

## Hepatitis B Virus PreS Variant and Hepatocellular Carcinoma

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**Hepatocellular carcinoma (HCC) is one of the most common cancers in the world. HCC is associated with multiple risk factors. Although the association between chronic hepatitis B virus (HBV) infection and HCC is well established, the underlying mechanism of HBV-related hepatocarcinogenesis still remains elusive. Viral proteins such as X protein and the truncated middle S protein have been implicated to be tranactivators. Recently, studies are focused on mutations within the HBV genome that may be associated with HCC. Deletions in the 3' end of preS1 and 5' terminal of preS2 may emerge during the course of chronic HBV infection which may potentially lead to impairment in immune clearance of these variants. The preS mutant proteins are localized in the endoplasmic reticulum (ER) and have been implicated in the induction of ER stress responses. The ER stress response induces a series of signal transduction pathways and oxidative DNA damage. Furthermore, preS2 mutant protein can upregulate cyclin A expression and induce nodular proliferation of hepatocytes. The findings in this review are important to study the correlation between HCC and preS deletion mutants.**

**Keywords:** Hepatitis B Virus, PreS, Hepatocellular Carcinoma

### Introduction

Hepatocellular carcinoma (HCC) is one of the most common primary cancers in the world <sup>(1)</sup>. It is the third most common cause of death from cancer in males and the seventh one in females <sup>(2, 3)</sup>. HCC affects approximately one million people around the world every year <sup>(4)</sup>. Chronic hepatitis B virus (HBV) infection is the primary risk factor for the development of hepatocellular carcinoma worldwide <sup>(5, 6)</sup>. After decades of chronic hepatitis, about 30-40% of patients develop liver cirrhosis, and of them, around 1-5% subsequently develop HCC <sup>(7)</sup>. Other recognized risk factors include chronic HCV infection, heavy alcohol consumption and exposure to aflatoxin B1 <sup>(8)</sup>.

It has been estimated that about 50-55% of HCC cases in the world are related to HBV with a higher incidence in Asia where the seroprevalence of hepatitis B surface antigen (HBsAg) in the population is high <sup>(7, 9)</sup>. In Southern Iran, it has been estimated that about 52.1% of HCC cases are related to HBV <sup>(10)</sup>. Moreover, the lifetime risk of

HCC has been shown to be increasing even in patients with occult HBV infection and after HBsAg clearance <sup>(11)</sup>. The epidemiological association between HBV and HCC is well established. In recent studies, it has been revealed that HBsAg carriers have 25-37 times increased risk of developing HCC as compared to non-infected people <sup>(1)</sup>. Although the relationship between chronic HBV infection and HCC has been well established, the exact role of HBV in the pathogenesis of HBV-related hepatocarcinogenesis remains to be elucidated.

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The hepatocarcinogenesis in HBV infection has been extensively analyzed, and multiple factors appear to play a role. Since the hepatocarcinogenic process involves interplays between HBV and host hepatocytes, both genomes may contribute to the final pathogenic outcomes, either individually or synergistically. It is therefore reasonable to speculate that apart from host factors, viral factors are very likely involved in HBV-related hepatocarcinogenesis.

### Virology of HBV

HBV genome consists of a partially double stranded DNA molecule of approximately 3200 nucleotides with four open reading frames encoding viral polymerase, core antigen, e antigen, HBx protein and preS/S gene encoding three surface antigens; large protein (preS1, preS2 and S), middle protein (PreS2 and S) and small protein (S only) (12).

### Viral oncology of HBV

At present, HBV-associated carcinogenesis can be seen as a multi-factorial process that includes both direct and indirect mechanisms which might act synergistically. It is generally considered that HBV has no direct oncogenic or cytopathological effect on the infected hepatocytes (13). In chronic HBV infection, viral DNA can be integrated into the host genome (14). Integrated HBV sequences have been observed in established hepatoma cell lines and in about 80% of human HBV-related HCCs (7, 13). Studies of different viral insertions in many human HCCs have revealed that integration can take place at multiple sites on various chromosomes; therefore, HBV integration is not specific and not associated with activation of any cellular proto-oncogenes (15-17). For this reason, in human HCCs, cis-activation of oncogenes by HBV promoter or enhancer insertion cannot be regarded as a general mechanism of transformation.

Based on the observations obtained from several isolates of human HCC tissues, the integrated HBV-DNA usually has truncated open reading frames coding for viral polymerase and the core antigens, and can only encode two gene products: the HBx and HBs proteins (18). Therefore, HBx and HBs proteins represent the two potential candidate proteins involved in HBV-related hepatocarcinogenesis. Although still controversial, the HBx protein has been studied extensively. This protein can inactivate or mix with cellular anti-oncogene product, P53, which is frequently disabled in HCC. However, P53 inactivation may occur in only a minority of HBV-

induced HCCs (19). P53 exhibits transactivating functions, and activates JAK1-STAT and the Ras-Raf-MAPK signal pathway (20, 21). In the case of HBs protein, the LHBs have been demonstrated to exert a tumor promoter-like function in the development of HCC (13, 22). The C-terminally truncated middle surface protein MHBst has been recognized as a transactivator and initiator of c-Raf-1/ Erk2 signaling (23). Moreover, a recent study demonstrated that the preS2 expressing cells also induced upregulation of human telomerase reverse transcriptase (hTERT) and telomerase activation (24).

In addition to the above-mentioned common viral factors, preS deletion of HBV has recently been shown to be associated with the progress of the liver disease and the development of HCC in HBV carriers. In our current sequencing results, approximately 40% of the HCC samples from Iran were found to contain a deletion or start codon mutation in the preS2 region (25). Sequencing analysis showed that the deleted regions were in the 5' terminus of preS2. Our data and results obtained from other countries indicate that patients with progressive liver diseases including HCC have a higher frequency of preS deletion (26-29).

### HBV preS deleted variants

HBV evolves rapidly because of its replication through the pregenomic RNA by reverse transcriptase, an enzyme prone to errors as it lacks proofreading activity. The annual nucleotide substitution rate per site for HBV has been estimated to be  $1.4$  to  $5 \times 10^{-5}$ , approximately the same as retroviruses ( $10^{-5}$ ) but  $10^4$  times higher than DNA viral genomes. At any time, the virus population can be composed of a number of different mutants referred to as "quasispecies". Some viral hot spots of mutation gradually develop during the natural course as a result of virus-host interaction (30). Many mutations and deletions in the HBV genome have been recently found during persistent viral infection (26, 27, 31). The presence of these viral mutants suggests the potential evolution of viral variants under immune pressure during HBV infection. The preS region (preS1 and preS2) carry potent T and B cell epitopes (32). Antibodies to preS1 and preS2 regions often appear early in patients who recover from acute HBV. Thus, these antibodies have important roles in viral neutralization and clearance (33).

There are two types of preS deletion variants: preS1 deletion variants that produce LHBs with deletion at the preS1 ( $\Delta$ S1-LHBs) and preS2

deletion variants<sup>(18)</sup>. The latter variants belong to two major types: those unable to synthesize M protein because of a mutation at the level of preS2 start codon, and those producing a shortened M protein because of in-frame deletions in the corresponding genomic region<sup>(33)</sup>. Deletion in the 3' end of preS1 and 5' terminal of preS2 can emerge during the course of chronic HBV infection that may potentially lead to impairment in immune clearance of these variants and will, therefore, be a possible mechanism for viral persistence.

### Biological characteristic of HBV preS variants

The preS deletion mutants have been shown to be competent for HBV replication although some of them may lose the ability of viral secretion which subsequently results in the accumulation of replicative intermediates in the cytoplasm<sup>(26, 27, 34)</sup>. It has been demonstrated that the ratio of large, middle and small surface proteins is very important for subviral particle secretion. In the active replicative phase, small and middle proteins are produced far in excess of that needed for viral morphogenesis and are rapidly secreted as 22-nm spherical particles. In contrast, the large surface protein usually assembles into long, branching, filamentous particles that become trapped in the endoplasmic reticulum (ER) and cannot be secreted in the absence of small surface proteins. A deletion in the pre-S region or the disruption of S promoter has been found to lead to a decreased synthesis of small surface antigen and subsequently results in the accumulation of surface proteins in the ER<sup>(34)</sup>.

### Accumulation of HBs proteins in ER and GGHs

Ground glass hepatocyte (GGH) represents a histological hallmark of chronic HBV infection and contains surface antigens in the ER. The GGHs at different replicative stages of chronic HBV infection are different in morphology and distribution in the liver. Two major types (types I and II) of GGHs have been recognized. Type I GGHs usually scatter sporadically in liver lobules and occur throughout the replicative phases. Typically, they have slightly eccentric nuclei with accumulation of ground glass substances or an inclusion-like expression of HBsAg in the cytoplasm. Type II GGHs usually emerge at late nonreplicative stage or at advanced stages of chronic HBV infection and are distributed in large clusters with a marginal expression of HBsAg. Different types of GGHs harbor specific preS deletion mutants. Type I GGHs consistently harbor mutant large surface proteins with deletions over the

preS1 region, whereas type II GGHs contain mutants with deletions over the preS2 region<sup>(35, 36)</sup>. This finding therefore demonstrates the role of type II or  $\Delta$ S2-LHBs in the pathogenesis of HBV-related tumorigenesis<sup>(27)</sup>.

### Mechanisms of accumulation of surface proteins in the ER

There are several mechanisms that explain the accumulation of surface proteins in the ER such as the disruption of the transcriptional regulation of the S gene. Preliminary studies using primer extension analysis of RNA transcripts revealed that the deletion of preS2 and the mutation at the translational start codon could disrupt the S promoter activity which led to the decreased synthesis of the small surface protein. This finding can explain molecular and virological bases of the intrahepatic accumulation and expression of marginal HBsAg at the late, non-replicative phase of chronic HBV infection. Then, defectiveness in the M protein production might induce an imbalance in the synthesis of all S proteins with an overproduction and an intracellular accumulation of the preS1 protein which in turn may interfere with the secretion of the Small protein and viral particles<sup>(27, 36)</sup>. Another reason for increased intracellular accumulation of envelope proteins is a paradoxical hyper modification phenomenon that occurs in the secreted middle envelope proteins containing a preS2 internal deletion. This phenomenon may be related to enhanced O-glycosylation<sup>(33)</sup>. Another proposed model that causes accumulation of preS mutant HBsAg in the ER is folding of these mutant proteins into improper conformations<sup>(37)</sup>.

### The role of preS mutant proteins in development of HCC

The preS mutant proteins accumulate in the ER and induce ER stress signals. The ER retention of preS mutant proteins may induce unfolded protein response (UPR) and may activate ER stress and other cellular signals, including apoptosis<sup>(38)</sup>, expression and activation of GRP78/94, PERK, and JNK signaling<sup>(35)</sup>. Both Grp78 and Grp94 are chaperones and have anti-apoptotic functions<sup>(39, 40)</sup>. It is believed that these molecules are involved in HCC<sup>(41, 42)</sup>. ER stress also induces cytoplasmic localization of P53 and blocks P53-dependent apoptosis<sup>(43)</sup>. It is proposed that the preS2 mutant proteins can directly or indirectly induce mutagenesis of P53 gene and inactivate the p53 tumor suppressor gene<sup>(44)</sup>.

The preS mutants may induce oxidative DNA damage and mutagenesis through ER stress signaling pathways<sup>(44)</sup>. The oxidative DNA damage caused by preS mutants may result in genomic instability and mutation of liver cells which ultimately leads to HCC. It has been shown that the preS2 mutant HBsAg causes a higher level of DNA damage than the wild-type or preS1 mutant HBsAg do. PreS2 mutant surface antigens induce degradation of cyclin-dependent kinase inhibitor p27 (Kip1) through c-Jun activation domain-binding protein 1. The preS2 mutants directly interact with Jun activation domain-binding protein 1 (JAB1). Association of preS2 mutants with JAB1 dissociates JAB1 from JAB1/IRE1 complex in ER. The free (active) JAB1 then translocates into cell nuclei and renders Cdk inhibitor p27 (Kip1) to cytosolic proteasome for degradation<sup>(45)</sup>. Also preS2 mutants induce hyperphosphorylation of tumor suppressor retinoblastoma (RB) via cyclin-dependent kinase 2 (Cdk2), a downstream molecule regulated by p27 (Kip1). This effect is independent of the ER stress signaling pathway. The transgenic mice carrying preS2 mutant genes also exhibit Cdk2 activation, p27 (Kip1) degradation, as well as RB hyperphosphorylation<sup>(18)</sup>. It has been reported that preS2 mutant proteins upregulate cyclin A expression and induce nodular proliferation of hepatocytes. The induction of cyclin A expression may occur via the specific transactivator function of pre S2 independent of ER stress<sup>(46)</sup>. Finally, preS deleted HBV may directly or indirectly stimulate hepatocyte proliferation and genomic instability, which surely enhances HCC development.

## Conclusions

Although the relationship between chronic HBV infection and HCC has been well established, the exact role of HBV in the pathogenesis of HBV-related hepatocarcinogenesis remains to be elucidated. HBV-associated carcinogenesis can be seen as a multi-factorial process that includes both direct and indirect mechanisms which might act synergistically. PreS deletion of HBV has recently been shown to be associated with liver disease progression and HCC development in HBV carriers. PreS mutant viruses lack potent B- and T-cell epitopes which normally exist in preS region. This variant could escape from immune system surveillance. These preS protein mutants accumulate in ER, resulting in ER stress. Then, ER stress not only can induce oxidative DNA damage and genomic instability, but also can induce

proliferation-related signal pathways in the hepatocytes. In this condition, cells may progress to tumor formation.

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