Review Article: Liver Immunology

# Hepatitis B virus large surface protein: function and fame

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**Abstract:** Chronic infection with hepatitis B virus (HBV) is the leading cause of liver cirrhosis and hepatocellular carcinoma worldwide. HBV life cycle begins with viral attachment to hepatocytes, mediated by the large HBV surface protein (LHBs). Identification of the sodium-taurocholate cotransporting polypeptide (NTCP) as a HBV receptor has revealed a suitable target for viral entry inhibition. Analysis of serum hepatitis B surface antigen (HBsAg) level is a non-invasive diagnostic parameter that improves HBV treatment opportunities. Furthermore, HBsAg plays an important role in manipulation of host immune response by HBV. However, observations in patients with chronic hepatitis B under conditions of immune suppression and in transgenic mouse models of HBV infection suggest, that in absence of adaptive immune responses cellular mechanisms induced by HBV may also lead to the development of liver diseases. Thus, the multifaceted pathological aspects of HBsAg predetermine the design of new therapeutical options modulating associated biological implications.

**Keywords:** Hepatitis B virus (HBV); sodium taurocholate cotransporting polypeptide; immune response; endoplasmic reticulum stress (ER stress); hepatocellular carcinoma (HCC)

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## Introduction

Chronic infection with hepatitis B virus (HBV) affects 350 to 400 million individuals worldwide and is the leading cause of liver cirrhosis and hepatocellular carcinoma (HCC) worldwide. More than 780,000 people die every year due to the consequences of hepatitis B (WHO 2014). Although much is known about HBV structure and replication cycle (1) the pathogenic mechanisms responsible for liver injury, cirrhosis development and malignant transformation during chronic HBV infection are not well understood. The inability to achieve a complete cure of chronic HBV infections is among the problems that still remain today.

## Hepatitis B virus (HBV)

Hepatitis B was recognized as a disease in ancient times, but the virus itself was identified not until 1965 (2). HBV is one of the smallest enveloped DNA viruses and the prototype member of the family of *Hepadnaviridae*. Infectious HBV has a spherical structure consisting of hepatitis B surface antigen (HBsAg) that envelops the viral nucleocapside, which is formed by the core protein (HBcAg). The encapsidated viral genome is organized as a relaxed circular partially double-stranded DNA (rcDNA) (3). Upon infection of hepatocytes, the HBV rcDNA is converted by cellular enzymes into a covalently closed circular DNA (cccDNA) inside nuclei of infected cells. Episomal HBV cccDNA persists in the hepatocyte as a stable minichromosome organized by histone and non-histone proteins. The viral minichromosome utilizes the cellular transcriptional machinery to produce all viral RNAs necessary for protein production and viral replication (4). The HBV genome contains four overlapping open-reading frames that encode the viral polymerase, HBV surface proteins, the structural core protein and the non-structural precore protein, also known as secreted e-antigen (HBeAg), and the X protein (1). HBV surface (HBs) proteins—the large (LHBs), middle (MHBs), and small (SHBs) can be distinguished by their different domains and glycosylation status (1). The carboxyterminal domain containing SHBs is

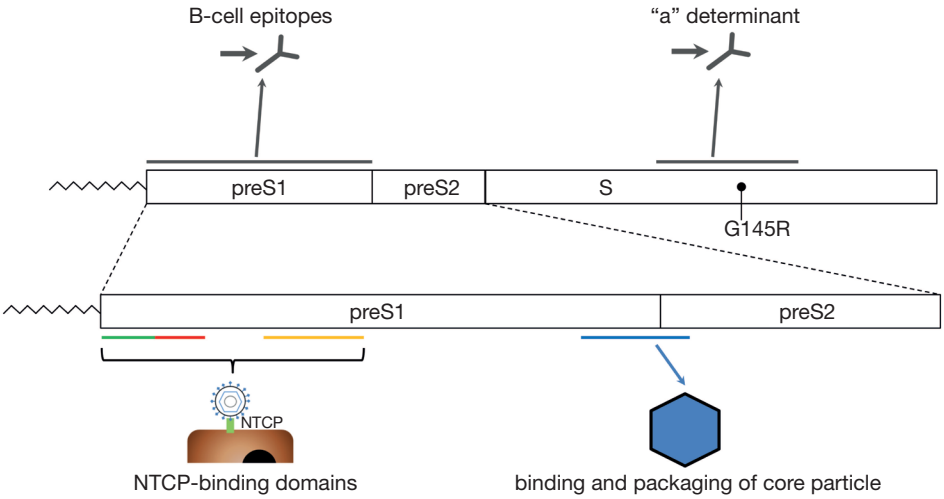


**Figure 1** Structure of HBV preS/S region [adapted from (1) and (6)]. HBV surface proteins are composed of three domains: S, preS2, and preS1. PreS1 contains 108, 118, or 119 amino acids (aa), depending on the genotype, preS2 domain is 55 aa long, and S-domain (S) contains 226 aa. NTCP-binding site includes essential (red line, 9-18 aa), and accessory (yellow line, 28-48 aa) domains. Infection-interfering myristoylated preS1 peptides (the signal domain for myristoylation is indicated (green line, 2-8 aa) overlap with the putative receptor-binding site. Major B-cell epitope sites for preS1 include epitopes for neutralizing antibodies. "a" determinant of S-domain (HBsAg) includes the major antibody neutralization domain of HBV (99-170 aa). G145R is a major vaccine-induced immune escape mutation. Dot line indicates an important mutation leading to escape from anti-HBs antibodies. Domain essential for binding and packaging of core particle is located at the junction of preS1 (92-108 aa) and preS2 (1-5 aa).

present in all surface-proteins, preS1 N-terminal extension only in LHBs, preS2 in LHBs and MHBs (5) (*Figure 1*). These three forms of HBV surface proteins represent HBsAg (2). Surface protein synthesis occurs in endoplasmic reticulum (ER) and could lead to the situation that amounts of protein produced exceed those required for virion assembly. Excess surface proteins undergo multimerization resulting in their budding from the ER/Golgi compartment as both non-infectious spherical and filamentous subviral particles (SVP) or as virions (3). The SVPs typically outnumber the virions, could be components of circulating immune complexes (7), and may induce immune tolerance by a mechanism of "viral apoptotic-like mimicry" (8).

## HBV receptor: sodium taurocholate cotransporting polypeptide (NTCP)

PreS extensions of LHBs and MHBs possess certain features that are very important for HBV infectivity such as binding the nucleocapsid during virus envelopment (3) as well as receptor recognition and binding (9,10). The best characterized determinant is a myristoylated motif within the N-terminal preS1 domain where myristoylation-deficient mutants assemble but are non-infectious (11,12). A mimic of preS1 peptide (Myrcludex B®) that inhibits HBV entry in hepatocytes is currently being evaluated in clinical trials for anti-viral activity in chronic HBV-infected patients (13). HBV binding and entry starts with a low-specific binding to heparan-sulfate proteoglycans (14-16), followed by binding to high-specific receptor that was unidentified recently (*Figure 1*) (17). Using HBV-susceptible primary hepatocytes from Asian tree shrews (*Tupaia belangeri*) in a biochemical approach, the authors detected NTCP as a binding factor for HBV preS1 peptides, and HBV or HDV (17). The identification of NTCP as a bona fide receptor has revealed a suitable target for HBV entry inhibition. NTCP receptor function is blocked by a variety of different agents including Myrcludex B®, a synthetic N-acylated preS1-derived lipopeptide that inhibits HBV entry in vitro and in vivo with high efficacy (18). Entry inhibitors in combination with nucleos(t)ide analogues could block re-infection and shield naive hepatocytes that emerge from natural liver turnover, opening up new therapeutic options.

## Clinical relevance of HBsAg

Primary treatment goals for patients with HBV infection are to prevent progression of the disease, particularly to cirrhosis,

 

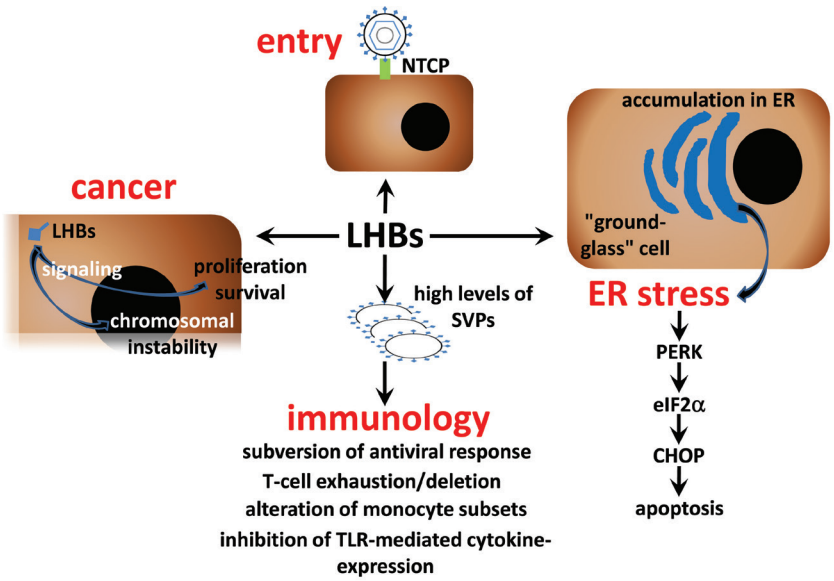


**Figure 2** Pathological implications of the LHBs. CHOP, C/EBP homologous protein; eIF2α, eukaryotic initiation factor 2α; ER, endoplasmic reticulum; LHBs, large Hepatitis B virus surface protein; NTCP, Na+-taurocholate cotransporting polypeptide; PERK, protein kinase RNA-like endoplasmic reticulum kinase; sVPs, subviral particles.

liver failure, and HCC (19). The clinical relevance of HBsAg levels is inferred from the relationship of this marker to the intrahepatic amount of cccDNA. There is a correlation between serum HBsAg concentrations and the intrahepatic levels of cccDNA, with the highest levels occurring in HBeAg positive hepatitis B and the lowest in patients with resolved hepatitis (20-23). Through this association, the amount of circulating HBsAg is thought to indirectly measure the control of infection by the immunological response independent from the antiviral response, which can be assessed by measuring HBV DNA levels in serum. HBsAg titres allow to monitor the natural history of HBV infection and to predict treatment outcome. HBsAg quantification can be used to differentiate between inactive carriers and HBeAg-negative chronic hepatitis B patients, who are likely to reactivate HBV infection and who can benefit from therapy (24). Early HBsAg monitoring could be also used during PEG-IFN therapy to develop a response-guided algorithm to stop or switch therapy at week 12 in poor responders, to continue standard 48-week treatment in most patients with a favourable response, and to extend therapy for intermediate on-treatment responders to improve the chances of response (25). The addition of entecavir (ETV) or tenofovir disoproxil fumarate (TDF) to PEG-IFN therapy has been shown to increase on treatment HBsAg decline, HBeAg seroconvertion and sustained virological response rate (26-28). Several studies suggest that baseline treatment and on-treatment HBsAg levels might help identify patients, who can stop therapy with a reduced risk of reactivation (25). In summary, analysis of serum HBsAg level is a non-invasive diagnostic parameter that improves HBV treatment opportunities.

It is recognized that genomic hyper-variability allows HBV to escape selection pressures imposed by antiviral therapies, vaccines and the host immune system (29,30). There is an increasing prevalence of mutant large surface antigens in serum. Although mutations occur throughout the HBV genome, they tend to cluster into mutational patterns in particular, the basal core promoter, the pre-core region, the polymerase gene and the “a” determinant of HBsAg (31) (*Figure 1*). Because HBsAg mutants with altered antigenicity most frequently variants selected under active or passive immunoprophylaxis or antiviral treatments could be viable and pathogenic, their spread would have substantial consequences for public health (6).

## HBV-modulated immune response

T cell exhaustion is a state of T cell dysfunction (*Figure 2*) that arises during many chronic infections and cancer. It is defined by poor effector function, sustained expression of inhibitory receptors, and a transcriptional state distinct from that of functional effector or memory T cells (32). A

 

**Table 1** Current concepts for preventive and therapeutic vaccination

| Concept for preventive or therapeutic vaccination strategy | Keywords | Reference |
|---|---|---|
| Co-delivery of viral proteins and a TLR7 agonist from polysaccharide nanocapsules: a needle-free vaccination strategy | Co-delivery; nanocapsules; mucosal vaccination | (56) |
| IL-12-based vaccination therapy reverses liver-induced systemic tolerance in a mouse model of HBV carrier | Robust hepatic HBV-specific CD8(+) T cell responses; HBV-carrier mouse model | (57) |
| Effective transcutaneous immunization against HBV by a combined approach of hydrogel patch formulation and microneedle arrays | CTB as an adjuvant; HBsAg loaded hydrogel formulation; FMA system | (58) |
| Phase I clinical trial in healthy adults of a nasal vaccine candidate containing recombinant HBV surface and core antigens | HBsAg-HBcAg vaccine candidate was safe; well tolerated and immunogenic; nasal vaccine candidate | (59) |
| HBV surface antigen-1018 ISS adjuvant-containing vaccine | HEPLISAV™; vaccine-hyporesponsive population | (60) |
| CpG increases vaccine antigen-specific cell-mediated immunity when administered with HBV vaccine in HIV infection | Effective in HIV infected individuals; immunostimulatory CpG | (61) |
| Adjuvanted HBV vaccine (Fendrix, Belgium) contains as active substance 20 µg recombinant HBsAg produced in Saccharomyces cerevisiae and the novel adjuvant system composed of aluminum salt and 3-O-desacyl-4'-monophosphoryl lipid A (AS04) | HBV-AS04 vaccine; earlier antibody response and higher antibody titres in pre and haemodialysis patients | (62) |
| Supercritical fluid extraction provides an enhancement to the immune response for orally-delivered HBsAg | HBsAg was produced in maize grain; bioencapsulation; oral vaccine; plant vaccine; supercritical fluid extraction | (63) |

CTB, cholera toxin B; FMA, functional MicroArray; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen.

particular characteristic of chronic HBV infection is the production of large quantities of SVPs containing only surface antigens and associated host-derived lipid (33). After long exposure to high levels of viral antigen in chronic hepatitis B patients, T cells either display an exhausted phenotype (34-37) or are deleted (38,39). SVPs are non-infectious and their enhanced production could be a way of subverting the antiviral response (40,41).

Peripheral blood monocytes (PBMC) supply peripheral tissues with macrophage and dendritic cell (DC) precursors and, in the setting of infection, also contribute directly to immune defence against microbial pathogens (42). Chronic HBV infection was shown to alter monocyte subsets frequencies depending on the clinical phase (43,44). HBV does not infect or productively replicate in human PBMCs (45). However, HBsAg particles are highly immunogenic, and DCs and macrophages from mice cross-present recombinant HBsAg particles to $CD8^+$ T cells in the absence of inflammatory signals (46-48). These studies have been performed in mice or in vitro model systems and demonstrated that HBV antigens have the ability to activate HBV-specific CD8+ T cells, which play a key role in HBV control (49). However, there is controversy on the effect of HBsAg on monocytes and DCs from chronic HBV-infected patients. It was reported that HBV has an inhibitory effect on TLR-mediated cytokine production by antigen-presenting cells (APC) and could affect innate immunity pathways (50-53). In contrast, other studies did not demonstrate any significant alterations in T cell stimulatory function of different APCs (54,55). More interesting, it was recently shown that CD14-positive monocytes retained an HBsAg depot and differentiation of $HBsAg^+$ CD14 monocytes from chronic patients to DCs induced cross-presentation of the intracellular reservoir of viral antigen. Thus, circulating HBsAg selectively captured by monocytes can be used advantage as a personalized antigenic reservoir to activate virus-specific T cells in chronic HBV patients (54).

Growing number of HBV mutants and recently discovered mechanisms of virus-mediated immune response modulation forced to look for new ways for immunoprophylaxis of HBV infection. Promising concepts for preventive and therapeutic vaccination are summarized in *Table 1*.

## Direct cytopathic effects of HBs

It is widely accepted that these events originate from persistent immune pathogenesis (15,64), but observations in patients with chronic hepatitis B under conditions of

immune suppression and in transgenic mouse models of HBV infection suggest, that in absence of adaptive immune responses cellular mechanisms induced by HBV may also lead to the development of these liver diseases (65-68). Chronic HBV carriers after administration of immunosuppressive drugs for an autoimmune disorder (69) or after liver (70-72), renal (73,74), or bone marrow (75) transplantation demonstrated increased viremia accompanied by high viral protein expression in infected hepatocytes. Some of these patients developed fibrosing cholestatic hepatitis (FCH), an aggressive and mostly fatal form of viral hepatitis. FCH is associated with increased viral replication (76) and is characterized by high intrahepatic expression of viral proteins, diffuse hepatocyte ballooning, the presence of ground-glass hepatocytes, prominent cholestasis, and periportal fibrosis (70-73,76,77). To examine whether HBV has direct cytopathic effects in immune compromised hosts, immune deficient mice (uPA-SCID) harboring human liver cells were infected with HBV. Histological analysis of the livers of long-term-infected uPA-SCID chimeras showed, that the majority of human hepatocytes had a ground-glass appearance, stained intensely for viral proteins, and showed signs of considerable damage and cell death (67). This histopathologic pattern closely resembles the picture observed in the livers of other transgenic mice that expressed HBV surface proteins (67,78-81). A relative increase in production of LHBs compared with that of SHBs (HBsAg) led to profound reduction of the HBsAg concentration in serum as a result of accumulation of both surface polypeptides in a relatively insoluble compartment within the cell (78). Furthermore, overproduction of LHBs resulted in the formation of extremely long (up to 800 nm), occasionally branching, filamentous 22-nm-diameter HBsAg particles, that accumulate within the ER of the hepatocyte and are not efficiently secreted. The hepatocytes become enlarged, hydropic, and eosinophilic and also display the characteristic features of "ground-glass" cells (*Figure 2*). As filament storage progresses, the ground-glass cells undergo coagulative necrosis and the mice develop an age-dependent lesion, whose severity is related to the intracellular concentration of surface proteins, that is characterized by focal hepatocellular degeneration and necrosis, lobular macrophagic inflammation, and increased serum transaminase activity. Thus, progressive intracellular accumulation of HBsAg, which can reach sufficiently high concentrations, could be directly cytotoxic to hepatocytes in this transgenic mouse system (79). Prolonged hepatocellular injury in these mice initiated a programmed response within the liver, characterized by inflammation, regenerative hyperplasia, transcriptional deregulation, and aneuploidy progressing to neoplasia. The incidence of HCC in this model corresponded to the frequency, severity, and age of onset of liver cell injury, which itself corresponds to the intrahepatic concentration of HBsAg and is influenced by genetic background and sex (80,81). Moreover, these mice were largely tolerant to the transgenic products (82) suggesting that the mouse expressing HBV surface proteins in the liver could be an excellent model for investigation of their direct cytotoxic effects. Since hepatic fibrosis constitutes the wound healing response to liver injury (83) this transgenic mouse-model began recently to draw an attention as model for investigation of liver fibrosis (84,85). Development of hepatic fibrosis after chemical liver injury is enhanced in BALB/c mice exhibiting a Th2 response compared to C57BL/6 mice, which demonstrated a primary Th1 response (86,87). As most studies were performed using HBV surface proteins expressing mice on fibrosis-resistant C57BL/6 genetic background, we backcrossed these mice to fibrosis-susceptible BALB/c genetic background (81). Despite the same level of HBV surface protein hepatic expression in transgenic mice on both genetic background comparative biochemical and immunohistochemical analysis revealed remarkable differences in liver pathogenesis between these two mouse strains. As expected we observed enhanced liver fibrosis in BALB/c mice that was a consequence of stronger liver injury (81). Previously it was demonstrated that over-expression of LHBs in human hepatoma cells (88) and accumulation of HBV pre-S mutants in the ER of transgenic mice hepatocytes resulted in induction of ER stress (89). As a response to ER stress induction by accumulation of misfolded proteins the unfolded protein response (UPR) is activated. Distinct branches of UPR are mediated by three different classes of ER-membrane transducers: inositol-requiring protein-1 (IRE1), activating transcription factor-6 (ATF6) or protein kinase-like endoplasmic reticulum kinase (PERK). PERK activation causes the phosphorylation of the alpha subunit of eukaryotic translation-initiation factor 2α (eIF2α) (90-92). From three UPR branches only PERK was activated in the liver of transgenic mice on both genetic backgrounds (81). However, the expression of the C/EBP homologous protein (CHOP), also known as growth arrest and DNA damage-inducible gene (GADD) 153, one of the downstream effectors of PERK pathway that mediates pro-apoptotic pathways emanating from the stressed ER (90,92) was strongly induced only in the liver of BALB/c transgenic mice. Thus, the response of the host to the expression of HBV surface proteins depends on its genetic background (81). Furthermore, two branches of UPR IRE1α and ATF6 were not activated in the liver of HBV transgenic

 

mice. PERK branch activation is largely sustained with unmitigated ER stress, whereas persistent ER stress attenuates IRE1α and ATF6 signaling (93). Therefore, permanent expression of HBV surface proteins leads to the activation of persistent ER stress in hepatocytes that induces PERK and impairs another branches of UPR. It is possible that this situation is common for chronic liver disease comprising ER stress induction. ER storage diseases (ERSDs) are a group of genetically based disorders in which mutant proteins fail to pass the ER quality control. ERSD may be caused indirectly by toxic effects of the misfolded protein or aggregates thereof on the cell. Additionally, the cell's reaction to the ER stress may include signaling pathways, which are ultimately detrimental (94,95). As transgenic mice expressing HBV surface proteins fit all of these criteria, these mice could be excellent model for study ERSDs.

## Carcinogenesis

Transgenic mice, that specifically overproduce LHBs in the liver, develop liver tumours (80). Initially, the development of HCC was considered to be caused by a permanent inflammatory process under conditions of LHBs overproduction (79). However, it was shown that LHBs is able to activate nuclear factor kappa B (*NF-κB*) and activator protein 1 (*AP-1*). This process is mediated by protein kinase C (PKC)-dependent activation of the c-Raf/MAP-kinase signal transduction pathway triggering cellular proliferation control (96). It is possible that continuous activation of these enzymes might additionally contribute to the development of HCC. The presence of several types of mutants in different replicative stages of chronic HBV infection suggests the potential evolution of virus under immune pressure during HBV infection. Naturally occurring preS mutants are detected frequently in serum obtained from patients with chronic HBV infection. The resulting mutants reveal shorter forms of LHBs proteins with internal deletions. Moreover, preS mutants were identified in ground-glass hepatocytes (GGH) representing a histological hallmark of chronic HBV infection (89). Two different types of GGHs have been defined to be associated with different stages of chronic HBV infection. Type I GGHs containing preS1 mutants are typical for the high viral replicative phase of chronic HBV infection, whereas type II GGHs harboring preS2 mutants are specific for the advanced stages of chronic liver disease (89,97). Furthermore, HBV preS mutants, especially preS2, were associated with an increased risk of HCC (97,98). It has been observed, that the ER stress response induced by preS mutated proteins is responsible for the enhanced expression of vascular endothelial growth factor-A (VEGF-A) and for the activation of Akt/mammalian target of rapamycin (Akt/mTOR) signaling in GGHs (99). In addition, it has been shown that preS2 mutated proteins may directly interact with the Jun activation domain-binding protein 1 (JAB1), thus triggering cyclin-dependent kinase (Cdk) inhibitor p27 degradation, Retinoblastoma hyperphosphorylation and cell cycle progression (100). The preS2 mutants may also induce the overexpression of both, cyclin A and cyclooxygenase-2, thereby leading to cell cycle progression, cell proliferation, and anchorage-independent growth. Furthermore, in livers of preS2 mutant-transgenic mice it has been shown, that cyclin A is located in the cytoplasm rather than in the nucleus. This aberrantly expressed form of cyclin A is implicated in centrosome over-duplication, which represents a potential mechanism for chromosome instability (6,101). Thus, carriers of preS mutants represent a high risk group of chronic HBV infected patients for HCC development. Specific therapeutic treatment should be applied to these patients to prevent development of HCC. In addition there is a potential association of HBsAg with HCC among patients with chronic HBV infection.

## Conclusions

HBV is the leading cause of liver cirrhosis and HCC worldwide. In spite of current treatments a complete cure of chronic HBV infections remains a challenge. We have discussed multiple pathological aspects of HBV surface antigens. HBV large surface antigen is becoming more important because of the identification of HBV receptor, the correlation of HBsAg levels with cccDNA in livers, the significance of HBV pre-S mutants in anti-viral treatment and HCC development, the exclusive identification of pre-S mutants in ground glass hepatocytes and the prediction of HCC development. In addition hepatitis B surface proteins could participate to ER storage diseases. An association between HBV preS mutated proteins and increased risk of HCC has been detected recently. Taken together, the multifaceted pathological aspects of the HBsAg predetermine the design of new therapeutical options modulating associated biological implications.

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