World Journal of Gastroenterology

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DOI: 10.3748/wjg.v22.i6.1943

World J Gastroenterol 2016 February 14; 22(6): 1943-1952
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

2016 Hepatocellular Carcinoma: Global view

Hepatocellular carcinoma and hepatitis B surface protein

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Author contributions: All authors equally contributed to the conception and design of the study, literature review, and analysis, critical revision, and editing of the paper; All authors approved the final version of the manuscript.

Supported by Science and Technology Planning Project of Guangdong Province, China, No. 2014A020212073.

Conflict-of-interest statement: The authors have no potential conflicts of interest.

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Received: April 11, 2015
Peer-review started: April 13, 2015
First decision: June 26, 2015
Revised: July 27, 2015
Accepted: November 9, 2015
Article in press: December 1, 2015
Published online: February 14, 2016

Abstract

The tumorigenesis of hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC) has been widely studied. HBV envelope proteins are important for the structure and life cycle of HBV, and these proteins are useful for judging the natural disease course and guiding treatment. Truncated and mutated preS/S are produced by integrated viral sequences that are defective for replication. The preS/S mutants are considered "precursor lesions" of HCC. Different preS/S mutants induce various mechanisms of tumorigenesis, such as transactivation of transcription factors and an immune inflammatory response, thereby contributing to HCC. The preS2 mutants and type Ⅱ "Ground Glass" hepatocytes represent novel biomarkers of HBV-associated HCC. The preS mutants may induce the unfolded protein response and endoplasmic reticulum stress-dependent and stress-independent pathways. Treatments to inhibit hepatitis B surface antigen (HBsAg) and damage secondary to HBsAg or the preS/S mutants include antivirals and antioxidants, such as silymarin, resveratrol, and glycyrrhizin acid. Methods for the prevention and treatment of HCC should be comprehensive.

Key words: Hepatitis B surface protein; Hepatocellular carcinoma; PreS/S mutants; Endoplasmic reticulum stress; "Ground Glass" hepatocytes

Core tip: The tumorigenesis of hepatitis B virus-associated hepatocellular carcinoma (HCC) has been widely studied. The preS/S mutants are considered "precursor lesions" of HCC. Different preS/S mutants induce various mechanisms of tumorigenesis, such as transactivation and an inflammatory response. The preS2 mutants and type Ⅱ "Ground Glass" hepatocytes represent novel biomarkers of HCC. The preS mutants may induce the unfolded protein response and endoplasmic reticulum stress-dependent and stress-

independent pathways. Treatments to inhibit hepatitis B surface antigen (HBsAg) and damage secondary to HBsAg or the preS/S mutants include antivirals and antioxidants. Methods for the prevention and treatment of HCC should be comprehensive.

Li YW, Yang FC, Lu HQ, Zhang JS. Hepatocellular carcinoma and hepatitis B surface protein. *World J Gastroenterol* 2016; 22(6): 1943-1952 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i6/1943.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i6.1943

INTRODUCTION

There are approximately 387 million carriers of hepatitis B virus (HBV) worldwide, and one-quarter of them will develop hepatocellular carcinoma (HCC)[1]. In HBV-endemic regions, chronic hepatitis B (CHB) is a primary risk for HCC[2]. Epidemiological studies have provided overwhelming evidence for a causal role of CHB infection in HCC development[3]. The risk for developing HCC increases from 6 to 37 times in patients with different statuses of HBV infection compared with control subjects[4-8]. In one study, the relative risk of HCC was increased by approximately 100-fold in HBV carriers compared with noncarriers[9]. Most of the HCC cases occur at the advanced stage or the anti-HBe-positive phase, with the peak incidence occurring in the sixth decade[10]. However, studies of HBV-induced tumorigenesis are widely debated[3].

STRUCTURE AND ROLE OF SURFACE PROTEINS IN HBV

The HBV genome of hepadnaviruses is a relaxed circular, partially double-stranded DNA (RC-DNA) structure[11]. The RC-DNA is converted into a template for the transcription of viral RNAs - covalently closed circular molecules - in the nucleus[12-14]. Three major unspliced transcripts, with sizes of 3.5 kb, 2.4 kb, and 2.1 kb, and a less abundant 0.8 kb transcript with a common polyadenylation site, all code for the viral proteins; the 3.5 kb pregenomic RNA also serves as a template for viral replication through a reverse transcription mechanism[14]. The 3.2 kb HBV genome has four overlapping open reading frames (ORFs)[3]: the preC/C ORF encodes the e antigen (HBeAg) and core antigen; the P ORF encodes the terminal protein (TP) and viral polymerase that possesses DNA polymerase, reverse transcriptase, and RNase H activities; and the X gene encodes hepatitis B x protein (HBx) for virus replication. The spliced viral transcript encodes a viral protein termed the hepatitis B spliced protein (HBSP)[15]. The preS/S ORF overlapping the HBV polymerase ORF encodes the three viral surface proteins. Three co-terminal envelope proteins termed large (LHBs, including the preS1 + preS2 + S domain), middle (MHBs, including the preS2 + S domain), and small (SHBs, the S domain alone) surface proteins, respectively[16]. Additionally, truncated and mutated preS2/S (the LHBs and truncated MHBs) or HBx proteins are produced by integrated viral sequences defective for replication[17,18]. The viral surface proteins are important for the HBV structure and life cycle because HBsAg reflects the transcriptional activity of the cccDNA[19]. The small surface proteins (SHBs) are major components of the virion envelope and the nucleocapsid-free subviral particles[20]. The HBV envelop proteins and capsids (with the HBV genome) are assembled on the viral particles in the endoplasmic reticulum (ER) and are discharged from the cell[21,22]. The viral surface proteins may interact with a host cell receptor to initiate the infection[3]. However, more than a dozen host-binding proteins to the preS1, preS2, or S domain have been identified[22]. PreS1, but not preS2, which is myristoylated at the glycine residue in position 2, is essential for virus infection[23,24]. The preS1 domain has a receptor binding site that contains the essential aa residues 9-18 and recognizes the asialoglycoprotein receptor on the surface of human hepatocytes or HCC cells[25-27]. HBV initially combines with heparan sulfate proteoglycans (HSPGs) that are trapped within the liver in the space of Dissé[20]. Sodium taurocholate cotransporting polypeptide (NTCP) is a binding partner for the myristoylated peptide 2-48 of the preS1 domain. Additionally, hepatitis delta virus uses HBV envelope proteins for its transmission[16]. However, the initial phases of HBV infecting hepatocytes, namely virion attachment, uncoating, and entry, are not completely identified[3]. Another important role of HBV surface proteins is in tumorigenesis, particularly in HCC, which has complex and heterogeneous features[3,28].

HBV PROTEINS AND GENOME IN HEPATOCARCINOGENESIS

HBV tumorigenesis involves inflammation and liver regeneration, HBV gene mutations, and viral (mutant) oncoproteins[4,13,29,30]. HCC-associated signaling pathways include the Wnt/b-catenin signaling[31], the p14ARF/p53 pathway[32], transforming growth factor alpha (TGF-α) signaling, Ras/mitogen-activated protein kinase (MAPK) signaling, and phosphatase and tensin homolog/Akt and mammalian target of rapamycin (mTOR) pathways[33].

Both HBeAg and HBV genotype C infections are considered risk factors of HCC[3,34]. Because reverse transcriptase lacks proofreading activity, HBV gene mutations occur more frequently than in other DNA viruses[35]. Various mutations can predict HBV-associated HCC during long-term infection[35]. HCC-associated mutations include the preS region in HBV genotype C, C1653T in enhancer Ⅱ, as well as T1753V and A1762T/G1764A in the basal core promoter in HBV genotype C. These mutations alone or in combination can predict

HCC development in 80% of all cases[36-38]. HBSP can promote carcinogenesis[39] as well as hepatoma cell motility and invasion[40]. Occult HBV infection (OBI, positive for HBV-DNA, negative for serum HBsAg) may produce proteins with transforming properties that contribute to hepatocellular transformation[41,42]. HBV DNA integration may frequently regulate key cellular pathways of carcinogenesis[42], contribute to cis- or trans-activation[3,43-47], and influence gene families that are involved in cell survival, proliferation, and immortalization[42].

Both HBx and HBs (mutant) proteins are designated "viral oncoproteins". The HBx protein has been studied extensively[3]. Tumorigenesis of the HBx protein may influence the regulation of the cell cycle, signaling pathways, DNA repair[1,48-50], chromosomal instability[51], cell transcription[52,53], proliferation, and inflammation and immune responses[54-56]. The HBs (mutant) proteins, primarily preS mutants, are recognized as "precursor lesions of HCC"[57] and a risk factor for the post-operative recurrence of HCC[58]. The tumorigenesis of HBs (mutant) proteins has also been widely studied.

CLINICAL ASSOCIATION BETWEEN HEPATITIS B SURFACE PROTEINS AND HCC

HBsAg has primarily been considered a marker of HBV infection. Since the two methods for HBsAg quantification, the Architect HBsAg assay (Abbott Diagnostics, Abbott Park, IL, United States)[59] and Elecsys HBsAg Ⅱ quant assay[60], first became available, serum HBsAg levels have been found to reflect the activity of intrahepatic cccDNA[61]. These methods may evaluate HBV replication more accurately.

HBsAg levels vary significantly according to the different courses of HBV infection[62]. They have proven useful in judging the natural disease course and guiding treatment in addition to HBV DNA and HBV envelope antigen and antibody[61]. CHB is classified into five phases: the "immune tolerant phase", "immune active phase", "inactive HBV carrier state", "HBeAg-negative CHB phase", and "HBsAg-negative phase"[63,64]. In HBeAg-positive patients, the HBsAg levels are associated with fibrosis and immune tolerance; lower HBsAg levels are associated with moderate to severe fibrosis[65,66]. In HBeAg-negative patients, an HBsAg level less than 1000 IU/mL was associated with a lower risk of HCC[67]. The absolute lowest risk with a cumulative risk for HCC decreased less than 1% over 9 years after the patients had anti-HBs and anti-HBe seroconversion[68]. However, the prediction[68] may not apply to preS mutants because these mutants may account for the different levels of HBsAg[61] and have a close association with HCC.

EPIDEMIOLOGY OF PRES MUTANTS

PreS mutants evolve in the long course of CHB, possibly for immune pressure, or antivirals[3,7]. The frequency of preS mutants increases successively in the different stages of CHB infection. A meta-analysis showed that it was approximately 10%, 20%, 35%, and 50% in asymptomatic HBsAg carriers, CHB patients, patients with liver cirrhosis, and HCC patients, respectively[36]. Su *et al*[69] reported that these mutants are present in up to 63% of HCC patients. The prevalence of the preS mutants varied in different countries and HBV genotypes; there was a higher prevalence in genotype B and C than in the other genotypes[70]. The mutants are located in both the preS1 and preS2 regions[69,71]. The preS2 mutants occur more frequently than the preS1 mutants[36], possibly because preS1 is essential for virus infection. The preS2 mutants often coincide with changes in human immune cell epitopes[71].

DIFFERENT PRES MUTANTS INDUCE VARIOUS MECHANISMS THAT CONTRIBUTE TO HCC

The preS1/preS2/S sequence encodes a transcriptional activator with potentially transforming properties[17,72,73]. These transcriptional effects can activate the protein kinase C-dependent c-Raf-1/MAP1-kinase signal pathway, thus promoting transcription factors to increase the proliferation rate of hepatocytes[17]. Only the carboxy-terminal truncation of LHBs or MHBs has transactivating properties[1]. The truncated LHB protein expressed in transgenic mice resulted in the development of HCC[74]. The transactivating effects of MHBs are mediated *via* sequence-specific binding to DNA[75,76], thereby stimulating promoter sequences of the c-myc, c-fos, and c-Ha-ras oncogenes[77,78]. Both LHBs and MHBs proteins have the same transcriptional effects[73,79].

Subsequent studies have reported tumorigenesis in several mutants. An HBV polymerase rtA181T/surface truncation mutant in a patient with advanced HCC transactivated the simian virus 40 and human c-Myc promoters; the tumorigenic effects of the mutant were identified in nude mice[80]. Three mutations, sL95*, sW182*, and sL216*, activated cell proliferation and transformational abilities; the sW182* mutant demonstrated potent tumorigenic activity. However, the three mutants could not promote ER stress[81]. A W4P/R mutation in the LHB region of HBV genotype C may contribute to HCC development in an interleukin (IL)-6-dependent manner only in male patients[82]. *In vitro*, the MHBst167 mutants interacted with the proteins related to tumor development/progression[83]. Because the preS mutants varied in different research

groups, it is difficult to investigate the corresponding management.

PRES MUTANTS INDUCE ER STRESS THAT CONTRIBUTES TO HCC

HBV proteins utilize the ER protein folding machinery and cellular secretory pathway[84]. Therefore, the underlying mechanisms of preS mutants that contribute to HCC are involved in ER stress. Both preS1 and preS2 mutants activate ER stress in hepatocytes[85]. Different preS mutants activate differential activities of ER stress in hepatoma cells with accumulated LHB proteins[86].

PreS1 mutants in HBV tumorigenesis

A previous study showed that HBV preSI mutants demonstrate hepatocarcinogenesis effects through the transactivation of the TGF-α gene[87]. PreS1 mutant activated higher levels of ER chaperones (Grp78 and 94), calcium release, cyclooxygenase-2 (COX-2), inflammatory cytokines, and oxidative stress intermediates, which tend to result in apoptosis[85]. The HBV preS2 mutant proteins play a more important role in ER stress.

"Ground glass" hepatocytes accumulated with preS mutants

"Ground glass" hepatocytes (GGHs) comprise abundant particles of surface antigens that accumulate in the ER during CHB infection[88-90]. Su *et al*[85] found that type Ⅱ GGHs distributed in large clusters express marginal HBs proteins that harbored preS2 mutants and usually emerge at the late nonreplicative stage or in cirrhotic liver; type Ⅰ GGHs, which accumulate with inclusion-like HBs (small surface protein) proteins and harbor preS1 mutants, are usually scattered sporadically during the replicative phases[86]. Type Ⅱ GGHs with preS2 mutants are suggested biomarkers of HCC and can predict recurrence and survival in HBV-infected HCC patients[91].

The mutated HBV surface proteins cannot be properly folded in the ER, possibly leading to the induction of the unfolded protein response (UPR)[90]. Both HBV surface proteins and HBx protein can trigger the UPR[92,93]. HBV SHBs activate the UPR and host autophagy to enhance HBV envelopment and replication[94]. However, HBV also activates the ER degradation-enhancing mannosidase-like proteins (EDEMs) to enhance the degradation of HBV surface proteins (terminally misfolded glycoproteins), thus relieving ER stress during the UPR[95]. The mechanisms might maintain the balance between viral loads and host cells to facilitate the persistence of HBV infection. This mechanism may direct us to a new treatment strategy. However, the pathogenic role of the UPR in HBV infection and HCC appears to be complex. A drug (similar to EDEM) that relieves ER stress to prevent HCC might facilitate persistent HBV infection and *vice versa*. These mechanisms require further study.

Pres2 mutants induce ER stress-dependent and stress-independent pathways

The preS2 mutant protein accumulated in the ER can trigger the ER stress-dependent vascular epithelial growth factor/Akt/mTOR and nuclear factor kappa B/COX-2 signal pathway[96]. Through this signal pathway, HBx and envelope proteins that accumulate simultaneously in GGHs can enhance the oncogenic effects in transgenic and human HCCs[97]. mTOR can suppress autophagy and regulate cellular metabolism[98]. The suppression of autophagy by mTOR[98] is in contrast to the induction of autophagy during UPR[94]. This might result in contradictory treatment for HCC and HBV infection[57].

The LHBs protein with preS2 mutants may also activate an ER stress-independent pathway, ultimately leading to a growth advantage in type Ⅱ GGHs. The pathway includes c-Jun activation domain binding protein 1 nuclear translocation and activation of p27/retinoblastoma/Cdk2/cyclin A, D pathways, which results in cell cycle progression, cell proliferation, and centrosome over-duplication[99-101].

Together, the data show that the preS2 mutant protein is a promising gene transactivator and an ER stress inducer, resulting in host genomic instability[85] and HCC development.

HBV SURFACE PROTEINS INDUCE AN IMMUNE INFLAMMATORY RESPONSE CONTRIBUTING TO HCC

HBV-induced chronic necroinflammation plays an indirect role in hepatocarcinogenicity in the preS/S transgenic mice[74]. The LHBs proteins in the ER triggered inflammation, abnormal regeneration and transcription, resulting in cancer development[102]. This mechanism is similar to the contribution of the W4P/R mutation to HCC development in an IL-6-dependent manner[84]. Another immune-associated carcinogenesis mechanism is viral immune escape induced by HBV mutants[103]. HBV escape from the host's immune surveillance may favor the clonal proliferation of hepatocytes with the preS mutants[104].

HBV surface proteins may be detected in immune cells. In dendritic cells, both HBV and HBsAg abrogated the CpG-A/TLR9-induced signal pathway and decreased the levels of co-stimulatory molecules and cytokines; this mechanism may contribute to HBV persistence[105].

In addition, microRNAs may contribute to carcinogenesis in HBV-induced HCC. Both HBx and HBs proteins can increase microR499a expression, which might play an oncogenic role by targeting MAPK6[106].

The duration of time between when HBV first enters a host and HCC development is long. A large

Table 1 PreS1/S2/S and hepatocellular carcinoma carcinogenesis

Function	PreS1/S2/S mutation	Ref.
A trans-activator function	Integration of HBV preS/S sequences	Caselmann *et al*[72] 1990, Kekulé *et al*[75] 1990
Transactivation of NF-κB, or AP and other transcription factors for transactivation, stimulating promoter sequences of the c-myc, c-fos, and c-Ha-ras oncogenes	MHBst	Meyer *et al*[77] 1992, Lauer *et al*[78] 1994
A transcriptional activator, activated tumor promoter pathways via the PKC-dependent c-Raf-1/ Erk2, MAP1- kinase signal pathway, increase the proliferation rate of hepatocytes	LHBs, the preS2	Hildt *et al*[17,73,79] 1996, 1998, 2002
Transactivator with DNA-binding properties	HBV surface (S)	Alka *et al*[76] 2000
Transactivated the simian virus 40 and human c-Myc promoters truncation mutant	Polymerase rtA181T/surface	Lai *et al*[80] 2008
Activated cell proliferation and transformational abilities	sL95*, sW182*, and sL216*	Huang *et al*[81] 2014
Interacted with the proteins related to tumor development/ progression *in vitro*	MHBst167	Li *et al*[83] 2014
Biomarkers of HCC and predictor of recurrence survival	GGHs harbored preS2 mutants in the ER	Malhi *et al*[90] 2011, Su *et al*[85] 2014
Trigger the ER stress-dependent pathway		
VEGF/Akt/mTOR and NF-κB/COX-2 signal pathway in GGHs		Yang *et al*[96] 2009
Enhance the oncogenic effects in transgenic and human HCCs	Co-expressing hepatitis B virus X protein and surface antigens	Wu *et al*[97] 2014
An ER stress-independent pathway		
Activate JAB1 nuclear translocation and activation of p27/ retinoblastoma/Cdk2/cyclin A, D pathways, results in cell cycle progression, cell proliferation, and centrosome over-duplication, a growth advantage in type Ⅱ GGs	LHBs with preS2 mutants	Wang *et al*[99] 2005, Hsieh *et al*[101] 2011, Wang *et al*[100] 2012
Immune-associated carcinogenesis		
HBV-induced chronic necroinflammation	Pre S/S transgenic mice	Chisari *et al*[102] 1989
The clonal proliferation of hepatocytes with the preS mutants	The HBV mutants escape from the host's immune surveillance	Zhong *et al*[104] 1999
Contributing to HCC in an IL-6-dependent manner only in male patients	A W4P/R mutation in the LHB region of HBV genotype C	Lee *et al*[82] 2015
Other		
Increase microR499a expression, which might play an oncogenic role by targeting MAPK6	HBs proteins	Xiang *et al*[106] 2014

HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; LHBs: Hepatitis B virus large surface protein; PKC: Protein kinase C; MHBst167: C terminally truncated surface antigen middle protein of hepatitis B virus; MAPK: Mitogen-activated protein kinase; ERK: Extracellular Signal-regulated Kinase signaling; GGHs: "Ground glass" hepatocytes; VEGF: Vascular epithelial growth factor; JAB1: c-Jun activation domain binding protein 1; LHBs: MHBs, large (including the preS1 + preS2 + S domain), middle (including the preS2 + S domain) surface proteins.

number of factors participate in carcinogenesis (Table 1). What are the key and special factors? For example, not all inflammation results in cancer. Additionally, are there any other special factors that promote HCC development in an IL-6-dependent manner?

TREATMENTS TO INHIBIT HBSAG AND DAMAGE SECONDARY TO HBSAG ANTIVIRALS

HBV-induced HCC was the first tumor to be prevented by universal immunization against the responsible virus[1]. The host's immune system can control HBsAg levels[61]. However, spontaneous HBsAg seroclearance is rarely achieved (an annual incidence of 1%-2% in CHB[107,108]). At an advanced stage of HCC, the levels of HBsAg are still high, while that of HBV DNA may be negative. This tumor type with a poor prognosis is refractory to chemotherapeutic regimens[109,110]. In chronic HBV infection, the prophylactic measures or therapy for HCC include suppression of HBV replication and recovery from molecular abnormalities due to HBV infection. Achieving HBsAg loss and anti-HBs positivity is the final aim of antivirals[61]. Nucleos(t)ide analog (NA) therapy has antiviral effects that may reduce HCC development and the post-operative recurrence of HCC[111]. NA treatment affects the reverse transcription of pregenomic RNA but does not affect cccDNA and subgenomic RNA that have translational activity associated with HBsAg levels[112]. Thus, the current NA therapy hardly clears HBsAg. With NA treatment, it may take 36 to 52 years to achieve HBsAg loss[113,114]. Lamivudine was the first licensed NA introduced to treat HBV, and it reduced the incidence of HCC when compared to no treatment. However, the development of HCC may increase in cases with lamivudine resistance. The current first-line NAs, entecavir and tenofovir, may also reduce HCC development, but the risk of HCC is not eliminated, even in the patients who remain in virological remission with this therapy[115]. NA resistance suggests antiviral failure.

Additionally, the drug-resistant HBV strains may have mutant surface proteins with oncogenic effects[116]. In our study, substitutions in the S region, but not the drug-resistant mutations, possibly resulted in the poor effects of adefovir[117]. Subsequent studies also showed that preS2 mutants induced resistance to NAs and predicted HCC development[118]. Treatment with interferon inhibits HBsAg or preS mutant proteins more than NAs[119-121].

Thus, due to cccDNA, HBV and HBsAg cannot be eliminated[61]. There are also no measures to manage the integrative HBV DNA. It is impossible to develop sequence-specific antivirals for so many envelope mutants. The rtA181T/surface mutant may occur spontaneously in the absence of antiviral therapy. Its oncogenic potential warrants careful re-evaluation of the current strategy of prolonged antiviral therapy[80].

However, there are many compounds in development for CHB up to June 22, 2015, such as Myrcludex B (entry inhibitor targeting NTCP), Rep 2139 (REP 9AC, HBsAg release inhibitor), TKM-HBV (HBsAg inhibitor), and BSBI-25 (cccDNA inhibitor)[122]. These compounds should improve the effects of antivirals and reduce the burden of drug resistance and HCC development[123].

TREATMENTS BASED ON ER STRESS

Drugs based on the theory of ER stress vary in the prophylaxis and treatment of HCC. The UPR is targeted as primary or adjuvant chemotherapy for HCC, *e.g.*, bortezomib for treating cancer *via* ER stress-induced apoptosis[124] and mTOR inhibition as a promising strategy for the clinical management of HCC[125]. However, mTOR inhibition may activate HBV replication in HBV-induced HCC[57]. mTOR activation may recruit the YY1-HDAC1 complex to feedback suppress transcription from the preS1 promoter (nucleotides 2812-2816)[57], thus partially explaining the low or negative HBV replication in the HCC stage. Therefore, mTOR inhibitors should be used in combination with antivirals.

To prevent HCC, targets to HBV-induced ER stress provide a strategy in high-risk CHB. Antioxidants may be such ideal agents because they reduce ER stress, thereby improving protein folding[126]. Natural products, such as silymarin and resveratrol, have been used in HCC. The two drugs can target the ER stress-associated signal pathways[85]. However, these findings require further verification.

Glycyrrhizin acid (GA) has multiple functions, such as effective hepato-protection and the reduction of elevated transaminases. Glycyrrhizin can suppress ER stress in acute liver injury[127]. Long-term treatment with glycyrrhizin prevents HCC development in chronic hepatitis C infection[128]. Glycyrrhizin treatment suppressed the sialylation of HBsAg and secretion of HBsAg in PLC/PRF/5 cells[129,130]. Therefore, it is also widely administered in CHB infection. A study to determine whether drugs such as GA and extracts from other herbs would influence the preS mutants is required.

Comprehensive prevention and treatment also include avoiding other risk factors such as aflatoxin B1 and alcohol intake. A prolonged battle against the damage induced by this virus is necessary.

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P- Reviewer: Li WH **S- Editor**: Yu J **L- Editor**: Filipodia
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ISSN 1007-9327