3 (SENP5 gene) chromosome 5 (TERT gene) chromosome 18 (ROCK1 gene) chromosome 19 (CCNE1, MLL4)

Genetic instability plays a crucial role in cancer initiation and progression. Meanwhile, the number of HBV integrations is associated with patient survival [18]. The recent development of efficient tools for genome-wide analysis of gene expression and genetic defects has allowed a comprehensive view of the multiple changes occurring in HCC.

HBV Gene Mutation

HBV has a relatively high mutation rate in its genome. YMDD mutation, also known as M204V/I mutation, is caused by the substitution of methionine by valine or isoleucine in the YMDD motif and is designated as the YVDD or YIDD variant. Previous research has suggested that lamivudine is the major cause of YMDD mutation. In recent years, spontaneous YMDD mutations have also been detected in patients with chronic HBV infection not previously treated with antiviral drugs. The spontaneous YMDD mutations are more likely to occur in genotype C strains, and the occurrence of spontaneous YMDD mutations in patients infected with genotype C strains may increase the risk of HCC [21].

Viral genomic mutations are closely related to the natural course of chronic HBV infection. In particular, the basal core promoter double mutation of A1762T and G1764A, the so-called BCP double mutation that has been implicated in the e-suppressive HBV phenotype [22], is important for HCC risk prediction in HBV genotype B or C carriers [23, 24]. Recent data suggest that accumulation of eight key mutations located in the X/preC regions of the HBV genome (G1613A, C1653T, T1753V, A1762T, G1764A, A1846T, G1896A and G1899A) is a risk marker for the development of HCC, and combination of BCP double mutation with these eight key mutations is significantly associated with HCC [25].

Microenvironment of Chronic Liver Injury in HCC Development

Inflammatory Factor and ROS

In chronic hepatitis B (CHB), liver injury is thought to be mediated by host immune responses, including HBV-specific T cells as well as infiltrating neutrophils, natural killer (NK) cells and non-antigen-specific lymphocytes that are attracted by the production of non-specific chemokines [26]. The release of inflammatory cytokines and chemokines also favors hepatocyte proliferation. Although virus-specific lymphocytes are readily detectable in inflammatory lesions in the liver, they are often not sufficient to clear virus infection. Many virus non-specific NK cells, NK T cells and polymorphonuclear leukocytes are also found in these lesions, mediating hepatocellular damage, but they are not effective in virus clearance [27].

Moreover, oxidative stress induced by inflammation and by accumulation of viral surface proteins in the endoplasmic reticulum (ER) leads to increased levels of intracellular reactive oxygen species (ROS), in turn enhancing genomic instability and allowing the accumulation

of genetic abnormalities and gene mutations. More specifically, ROS may trigger the activation of intracellular signaling pathways such as the mitogen-activated protein kinase and nuclear factor- κ B (NF- κ B) pathways that play an important role in hepatic carcinogenesis [28].

Dysfunction of Innate Immunity against HBV

Innate immunity has evolved to rapidly recognize viral nucleic acids, viral proteins and tissue damage. It induces an antiviral state on infected cells by producing type I interferons (IFNs), decreases the pool of infected cells by directing NK cell-mediated killing of viral infected cells, and supports the efficient maturation and site recruitment of adaptive immunity through production of pro-inflammatory cytokines and chemokines [29].

The apparent lack of induction of IFN-I response was interpreted as an ability of HBV to escape innate recognition. This could be the result of the replication strategy of HBV, which uses a transcriptional template, covalently closed circular DNA (cccDNA), that is sequestered within the nucleus of infected cells, where it might not be detected by the innate DNA sensing cellular machinery, and produces polyadenylate viral mRNA that resembles the normal cellular transcripts. Moreover, newly transcribed genomes are protected within viral capsids in the cytoplasm [30]. HBV can actively suppress instead of escaping the innate immunity through the action of different viral proteins. HBV polymerase can inhibit IFN- β induction by interfering with IFN regulatory factor signaling [31, 32].

A major signaling molecule in innate immunity is NF- κ B, which induces pro-inflammatory and hepatoprotective gene expression [33]. HBx activation of NF- κ B [34] may suppress innate immunity by switching NF- κ B signaling to hepatoprotection, thus promoting resistance to immune-mediated damage. Intrahepatic NK cell function is also skewed towards cytolytic activity without IFN- γ production, suggesting that hepatocellular killing occurs without virus clearance [35]. Murine models of hepatocarcinogenesis have highlighted the role of interleukin 6 (IL-6) and of the NF- κ B pathway in HCC development. Moreover, microRNAs involved in inflammatory signaling (miR-124 and miR-24/miR-629) have been shown to disturb the IL-6/Stat3 and the Stat3/HNF4- α axis, mechanistically linking inflammation and liver cancer in a self-reinforcing feedback circuit [36].

Dysfunction of Adaptive Immunity against HBV

Adaptive immunity acts through functional maturation and expansion of distinct B and T cell clones able to specifically recognize the infectious agents, a process that necessitates time. This process leads to the control of infection and generates a memory response which increases the host's ability to block subsequent infections with the same pathogens. However, T cells are more dysfunctional within the infected liver. HBV-specific T cell dysfunction is responsible for hepatoma cell survival, while inflammation-mediated alteration of specific signaling pathways contributes to tumorigenesis.

Dendritic cells (DCs), which link innate immunity with adaptive immunity and which may be infected with HBV, are also defective in chronic HBV infection, resulting in poor adaptive immunity [37]. Regulatory T cells (Tregs) help maintain a tolerogenic environment in the liver. These Tregs are induced by HBx-stimulated production of transforming growth factor- β 1 (TGF- β 1) [38]. Elevated TGF- β activity, associated with the persistent presence of HBV in the liver tissue, suppresses the expression of miR-34a, leading to enhanced production of chemokine CCL22, which recruits Tregs [39]. In adaptive immunity, there are also defects in HBV-specific CD8⁺ T cells [40], which are characteristic of T cell exhaustion. Li et al. [41] further showed that the Tim-3/galectin-9 signaling pathway mediates T cell senescence in HBV-associated HCC. These findings provided some mechanisms for HBV-induced immune dysfunction in the development of HBV-associated HCC.

Impact of Viral Proteins in HBV-Induced HCC

The HBV genome has a compact, entirely coding organization, with four overlapping reading frames that encode the viral proteins [42]. HBV-encoded proteins alter host gene expression and cellular phenotypes that are recognized as hallmarks of cancer. These changes promote growth factor-independent proliferation, resistance to growth inhibition, tissue invasion and metastasis, angiogenesis, reprogramming of energy metabolism, and resistance to apoptosis in the face of persistent immune attack and during therapeutic intervention [43].

HBx

HBx is critical for viral pathogenesis and oncogenesis in HBV-infected livers [44, 45]. The HBx gene is the most frequently integrated viral sequence in HCC, and HBx protein is detected in most patients with HBV-related HCC, even in the absence of viral DNA replication [46–48]. HBx has been implicated in mediating multiple viral and cellular events in HBV-infected cells, including viral replication, transactivation of transcription factors, signal transduction, cell cycle progression and cell death. HBx also interacts with the anti-apoptotic proteins Bcl-2 and Bcl-xL through a Bcl-2 homology 3-like motif to promote cytosolic calcium elevation, cell death and viral replication during HBV pathogenesis [49].

HBx truncation is caused by HBV integration. Upon integration, the 3' end of HBx is often deleted [50]. Meanwhile, the C-terminal region of HBx produced by HBx truncation also contributes to HCC development. The C-terminal region of HBx was found to be important for HBV replication [51] and suggested to be required for ROS production and 8-oxoguanine formation, which are biomarkers of oxidative stress and play an important role in HCC development [24]. Other studies have found that the C-terminal truncation of HBx plays a role in enhancing cell metastasis [52] and directly regulates microRNA transcription and in turn promotes hepatocellular proliferation [53].

Hepatitis B Surface Proteins

The envelope of HBV is formed by three different surface proteins (HBs) termed L (large), M (middle) and S (small), with a ratio of 1:1:4 [54]. On biogenesis, the HBV L protein, together with the structurally closely related M and S envelope proteins, is expressed from a single open reading frame of the viral genome by differential translation initiation. As a consequence, the entire sequence of S is repeated at the C terminus of M and L, which contain the additional pre-S2 domain or pre-S2 and pre-S1 domains, respectively [55]. In the late 1990s, two major types of pre-S deletion mutant LHBs were identified and associated with HCC [56, 57]. In the two types of pre-S mutant LHBs, pre-S1 and pre-S2 mutant LHBs, the pre-S1 and pre-S2 regions, respectively, are partially deleted [58, 59]. They accumulate in the ER and induce strong ER stress [60]. Through an ER stress-mediated pathway, they cause oxidative stress and DNA damage [61]. Through an ER stress-independent pathway, however, pre-S2 mutant LHBs contribute to the increased proliferation of hepatocytes [62]. Pre-S2 mutant large HBs expressed in hepatocytes cluster into groups and exhibit clonal expansion and growth advantage. Based on the findings of these previous studies, pre-S mutant LHBs, especially the pre-S2 type, are believed to be crucial in HBV-associated hepatocellular carcinogenesis.

The S protein is expressed at the highest levels and is predominant in virions and subviral particles. The interacting role between host proteins and S protein is another important mechanism leading to HCC. These proteins include JTB, ECHS1 and ALDOA, which refer to cell proliferation, cell motility and cell apoptosis [63–65]. More mechanisms need further research.

HBV Polymerase

HBV polymerase can inhibit IFN- β induction by interfering with IFN regulatory factor signaling [31, 32]. Mechanistically, polymerase was shown to interact with DDX3, a transcriptional factor of the IFN- β promoter. Since DDX3 acts downstream of the activation cascade triggered either by Toll-like receptor 3 (TLR3) (through TRIF) or by RIG-I (through IPS-1), the results of these studies show that polymerase can block the activation mediated by these two receptors recognizing dsRNA in the endosomes (TLR3) or in the cytosol (RIG-I).

Hepatitis B e Antigen

Hepatitis B e antigen (HBeAg) is the major product of the pre C-C gene and is a secreted, non-particulate form of the viral nucleocapsid [66, 67]. The HBeAg is regarded as an accessory protein of HBV and is not required for viral replication or infection. The natural history of CHB is typically divided into two phases: HBeAg-positive and HBeAg-negative. Patients with HBeAg-positive CHB have significant downregulation of TLR2 on the surface of monocytes circulating in the peripheral blood and also on hepatocytes and Kupffer cells in their livers. In contrast, HBeAg-negative infection results in upregulation of TLR2 and cytokine expression. Exposure to HBeAg is able to downregulate TLR2 expression but not TLR4 expression on monocytes and results in downstream effects on cytokine production [68]. This inhibition of cytokine production is mediated through TLR signaling pathways. The proposed interaction between the precore protein and the TLR2 pathway may broaden its immunological role in hepatitis B pathogenesis [69].

Immunotherapeutic Interventions for CHB

The two therapeutic approaches available for the suppression of HBV replication include antiviral agents (nucleoside analogues) and immune-based therapies (IFN- α or pegylated IFN- α). However, the rates of HBsAg loss and seroconversion to HBsAb are very low [70], and drug resistance or poor response rate are accompanied by numerous side effects. The goal of therapy is to improve quality of life and survival by preventing progression to cirrhosis, decompensated cirrhosis, HCC and death through sustained suppression of HBV replication [71]. Various therapeutic interventions have been tried as adjuvants to inhibit HBV replication, such as immunotherapeutic interventions, in the treatment of CHB (table 2).

Impaired DC function in patients with CHB may lead to insufficient T cell response to HBV, which may be associated with persistent viral infection. HBV particles and purified HBsAg may both contribute to the dysfunction of myeloid DCs [72] and directly inhibit the production of IFN- α [73]. Recent research suggested that HBsAg/hepatitis B core antigen (HBcAg)-pulsed human blood DC of CHB patients and DC from HBV transgenic mice induced HBsAg-specific and HBcAg-specific cytotoxic T lymphocytes (CTLs) [74]. Activated pulsed DCs acted synergistically with HBcAg-pulsed monocyte-derived DCs in the induction of HBsAg- and HBcAg-specific CTL response [75]. Therefore, a DC-based immunotherapeutic approach may disrupt tolerance against HBV and restore functional antiviral immunity, which is critical for the control of the virus in chronic HBV infection.

A low frequency of T cell immune responses to HBsAg was found in Chinese subjects with HBsAg seroclearance following antiviral therapy, which indicated that HBsAg-specific immune responses were not responsible for HBsAg seroclearance [76]. Robust anti-core T cell responses were found in patients with reduced HBsAg serum levels, suggesting that core-specific T cell responses mediated a protective effect in HBV control [77]. These results suggested that expansion of HBV-specific T cells in vitro through HBcAg stimulation may be one immunotherapeutic intervention.

Table 2. Strategies designed to increase the efficacy of immune-based therapy

Strategy	Effect
DC and HBV-specific cytotoxic T lymphocytes (CTLs)-based immunization	Disrupting tolerance against HBV and restoring functional antiviral immunity
NK cell-based therapy	It was restored following blockade of these immunosuppressive cytokines
Treatment with cytokines	Restoring functional HBV-specific CTLs
Blockage or depletion of immunoinhibitory cells	Increasing CD8+ T cell proliferation and IFN- γ and IL-2 production
Activation of signaling pathways leading to IFN- α production (TLR agonist)	Producing large amounts of type I and III IFN
Therapeutic vaccines	Inducing reduction in viremia and cccDNA and boosting the hepatic immune response

In CHB patients, the cytotoxic capacity was retained, but NK cell activation and subsequent IFN- γ and tumor necrosis factor alpha (TNF- α) production, especially of the CD56 subset, were strongly hampered. This selective defect in NK cell function may be attributed to the influence of IL-10 and TGF- β in the liver, since it was restored following blockade of these immunosuppressive cytokines in vitro [78]. Restoration of the NK cell cytokine-producing capacity by viral load reduction, therefore, contributed to clearance of the virus [79].

There are many important cytokines involved in the immune responses to HBV infection. Compared with lamivudine alone, cytokine (IFN- γ + TNF- α) treatment and sequential therapy with cytokine and lamivudine showed a stronger inhibition of HBV cccDNA [80]. IL-12 is an immunomodulatory cytokine that promotes cellular immunity. IL-12 stimulation can also lead to downregulation of the co-inhibitory molecule PD-1 [81] and has a pleiotropic effect in restoring functional HBV-specific CTLs. Therefore, IL-12 could be used as an adjuvant agent to break immunological tolerance in CHB treatment.

Tregs showed an immunosuppressive effect against HBV-specific T helper cells. The presence of HBV-specific Tregs contributed to inadequate immune response against the virus, leading to chronic infection [82]. Tregs determine the disease prognosis by reflecting infection progression and impaired immune response. Tregs are therapeutic targets for immunotherapy of HBV infection. The PD-1/PD-1 ligand 1 (PD-L1) system may play a role in the negative regulation of T cell functions in HBV infection. Intrahepatic HBV-specific CD8+ T cells expressed higher levels of PD-1 and lower levels of CD127 than their peripheral counterparts [83]. PD-1 expression in CTLs may be related to the degree of liver damage in CHB patients [84]. Blockade of PD-1/PD-L1 interaction increased CD8+ T cell proliferation and IFN- γ and IL-2 production by circulating intrahepatic lymphocytes, even though anti-PD-L1 showed a stronger effect on intrahepatic compared with peripheral T cells [85].

TLRs are evolutionarily conserved pattern recognition receptors. They play a crucial role in early host defense in a range of microbes, including HBV. Stimulation of TLR9 dramatically increased the CTL responses [86]. Activation of pulsed DCs using synthetic TLR7 and TLR9 ligands or agonists is capable of producing large amounts of type I and III IFN in response to many viruses, including HBV, which contributes to the suppression of HBV replication [87]. These data suggested that the combination of TLR agonists, especially TLR9 agonist, and appropriately timed immune therapy may be a promising approach in the treatment of HBV infection [88].

Therapeutic vaccines, as an alternative to long-term antiviral treatment or to support only partially effective therapy, are currently being developed for chronic HBV infection. Buchmann et al. [89] evaluated a novel vaccine formulation comprising particulate HBsAg and HBcAg, and the saponin-based ISCOMATRIX™ adjuvant for its ability to stimulate T and B cell responses in C57BL/6 mice. The results suggested that the vaccine induced multifunctional HBsAg- and HBcAg-specific CD8+ T cells as well as high antibody titers against both antigens. DNA vaccine encoding HBV large envelope and/or core protein was shown to induce reduction in viremia and cccDNA in the liver in a duck model [90]. The latter was achieved by boosting the hepatic immune response [91]. These data indicate that DNA vaccines combined with viral antigens were potential HBV therapeutic vaccines.

Future Challenges

Although many risk factors have been identified for HCC and tremendous progress has been made in the understanding of the mechanism of HBV-induced HCC, we are still uncertain as to which risk factor takes the major responsibility for hepatocarcinogenesis. What is clear is that HBV-induced hepatocarcinogenesis is a multifactorial process. HBV integration may directly lead to malignant transformation through altering the function of certain key genes. Meanwhile, HBV products and HBV mutation may disrupt normal cellular signaling pathways, thus contributing to HBV-induced HCC. Antiviral therapy of chronic HBV infection has improved dramatically during the last decades, and several new approaches have achieved preclinical validation, but a completely effective treatment is still not available. Thus, functional cure of chronic HBV infection remains an important therapeutic challenge.

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Disclosure Statement

The authors declare no conflict of interest.

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