Review

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# Hepatitis B virus PreS/S gene variants: Pathobiology and clinical implications

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## Summary

The emergence and takeover of hepatitis B virus (HBV) variants carrying mutation(s) in the preS/S genomic region is a fairly frequent event that may occur spontaneously or may be the consequence of immunoprophylaxis or antiviral treatments. Selection of preS/S mutants may have relevant pathobiological and clinical implications. Both experimental data and studies in humans show that several specific mutations in the preS/S gene may induce an imbalance in the synthesis of the surface proteins and their consequent retention within the endoplasmic reticulum (ER) of the hepatocytes. The accumulation of mutated surface proteins may cause ER stress with the consequent induction of oxidative DNA damage and genomic instability. Viral mutants with antigenically modified surface antigen may be potentially infectious to immune-prophylaxed patients and may account for cases of occult HBV infection. In addition, preS/S variants were reported to be associated with cases of fulminant hepatitis as well as of fibrosing cholestatic hepatitis, and they are associated with cirrhosis and hepatocellular carcinoma development.


## Introduction

Despite the availability of an effective vaccine, hepatitis B virus (HBV) infection remains a major health problem worldwide with estimates of nearly 400 million chronic HBV surface antigen (HBsAg) carriers. HBV infection may be associated with a large spectrum of clinical manifestations, ranging from very mild and asymptomatic clinical forms to the most severe liver diseases including fulminant hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) [1]. The variation of the natural course of the infection and related disease is determined by the interaction between virus and host factors. The HBV replication cycle is not directly cytopathic and host immune responses against viral antigens is considered the main cause of hepatocellular injury [2]. However, several lines of evidence indicate that a certain number of HBV genetic variants, apparently provided with higher pathogenicity, may emerge during the course of the infection under endogenous (host immunity) and/or exogenous (immunoprophylaxis and antiviral therapies) selection pressures [3]. In this context, a growing number of studies performed in different geographic areas – thus evaluating different HBV genotypes – are pointing out the considerable importance of HBV envelope protein mutants (preS/S variants) including those able to escape the vaccine-induced anti-HBV neutralizing antibodies as well as those frequently associated with severe forms of acute and chronic liver disease and hepatocellular carcinoma (HCC) development [4–16]. Aim of this review was to revise the collection of data on the biological and clinical impacts of preS/S HBV variants, also stressing the aspects that are widely accepted by the scientific community and those that are still debated.

### Key Points

- Selection and emergence of naturally occurring or therapeutic induced HBV variants with mutations in the preS/S genomic region is a frequent event in chronically HBV infected patients
- S-escape variants may be undetectable by the commercially available HBsAg assays and are potentially capable of infecting properly immune-prophylaxed patients, and they may also account for cases of occult HBV infection
- Several specific mutations in the preS/S gene may induce an unbalanced production of envelope proteins that accumulate in the endoplasmic reticulum (ER) of the hepatocytes, and may activate the ER stress-signaling pathways with consequent induction of oxidative DNA damage and genomic instability
- The cytotoxic effects exerted by the intracellular accumulation of surface proteins can contribute to liver damage, favoring the progression of the disease toward cirrhosis and the development of HCC

Keywords: HBV preS/S genetic variability; Vaccine escape variants; Occult HBV infection; Endoplasmic reticulum stress signaling pathway; Ground glass hepatocytes; Hepatocellular carcinoma.
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## HBV virology

HBV is one of the smallest viruses in nature and its genome presents a highly compact genetic organization. It consists of a relaxed circular partially double-stranded DNA (rcDNA) of approximately 3200 nucleotides and comprises four partially overlapping open-reading frames (ORF): preS/S, preCore/Core, Pol, and X. There are no non-coding sequences in the viral genome and all regulatory regions (enhancer II/basal core promoter, preS1 promoter, preS2/S promoter, enhancer I/X promoter) are also part of protein-encoding sequences. The PreS/S ORF encodes the three different, structurally related envelope proteins, which are synthesized from alternative initiation codons and are termed Large (L), Middle (M), and Small (S) protein, respectively. The three proteins share the same carboxy-terminus part but have different amino-terminal extensions. In particular, the S-protein – corresponding to the HBsAg – consists of only 226 amino acids (aa), the M-protein contains an extra N-terminal extension of 55 aa, whereas the L-protein has a further N-terminal sequence of 108 or 119 aa – depending on the genotype – compared to the M-protein [17] (Fig. 1).

Envelope proteins contain the major viral antigenic domains. In particular, the immunodominant determinant bearing the anti-HBs neutralization domain, termed "a" determinant, has been mapped to amino acids 99–170 of the S-protein [17,18]. The preS/S ORF completely overlaps with the Pol ORF, which encodes the viral polymerase, a multifunctional protein that possesses reverse transcriptase, DNA-dependent DNA polymerase, and RNase H activities, and also functions as a terminal protein for priming. The pre-Core/Core and X ORFs overlap with the Pol ORF only in part. The pre-Core/Core region encodes the structural protein of the viral nucleocapsid (the hepatitis B core antigen, HBcAg) and the non-structural secreted hepatitis B e protein (HBeAg). These two viral proteins also derive from alternative initiation of translation at two in-frame initiation codons. The X region encodes the small regulatory X protein, which is essential for viral replication, and can directly and indirectly modulate host and viral gene expression [17,19].

Upon infection of hepatocytes, the HBV rcDNA is converted by cellular enzymes into a covalently closed circular DNA (cccDNA) inside the cells nuclei. 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, which requires reverse transcription of the pregenomic RNA (pgRNA) [20]. Unlike retroviruses, HBV does not need to integrate its DNA into the host genome to replicate. Nonetheless, HBV DNA integration occurs frequently during the course of viral infection, and the integrated viral sequences, being usually deleted or rearranged, are replication-incompetent and differ from each other in size and structure [21]. Following transcription, all viral RNAs are transported to and translated in the cytoplasm. The nucleocapsid protein HBcAg, the soluble secreted HBeAg and the Pol protein are produced from the 3.5-kb pgRNA/pre-Core RNAs; the L envelope protein from the 2.4-kb RNA; the M and S envelope proteins from the 2.1-kb RNA; the X-protein from the 0.7 kb RNA. In the cytosol, the 3.5 kb pgRNA – apart from being transcribed – is selectively incorporated into progeny nucleocapsids and reverse transcribed by the co-assembled viral polymerase into new HBV genomes [19]. The mature rcDNA-containing nucleocapsids can then either re-deliver their genomes to the nucleus to build up a pool of around 10–100 copies cccDNA [22] or can interact with the envelope proteins at ER/Golgi apparatus and be secreted as new infectious virions [23]. Envelope protein synthesis follows a pathway that is distinct from viral replication. It occurs in the ER and leads to amounts of proteins that far exceed those required for virion assembly. Excess envelope proteins undergo dimerization and multimerization resulting in their budding from the ER/Golgi compartment as both non-infectious spherical and filamentous subviral particles (SVP) or as virions. The SVPs typically outnumber the virions by a factor of 1,000- to 10,000-fold, they may be components of circulating immune complexes [24], and may induce immune tolerance by a mechanism of "viral apoptotic-like mimicry" [25]. HBsAg is the most abundant protein in SVPs as well as in virions, whilst the M- and L-protein constitute approximately 20% of the total envelope proteins present in the HBV particles [26]. Of note, the commercial HBsAg quantification assays available target all forms of circulating envelope proteins, since the antibodies used in the quantitative immunoassays identify epitopes in the S domain, and are not capable of distinguishing between virion-associated HBsAg and subviral particles or HBsAg produced by possible integrated HBV sequences [27].

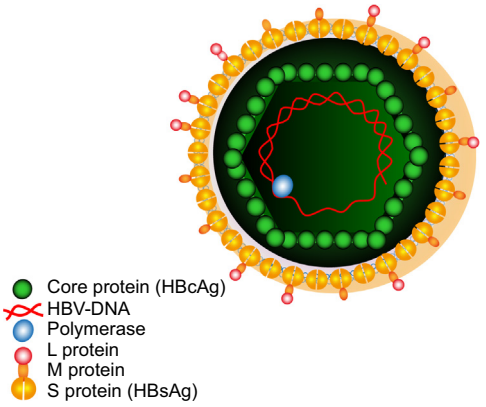


**Fig. 1. Schematic representation of the hepatitis B virion.** The virion consists of an envelope containing three related surface proteins (S-, M-, and L-proteins) and lipids, an icosahedral nucleocapsid that is constituted by the core protein (HBcAg) and that encloses the viral DNA genome covalently linked to the terminal protein of the viral polymerase.

The L, M, and S proteins perform different functions during viral morphogenesis and release, and their specific roles appear to be strictly related to their transmembrane topology. In particular, the L envelope protein shows two transmembrane topologies. In fact, the preS1 domain of the L protein can be either projected towards the cytoplasm or oriented towards the ER lumen. This is consistent with the different and essential functions fulfilled by the preS1 domain in the HBV life cycle, given that the cytoplasmic fraction performs a matrix-like function in nucleocapsid envelopment, and the fraction that faces the ER lumen ends up exposed on the viral surface of mature viral particles and is involved in the attachment of HBV to hepatocytes [23,28]. HBV polymerase lacks a proofreading function, thus the reverse transcription step results in the selection of HBV quasispecies containing several mutations within their viral genome. Indeed, HBV exhibits a mutation rate more than 10-fold higher than other DNA viruses. Mutations accumulating in individual genomes reflect both the duration of active HBV infection and the strength of the immune response. Moreover, apart from viral and host factors, exogenously induced selection

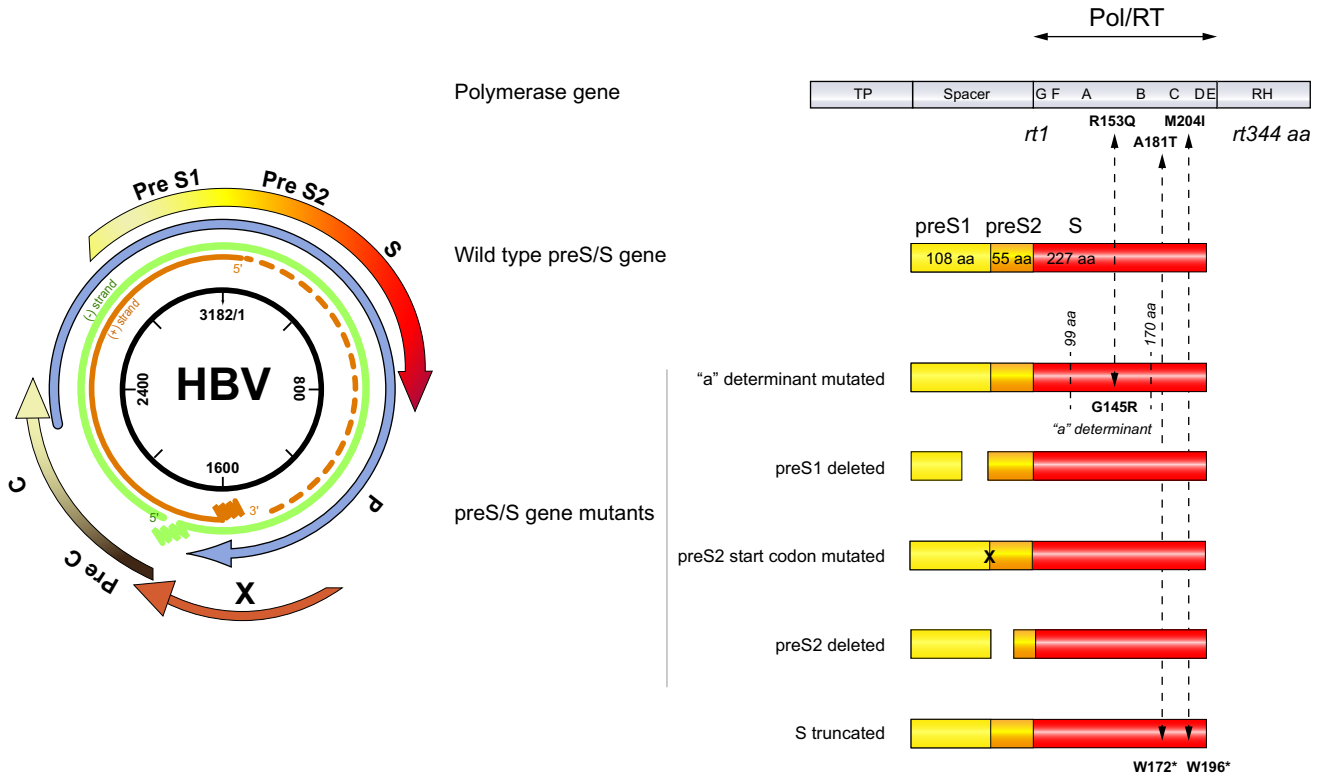


**Fig. 2. Types and positions of mutations affecting the preS/S region that are selected under the pressure of host immunity, immunoprophylaxis, and antiviral therapy.** At the top is a schematic representation of the overlap between the polymerase and the envelope open reading frames, showing that mutations in one reading frame can affect the other. The numbers indicate amino acid (aa) sites. Numbering is according to genotype D. The "a" determinant of HBsAg that is located between aa 99 and 170, and which includes the major antibody neutralization domain of HBV, is indicated. Deletions are shown by dashed boxes. Up-down dashed arrows indicate resistance mutations in the polymerase gene that result in viral envelope changes leading to escape to anti-HBs antibodies and altered virion secretion. The X symbol indicates the abolition of the preS2 start codon. The ∗ symbol indicates a stop codon.

pressures (immunoprophylaxis and antiviral therapy) may strongly affect substitution rates in HBV [3]. Some of these mutations are detrimental to the virus while others may provide the virus with a survival advantage. Several studies have shown that these HBV mutations are not distributed randomly over the entire genome but rather tend to cluster in particular portions of the viral DNA as the basal core promoter (BCP)/preCore region and the preS/S region [29].

In particular, the preS1 and preS2 regions appear to be the most variable sequences of the viral genome. Indeed, HBV variants with point mutations, deletions, or insertions in the preS sequences are often found in patients with long-lasting chronic hepatitis B (CHB) [29] (Fig. 2). Among these variants those defective in synthesizing the M protein appear to be the most frequently selected [4–10,30–32]. Actually, whereas L and S proteins are essential for the HBV life cycle, the M protein is unnecessary for virion formation, secretion, and infectivity, as shown both by *in vitro* and *in vivo* studies [10,33,34]. In fact, the preS2 genomic region overlaps the spacer domain of the P gene, which can tolerate mutations and large deletions without affecting the polymerase activity [35]. In this context, it has to be remembered that the complete overlap of the preS/S ORF by the polymerase gene may create conditions in which changes within the S gene – naturally selected or selected as a consequence of immunotherapies such as HBV vaccination or prophylaxis with hepatitis B immune globulin (HBIg) – can cause functionally significant alterations of the viral polymerase possibly affecting viral replication fitness (Fig. 2). Similarly, changes selected within the polymerase coding region as a consequence of nucleos(t)ide analogue (NA) therapy may result in structural changes in HBsAg, with consequent modifications of the antigenic properties of this protein [36]. Furthermore, it is worth mentioning that the process of intracellular recycling of newly synthesized rcDNA-containing nucleocapsids [37,38] apart from involving rcDNA molecules with naturally selected mutations arisen in the course of error-prone reverse transcription can also enclose those molecules selected under immune and/or drug pressure which all end up being archived into the nuclear cccDNA pool [39] (Fig. 2).

It has recently been demonstrated that infection with preS/S mutants may be associated with significantly lower levels of circulating HBsAg in CHB patients, and that cells replicating preS/S mutants isolated from patients, in addition to harboring envelope proteins in the ER, had reduced amounts of HBsAg in the supernatant and an increased accumulation of cccDNA molecules into the nuclei [40]. Actually, a large amount of evidence indicates that a modified ratio between envelope proteins is associated with an impaired secretion of virions and SVPs [41–44] and with a significant increase of cccDNA copy numbers per hepatocyte [45–48]. A determined relative molar ratio of L to S protein is, in fact, essential for correct assembly and release of replicating viral particles [44,45,49] as well as for the control of cccDNA

formation rates [45–50]. Of importance, it has been shown that failure in the regulation of the size of cccDNA pool may result in hepatocyte death [48,51,52]. Therefore, a balanced expression of envelope proteins appears to be vital for the HBV life cycle as well as for maintenance of the non-cytopathic virus-cell relationship.

## Clinical significance of HBV preS/S genetic variants

### *PreS/S mutations as cause of immune escape HBV and occult HBV infection*

The beneficial effect of the available anti-HBV vaccine in preventing new infection is evident worldwide and particularly in highly endemic areas [36,53,54]. Analogously well recognized, however, is the possible emergence of HBV genetic variants that are able to elude the control of the specific vaccine-induced anti-HBs antibodies [55–57]. The identification of HBV vaccine escape mutants was firstly reported in the early '90s in an Italian child born to an HBsAg/HBeAg positive mother [12]. This child developed chronic HBV infection despite having received correct passive-active immunoprophylaxis and having developed anti-HBs antibodies, thus being theoretically protected against the virus. Many subsequent studies from different areas of the world largely confirmed that first observation, reporting the identification of individuals who developed HBV infection in spite of having had vaccine-induced circulating anti-HBs antibodies [11,58–60]. In the majority of cases, the HBV vaccine escape variants carry point nucleotide mutations causing amino acid substitution in the "a" determinant of the HBsAg and particularly the arginine to glycine substitution at aa position 145 [61,62]. In other cases, additional mutations have been recognized, and in particular a unique in-frame insertion of 2 to 8 aa between codon position 121 and 124 – just upstream of the first loop of the "a" determinant – has been detected, an insertion that may considerably modify the conformational status of the "a" determinant [63–65]. The same escape variants may emerge and take over from patients with graft infection after liver transplantation for HBV-related diseases who receive immunoprophylaxis with high doses of monoclonal or polyclonal hepatitis B specific HBIg [15,16,66–68]. As mentioned above, the Pol gene completely overlaps the S gene, and consequently, drug-resistant HBV mutants emerging under antiviral therapy with NA inhibitors of the viral reverse transcriptase may also carry mutations in the S protein causing alteration of its antigenicity [3,36,53,69–73]. Of note, although HBV mutants with altered envelope antigenicity are mostly "therapeutically" induced variants (since selection occurs under active or passive immunoprophylaxis or antiviral treatments), they may also emerge spontaneously because viral strains with mutations in the "a" determinant or surrounding it have also been isolated from individuals naïve to any immunoprophylaxis or antiviral treatment [74–77]. In this context, however, the possibility cannot be ruled out that some of these individuals have been infected with HBsAg-mutated viruses from subjects – the source of the infection – in whom the mutants had emerged under therapy. In fact, there is considerable evidence that the "a" determinant variants may be viable and pathogenic [78,79] and that they may infect properly vaccinated people [59,80–84]. At present, contrasting data are available on the incidence of infection with the S-escape variants [11,13,85], but it is clear that their possible spread would have considerable consequences in terms of public health, and the establishment of surveillance programs to monitor the emergence and possible clinical effects of these variants has thus been recommended [53,72].

Furthermore, apart from the ability to avoid neutralization by both vaccine-induced and HBIg anti-HBs, these HBV variants may also account for cases of occult HBV infection (OBI). In fact, although OBI is more often related to suppression of HBV replication and gene expression by host defense mechanisms [86], there is also much evidence of occult HBV infection due to actively replicating viruses able to elude the recognition by the commercially available HBsAg detection kits in different settings of HBsAg-negative patients [87]. This issue is of utmost importance for the risk of transmission of HBV infection [61,86,88,89]. Noteworthy, the main cause of the residual cases of HBV transmission by blood transfusion appears to be essentially related to the lack of recognition of an infection with HBV S-escape mutants of the donor [90–95]. Even more importantly, HBsAg-mutant strains were reported to be involved in the cases of *de novo* HBV infection occurring in properly vaccinated children who underwent liver transplantation [96], as well as in many cases of OBI in children born to HBsAg-positive mothers in spite of effective prophylaxis with vaccination and HBIg [97]. Of course, long-term follow up of these children is needed to evaluate the possible clinical relevance – if any – of this conceptually intriguing observation [98].

### *PreS/S mutants in acute and chronic hepatitis B*

S and much more frequently preS HBV mutants have been found in association with different, severe forms of acute and chronic liver disease, including fulminant hepatitis (FH), fibrosing cholestatic hepatitis (FCH) and cirrhosis. Actually, emergence and selection of preS variants is a fairly common event in HBV chronically infected patients. The genetic defects are usually due to (a) *in-frame* deletions of different sizes involving the carboxy terminus of the preS1 region or (b) the middle part of the preS2 region, (c) to point mutation(s) involving the start codon of the preS2 genomic region with consequent complete abolishment of the M protein synthesis [4–6,10,30,31,40,99–101].

Transmission of HBV preS-mutants, particularly those unable to synthesize the M protein, has been reported to be associated with cases of FH. In this context, in the late '90s we had the chance to examine isolates from serially collected serum samples from a surgeon and his mother, who was accidentally infected by the son: both died of FH. The infecting viruses were genetically almost identical in both patients and carried a double nucleotide mutation in the start codon of the preS2 region that prevented the synthesis of the corresponding protein, as also confirmed by immunoassay experiments (of note, the virus isolates from both patients had wild-type preCore and BCP sequences in the early phases of the infections) [10]. Moreover, we detected preS2-defective mutants in additional cases of FH and similar findings were subsequently reported by other authors [102,103]. Although experimental studies definitively confirming the suspected role of preS2-defective HBV in the pathogenesis of the acute liver failure are still lacking, it is noteworthy to mention that intracellular retention of HBV surface proteins was found to be associated with FH in a transgenic mouse model showing panlobular necrosis and hepatic failure as a consequence of the extreme sensitivity of hepatocytes with HBV surface protein retention to interferon-gamma produced by the cytotoxic T

lymphocytes [104]. In analogy, one might hypothesize that similar events may also occur in patients newly infected with viruses unable to encode the M protein and then overproducing and accumulating L protein in the hepatocytes. Still speculating about the possible involvement of preS2-defective mutants in the severe course of acute hepatitis B, one might also hypothesize that, since the T- and B-cell immunities specific for the M protein are important early events in the human immune response to HBV infection [2], the absence of this protein may result in inefficient neutralization of the virus, favoring the more severe course of the infection.

In analogy, infection with preS variants causing an imbalance of surface protein synthesis and consequent intracellular accumulation of proteins and viral particles has been found to be associated with cases of FCH a very severe and rapidly progressive form of cholestatic liver disease that may occur in HBV infected immunocompromised patients including liver, kidney, and bone-marrow transplanted individuals [105–107]. The histological pattern characterized by a negligible inflammatory component and a massive presence of viral antigens in the hepatocytes has led to the hypothesis of a direct cytopathic effect of the virus in these cases [108–111], a hypothesis that has also been confirmed by *in vitro* experiments [106,112]. Noteworthy, however, the advent of specific treatment preventing active HBV reinfection in individuals undergoing liver transplantation has proved to be very effective in also preventing FCH development in these patients.

The intracellular accumulation of viral proteins and replicative intermediates that may follow the emergence and takeover of preS mutants may therefore be cytotoxic in itself representing an additional, possible mechanism of liver injury that may contribute to worsen the outcome of the CHB [113]. In fact, a fairly large number of studies have reported a significant association between infection with preS mutants and cirrhosis [9,114], also confirmed in the few prospective studies performed in the field so far [7]. In this context, the evidence suggesting that "complex HBV variants" with combined mutations at level of the preS and BCP regions are a stronger risk factor than variants mutated in a single region for CHB progression toward cirrhosis appears of relevance [6,7].

*PreS/S mutants and HCC*

A recent meta-analysis that included 43 studies and evaluated a total of 11,582 HBV-infected patients has demonstrated that infection with preS mutants is associated with 3.77-fold increased risk of HCC [32]. Actually, data from a prospective study had already revealed the predictive value of the preS deletion mutants in the development of HCC [7], and a large number of experimental and clinical studies had provided strong evidence on the potential role of HBV preS mutants in the pathogenesis of HCC (Fig. 3) [7,9,14,115–128].

HBV variants more commonly associated with HCC include preS2-defective viruses with mutations at the level of the preS2 start codon and/or deletions in the 5′-terminal half of the preS2 region and preS1 mutated viruses with deletions in the 3′-terminal half of the preS1 region [9,14,129,130]. The deleted sequences often correspond to viral regions containing B or T cells epitopes [2], therefore preS HBV mutants may represent selected immune escape HBV variants [3,130]. As mentioned above, both preS1 and preS2 mutations can determine an unbalanced production of envelope proteins with a consequent accumulation of mutated L protein in the ER of hepatocytes, resulting in the activation of the ER stress signaling pathway [14,41, 42,44,113,115,117,124, 131–134]. It has been proposed that, through the ER stress, large amounts of reactive oxygen species are generated and cause oxidative DNA damage, and genomic instability, and ultimately result in HCC development [14,116,126,135,136]. Numerous data from experimental studies in transgenic mice and cell cultures appear to confirm the potential pro-oncogenic role of mutated envelope proteins [14,115,118–120,124,135,137,138]. Interestingly, ground-glass hepatocytes (GGHs) characterized by marked accumulation of viral surface proteins in ER and considered to be the histological hallmark of chronic HBV infection [139] have been recently recognized as precursor lesions of HCC [14, 131–134,137]. At least two different types of GGHs have been defined and associated with different stages of chronic HBV infection. Type I GGHs – harboring mutated L protein with deletions over the preS1 region – display a globular or inclusion-like immunostaining pattern of HBsAg and are typical of the high viral replicative phase of chronic HBV infection, whereas type II GGHs – harboring mutants with deletions over the preS2 region or lacking preS2 starting codon (preS2 mutants) – show marginal staining patterns of HBsAg, are distributed in large clusters because of their higher proliferative activity and are characteristic of the advanced stages of chronic liver disease [14]. 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 [137]. Apart from the induction of ER stress signals, 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 [136]. 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 [124,126,135]. 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 and that this aberrantly expressed form of cyclin A is implicated in centrosome overduplication, which represents a potential mechanism for chromosome instability [124,126] (Fig. 3).

The preS/S genomic region when deleted in the C-terminus portion (which includes the viral transmembrane hydrophobic region III of the S domain) may produce C-terminally truncated M proteins ($MHBs^t$) that are retained and accumulated in the ER, and may display transcriptional activation properties and transforming potentials [118–120]. It is noteworthy that preS/S sequences deleted at the 3'-end and producing functionally active $MHBs^t$ have been found in many viral integrates from HBV-associated HCCs [121,122,140–142], and experimental data from transgenic mice and hepatoma cell cultures have shown that $MHBs^t$ proteins retained in the ER can trigger a PKC dependent activation of c-Raf-1/MEK/Erk2 signal transduction cascade, which leads to the induction of AP-1 and NF-κB transcription factors, and to an enhanced proliferative activity of hepatocytes [119,143]. $MHBs^t$ proteins may also induce an increased cleavage of procaspases-3 and -9, thus rendering hepatocytes more susceptible to TRAIL-induced apoptosis [144]. Of interest, the activator function of the $MHBs^t$ regulatory protein does not appear to

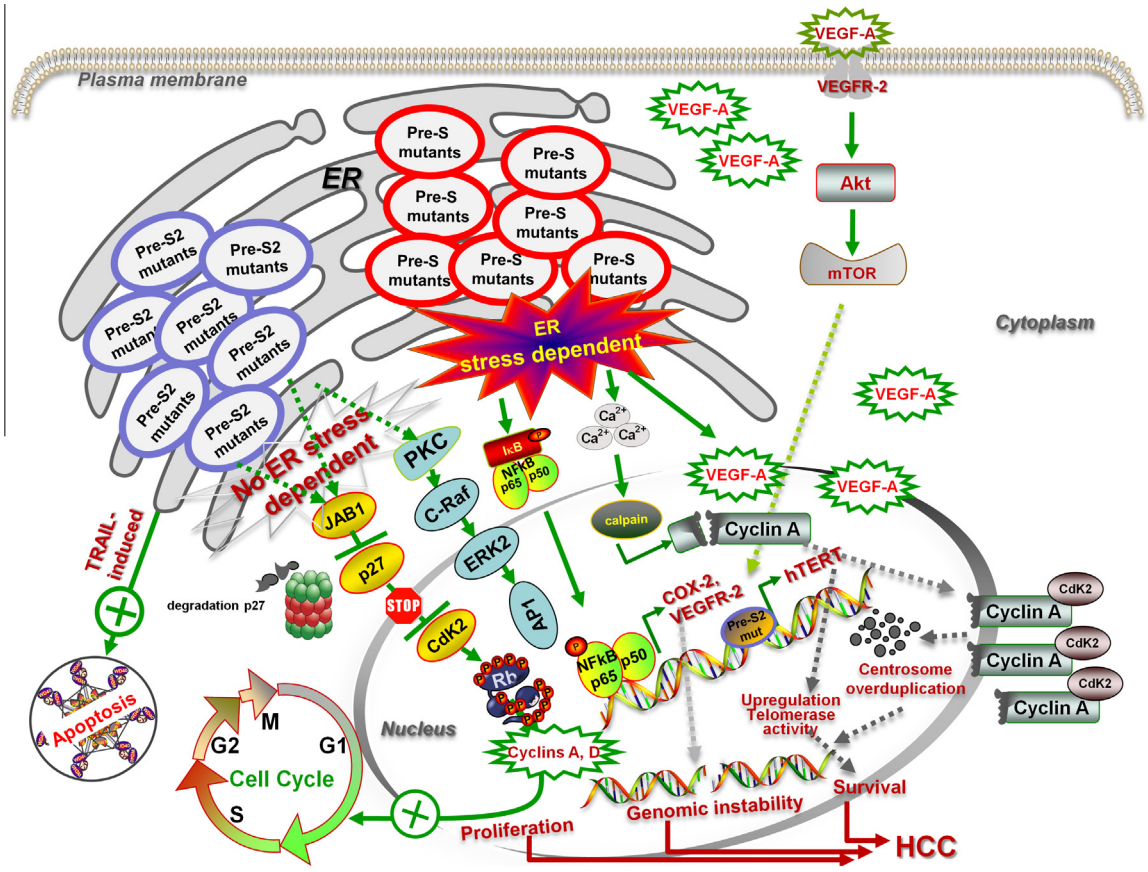


**Fig. 3. Schematic representation of the proposed models for hepatocellular carcinogenesis associated with the HBV preS/S mutant proteins.** The preS mutants can activate both endoplasmic reticulum (ER) stress-dependent and ER stress-independent signals. PreS1 and preS2 mutations may cause overproduction and accumulation of mutated envelope proteins in the ER, resulting in significant ER stress that may induce DNA damage and genomic instability. The ER stress initiated by the preS mutants can activate the calcium-dependent protease μ-calpain that in turn causes the cleavage of cyclin A resulting in an N-terminus-truncated product that translocates into the cytoplasm and causes centrosome overduplication. The preS mutants-induced ER stress may also activate different signal pathways involving both nuclear factor NF-kB to upregulate cyclooxygenase-2 (COX-2) and vascular endothelial growth factor (VEGF) to activate Akt/mammalian target of Rapamycin (mTOR) signaling. Moreover, pathways protecting the hepatocytes from apoptosis can be also activated by preS mutants. The preS2 mutants can additionally promote hepatocyte proliferation by inducing an ER stress-independent activation of a signal transduction pathway that involves the Jun activation domain-binding protein 1 (JAB1), the cyclin-dependent kinase (Cdk) inhibitor p27, and the Retinoblastoma tumor suppressor. Finally, truncated preS2 protein – functioning as a transactivator – can directly interact with the hTERT promoter and up-regulate telomerase activity or may activate ERK2 and render hepatocytes susceptible to TRAIL-induced apoptosis.

be linked to the ER stress response but rather to the peculiar topology of its preS2 domain [143]. Indeed, whereas the preS2 domain of the structural M protein normally faces the lumen of the ER, the preS2 domain of the MHBs$^t$ activator protein is directed toward the cytoplasm [145]. It has been shown that the preS2 domain facing the cytoplasm may directly interact with cytosolic binding proteins, thereby triggering intracellular signal transduction cascades [28,119,145]. Of interest, it has also been reported that MHBs$^t$ may upregulate human telomerase reverse transcriptase (hTERT) expression. Intriguingly, a truncated form of preS2 protein appears to be able to directly interact with a preS2-responsive DNA region and can activate the hTERT promoter, resulting in the upregulation of telomerase activity and in the promotion of HCC development [120] (Fig. 3). Importantly, hTERT has been found highly expressed in preS2-positive human HCC samples [120].

It is worth mentioning that apart from the chromosomal integrated HBV sequences, C-terminally truncated surface proteins can also be produced by non-integrated viral variants that emerge under the selective pressure of the host's immune response and/or antiviral treatment [40,69,75,138,146–148]. In particular, various studies have reported that infections with mutated HBV strains with premature stop codon at position 172 or 182 of the S gene are significantly associated with cirrhosis and HCC [69,138,146,148]. In addition and of particular interest, in patients who underwent prolonged antiviral therapy with NAs such as lamivudine or adefovir the possible selection of a resistant HBV variant showing the A181T mutation in the viral polymerase and a stop codon in the overlapping S gene at amino acid 172 (sW172*) has been described, which results in the deletion of the last 55 amino acids of the C-terminal hydrophobic region of the surface proteins [69,138,147,148] (Fig. 2 and Table 1). Various studies have demonstrated that this variant has a secretory defect and may transactivate different oncogene promoters [138,148]. Of interest, it has also been shown that NIH3T3 cells stably expressing the sW172* preS/S mutant have increased tumorigenicity in nude mice [138,148]. The direct oncogenic potential of the rtA181T/sW172* HBV variant has been invoked to explain the fact that long-term therapy with NAs (particularly, when started with lamivudine monotherapy) may reduce, but does not definitely eliminate, the

**Table 1. Major drug-resistant mutations selected in the HBV reverse-transcriptase (rt) and leading to the generation of stop codons in HBsAg.**

| Nucleos(t)ide analogs | Resistance mutations | S gene nonsense mutations |
|---|---|---|
| Adefovir<br>Lamivudine<br>Telbivudine | rtA181T | sW172- stop |
| Entecavir | rtT184M | sL176-stop |
| Lamivudine<br>Telbivudine | rtM204I | sW196-stop |

risk of HCC development in CHB patients [149,150]. Indeed, there is evidence indicating that the rate of HCC is particularly high in lamivudine resistant patients and that among cirrhotics, the incidence of HCC is significantly higher in patients with lamivudine resistance than in those who are NAs naïve [149]. Of interest, some recent studies have reported that deletions in the preS2 genomic region tend to be co-selected with the drug resistance–associated mutations in lamivudine treated patients, and that the preS2 genomic changes do not confer drug-resistance but play a supportive role in the replication of lamivudine-resistant viruses [30,151].

In this context, the observation that CHB patients – in particular if cirrhotics – maintain a quite high risk of developing HCC even under efficient NA therapy with persistent and long-lasting negative serum HBV DNA is of great relevance [152–155]. As mentioned above, NAs suppress the viral replication but have no effect on the S gene transcription and surface protein synthesis, thus making it possible to speculate that the persistence of HBV oncogenicity in the context of an efficient antiviral therapy might be related to the persistence of the oxidative stress exerted by the surface proteins accumulated in the hepatocytes. Prospective studies are needed to verify this hypothesis.

## Conclusions

Selection of naturally occurring or therapeutic induced HBV variants carrying mutations at the level of one or more of the three *in frame* coding regions of the viral preS/S gene is a fairly frequent event in chronically HBV infected patients. These mutations are invariably associated with changes in the HBV Polymerase gene that completely overlaps the S-ORF. Several of these variants may have important clinical impacts. Mutations at the level of the "a" determinant of the S protein may lead to the production of an antigenically modified S protein that may not be recognized by the specific anti-HBs neutralizing antibody, thus rendering these S-escape variants potentially capable of infecting properly immune-prophylaxed patients. In addition, these variants with an antigenically modified S protein may be undetectable by the commercially available HBsAg assays and they may account for cases of occult HBV infection. Deletions in the preS1 or in the preS2 genomic regions, mutations abolishing the preS2 start codon or inserting stop codons in the S genomic region may determine an unbalance in the synthesis of all envelope proteins with an overproduction and an intracellular accumulation of the L protein, which in turn may interfere with the secretion of the S protein and the viral particles. In this context, the observation that accumulation of L protein in the hepatocytes might have a direct and deleterious cytopathic effect both in transgenic mice (which develop FH) and in liver transplanted patients (who may develop FCH) assumes great relevance. The cytotoxic effects exerted by the intracellular accumulation of surface proteins might contribute to liver damage, favoring the progression of the disease toward cirrhosis. In fact, relevant evidence has demonstrated that frequencies of the preS mutations consecutively increase during the progression of chronic HBV infection from the asymptomatic HBV carrier state to liver cirrhosis or HCC. It has been shown that hepatocytes harboring modified L and M surface proteins have a potential growth advantage. They assume the typical aspect of type II GGHs, which frequently cluster in nodules because of their higher proliferative activity and potential clonal expansion. There are several possible hepatocarcinogenic effects of preS/S mutations. The overproduction and accumulation of mutated L and M surface proteins in the ER is the cause of significant ER stress with the consequent induction of oxidative DNA damage and genomic instability. Furthermore, truncated preS2/S sequences have been proposed to enhance tumor development by encoding a protein with transcriptional transactivation activity. Therefore it appears of the utmost importance that patients with chronic hepatitis B are screened for the presence of preS/S mutant infection. The detection of these specific HBV variants may indeed be useful for the identification of those patients requiring a preventive and appropriate treatment and a more intensive follow-up strategy for early detection of HCC.

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## Conflict of interest

The authors declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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