Pre-S2 and HBV associated hepatocellular carcinoma

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Abstract
Hepatitis B virus (HBV) infection is a primary cause of hepatocellular carcinoma (HCC). Under selection pressures of host immunity and/or immunoprophylaxis and antiviral therapies, HBV evolves by accumulating mutations in its genome. Several studies highlighted the considerable importance of HBV surface (HBs) protein mutants (pre-S/S variants) in tumorigenesis. Among those mutants, pre-S2 mutants have been recognized as "precursor lesions of HCC" and as risk factors for post-operative recurrence of HCC. Pre-S2 mutants play important roles in tumor progression and induce various mechanisms of tumorigenesis. These roles include that the cytoplasmic orientation of the pre-S2 domain is essential for the transcriptional activator C-terminally truncated middle surface protein (MHBst) which participates in the development of hepatocellular carcinoma. Pre-S2 mutants may also play important roles in HBV tumorigenesis by inducing both endoplasmic reticulum stress-dependent and endoplasmic reticulum (ER) stress-independent pathways. Because HCC has poor prognosis and its incidence is increasing, methods for the prevention and treatment of HCC should be comprehensive. Emerging treatments based on ER stress may provide a new strategy.

Keywords: Pre-S2, hepatocellular carcinoma, hepatitis B virus, endoplasmic reticulum stress

INTRODUCTION
More than 240 million individuals worldwide are infected with chronic hepatitis B virus (HBV) [1]. Chronic HBV infection progresses to cirrhosis in up to 40% of untreated patients, and there is an associated risk of decompensated cirrhosis and hepatocellular carcinoma [2-4]. Several hypotheses have been proposed to...
explain the mechanisms of HBV related to tumorigenesis, including inflammation, liver regeneration associated with cytotoxic immune injuries and transcriptional activators of mutant HBV gene products [7-10]. The HBV genome consists of a circular, partly double-stranded DNA with four overlapping open reading frames: (1) the pre-S/S open reading frame (ORF) encodes three viral surface proteins [including hepatitis B surface antigen (HBsAg)/HBV surface (HBs)], (2) the pre-C/C ORF encodes the hepatitis B e antigen (HBeAg) and the hepatitis B core antigen (HBcAg), (3) the P ORF encodes the terminal protein (TP) and the viral polymerase that possess DNA polymerase and reverse transcriptase and RNaseH activities, and (4) the X gene encoding for a transcriptional transactivator, hepatitis B virus X protein (HBx), which is essential for virus replication [11,12].

Among the four functional proteins encoded by HBV (X, surface, core, and polymerase), HBx and HBs (mutant) proteins are designated “viral oncoproteins” [13]. The pre-S/S mutants of HBV are considered “precursor lesions” of hepatocellular carcinoma (HCC) [14] and as risk factors for the post-operative recurrence of HCC [15,16]. Various pre-S/S mutants contribute to HCC tumorigenesis via various mechanisms, including transactivation of transcription factors, the endoplasmic reticulum (ER) stress-dependent pathway, the ER stress-independent pathway, and others. Among these mutants, pre-S2 mutants showed significant correlations with HCC and have been widely considered novel biomarkers of HBV-associated HCC [13,17]. The malignant transformation potential of pre-S2 mutation has been confirmed in an immortalized human hepatocyte line HH411 [18]. In transgenic mice, pre-S2 mutants induced dysplasia of hepatocytes and development of HCC [19], suggesting that pre-S2 plays a key role in HCC tumor progression.

In this mini-review, we discussed the relationship between pre-S2 mutations and HCC, as well as the underlying molecular mechanisms and treatments based on HBV tumorigenesis induced by pre-S2.

**STRUCTURE AND ROLE OF PRE-S IN HBV**

HBV is a small, enveloped 3.2-kb DNA virus with four open reading frames. The HBV envelope is composed of three forms of HBsAg, including the large (encoded by the pre-S1/S2/S gene), middle (pre-S2/S gene) and small (S gene) envelope proteins [20,21]. In addition, truncated and mutated pre-S2/S [the large HBV surface protein (LHBs) and truncated middle surface protein (MHBs)] or HBx proteins are produced by integrated viral sequences [22-24]. The pre-S region has been reported to mediate hepatocyte attachment of the virus, containing B cell and T cell epitopes [25,26], a binding site for neutralizing anti-pre-S2 antibody [27,28], and an S promoter for controlling the production of middle and small HBs proteins. Under endogenous (host immunity) and/or exogenous (immunoprophylaxis and antiviral therapies) selection pressures, HBV evolves by accumulating mutations in its genome, resulting in HBV variants with altered epitopes providing higher pathogenicity [29-31]. In this context, a growing number of studies were performed to evaluate various HBV genotypes; these pointed out the considerable importance of HBV envelope protein mutants (preS/S variants) [32,33]. Naturally occurring pre-S mutations are frequently detected in serum obtained from patients with chronic HBV infection [34]. Furthermore, pre-S mutations were more common in chronic HBV infection and were related to disease progression and HCC. Currently, the most frequently reported variations are the pre-S deletion mutation and the pre-S2 start codon mutation [35-37]. In particular, the pre-S2 mutation often coincides with changes in human immune cell epitopes [38] and is more significantly correlated with HCC than pre-S1 mutation [39].

**THE ASSOCIATION BETWEEN PRE-S MUTATIONS AND HCC**

The notion of pre-S/S mutations as causes of HBV immune escape was supported by the identification of individuals who developed HBV infection in spite of having vaccine-induced circulating anti-HBs antibodies [31,32,40]. Apart from the ability to avoid neutralization by vaccine-induced anti-HBs, these pre-S/S mutations may also have accounted for cases of occult HBV infection [31,41]. Furthermore, pre-S/S mutations
have been found in association with various forms of acute and chronic liver disease, including fulminant hepatitis (FH), fibrosing cholestatic hepatitis (FCH) and cirrhosis. Both pre-S1 and pre-S2 mutants led to defective secretion of mutant large surface antigens which then accumulated in ER, leading to ground glass hepatocytes (GGH) formation in chronic HBV infection. Under electron microscopy, GGHs were characterized by an abundance of ER, and overloaded ER made the cytoplasm of GGH become “foggy” or “glassy”. GGH was recognized as a risk factor for HCC, in particular, type II GGHs that harbor pre-S2 mutations accumulated on the ER of hepatocytes were considered biomarkers of HCC and were helpful in predicting recurrence and survival in HBV-infected HCC patients. Previous studies reported several tumorigenic mutants, including sL95*, sW182*, and sL216*, that did not promote ER stress but rather activated cell proliferation and transformational abilities; the sW182* mutant was demonstrated to have potent tumorigenic activity; MHBst167 mutants have been shown to interact with proteins associated with tumor progression/progression in vitro. A recent study reported that a pre-S2 start codon mutation of HBV subgenotype B3 affected nuclear factor κB (NF-κB) expression and activation in Huh7 cell lines.

The frequency of pre-S mutations increased successively in the various stages of chronic hepatitis B (CHB) infection. A meta-analysis showed that the frequency of pre-S mutants was approximately 10%, 20%, 35%, and 50% in asymptomatic HBsAg carriers, CHB patients, patients with liver cirrhosis and HCC patients, respectively. The prevalence of pre-S mutants varied among countries with endemic HBV genotypes with a higher prevalence of genotypes B and C. Pre-S deletion mutants detected in serum were also reported to increase the risk of post-operative recurrence of HCC. To efficiently detect pre-S deletion mutants in serum, Su et al. successfully developed an oligonucleotide pre-S gene chip to detect pre-S deletion mutations in sera as a predictive hallmark of HCC. Combined detection of pre-S mutations and other markers of HBV replication such as HBeAg and viral loads may offer a reliable method for predicting HCC risks in chronic HBV carriers. Among those mutants, the pre-S2 mutation in particular was found to be significantly associated with the risk of HCC development.

**VARIOUS MECHANISMS OF PRE-S2 CONTRIBUTING TO HCC**

**Pre-S2 transcriptional activator proteins**

During the infectious process, HBV DNA integrates into hepatocellular chromosomes and encodes two transcriptional activators: the HBV X protein and the family of the pre-S2 activator proteins of HBV, including the LHBs and C-terminally MHBst. The pre-S/S genomic region, when deleted in the C-terminus portion (including the viral transmembrane hydrophobic region III of the S domain) produces C-terminally truncated middle surface protein. HBs transactivators (LHBs and MHBst) function based by cytoplasmic orientation of the pre-S2 domain. Unlike full-length MHBs, truncated MHBst is retained in the endoplasmic reticulum and is not secreted. Therefore, the pre-S2 region of MHBst can interact with the cytoplasmic protein in the cytoplasmic region, resulting in transcriptional activation.

The discovery of transactivating functions exerted by LHBs and MHBst supports the notion that transactivation of cellular gene expression could be relevant to hepatocarcinogenesis. Pre-S2 activators LHBs and MHBst exerted tumor promoter-like functions by activating c-Raf-1/Erk2 signaling in transgenic mice, leading to enhanced proliferative activity of hepatocytes. Liang et al. found that overexpressing MHBst in hepatoma cells enhanced TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis. In addition, a study showed that pre-S2, functioning as a transcriptional activator, promoted the development of hepatocellular carcinoma by activating oncogenes, including c-myc, human telomerase reverse transcriptase (hTERT) and forhead box P3 (Foxp3). Another recent study provided evidence that HBV protein pre-S2 was responsible for reactivation of two oncogenes, alpha-fetoprotein (AFP) and glypican 3 (GPC3), in HCC. Other studies reported that pre-S2 increased protein levels of transcriptional co-activators with
PDZ-binding motifs (TAZ), thereby playing oncogenic roles in HCC cells by repressing miRNA-338-3p expression, implicating hepatocarcinogenesis\(^{64-66}\).

**Pre-S2 mutants**

Both pre-S1 and pre-S2 mutants led to defective secretion of mutant large surface antigens that then accumulated in the ER, leading to GGH formation in chronic HBV infections. As mentioned above, type II GGHs that harbored pre-S2 mutations accumulating on the ER of hepatocytes were considered biomarkers of HCC\(^{47}\). HBV proteins utilize the ER protein folding machinery and cellular secretory pathway\(^{67}\). Therefore, the underlying mechanisms of pre-S mutations contributing to HCC may be involved in ER stress\(^7\). ER stress, also called the UPR in mammalian cells, is a cellular defense mechanism that responds to unfolded viral proteins or perturbed ER functions\(^{69}\). Expression of viral gene products is detected by three UPR sensors, including two ER transmembrane kinases (IRE1 and PERK), and one ER transmembrane transcription factor (ATF-6). The three UPR sensors are associated with ER chaperone GRP78/BiP at rest, and are dissociated from GRP78 upon ER stress\(^{64}\). Induction of GRP78 prevented cells from apoptosis, and ER stress-regulated translation increased tolerance to extreme hypoxia and then promoted tumor growth\(^{69,70}\). The activation of ER-stress downstream molecules such as ATF-6, GRP78 and XBP-1 is believed to be involved in hepatocarcinogenesis\(^{71}\).

Both types of pre-S mutants cause overproduction and accumulation of mutated envelope proteins in the ER, and the accumulation of mutant or unfolded proteins cause stress in the ER that is sensed by the glucose-regulated protein 78 (GRP78). Unfolded proteins sequester GRP78 and dissociate from three ER transmembrane transducers leading to their activation; this leads to significant ER stress that may lead to oxidative stress and DNA damage\(^{72}\), resulting in genomic instability\(^{73}\) and ultimately development of HCC\(^{74,75}\). A detailed study aimed at delineating the molecular mechanisms of pre-S mutant-induced genomic instability suggested that pre-S2 mutant large surface protein inhibited DNA double-strand break repair and led to genome instability in hepatocarcinogenesis; this represented a promising high-risk HCC biomarker in chronic HBV carriers\(^{76}\). The ER stress initiated by the pre-S mutants activated two pathways that protect hepatocytes from apoptosis, one involving nuclear factor (NF)-κB to upregulate cyclooxygenase-2 (COX-2)\(^{45,77}\) and the other involving vascular endothelial growth factor to activate AKT/mammalian target of rapamycin (mTOR) signaling\(^{24}\). The mammalian target of mTOR is a highly conserved serine/threonine kinase that controls cell growth and proliferation\(^{78}\). Pre-S2 mutations promoted tumorigenesis by sustaining high activation rates of aerobic glycolysis through the mTOR signal cascade\(^{69}\). In addition, the pre-S2 mutation LHBs induced an ER stress-independent c-Jun activation domain binding protein 1 (JAB1)/p27/retinoblastoma (Rb)/adenovirus E2 promoter binding factor/cyclin A signal to initiate cell cycle progression\(^{74}\). These studies suggested that the combined effects of genomic instability and cell proliferation potentially resulted in carcinogenesis\(^{79}\).

**TREATMENT STRATEGIES BASED ON ER STRESS**

One of the strategies used to prevent HBV-associated liver diseases and HCC is vaccination\(^{80}\). The effectiveness in preventing blood-borne transmission from an infected mother to her newborn was about 90%\(^{81}\), however therapeutic vaccines for the treatment of established HBV infection are not available\(^{82,83}\). Two antiviral therapies have been approved: pegylated alpha interferon and nucleoside/nucleotide analogues (NA)\(^{44}\). NA therapy has antiviral effects that reduce HCC development and post-operative recurrence of HCC\(^{49}\). NA treatment affects the reverse transcription of pregenomic RNA but does not affect cDNA and subgenomic RNA that have translational activity associated with HBsAg levels. Thus, current NA therapy can hardly clear HBsAg\(^{31}\). Subsequent studies also showed that pre-S2 mutations induced resistance to NAs and predicted HCC development\(^{66}\). Related studies showed that interferon treatment, more than NA treatment, inhibited HBsAg and pre-S mutant protein\(^{51,57,84}\). However, these antivirals therapy often failed to eradicate the virus completely, and their efficacy in preventing liver cirrhosis and HCC was limited\(^{69,90}\).
Thus, it is necessary to clarify the details of the host-virus relationship during HBV infection to facilitate the development of efficient therapeutic strategies for HBV infection.

To prevent HCC, targeting HBV-induced ER stress may provide novel strategies in high-risk CHB. Antioxidants may be such ideal agents, because they reduce ER stress, thereby improving protein folding\textsuperscript{[91]}. Natural products, including silmarin and resveratrol, have been used in HCC. The two drugs target ER stress-associated signal pathways\textsuperscript{[7]}. The pre-S2 mutant initiated an mTOR-dependent glycolytic pathway to activate the solute carrier family 2 member 1 (SLC2A1), contributing to aberrant glucose uptake and lactate production in advanced stages of pre-S2 mutant transgenic tumorigenesis; the mTOR signaling cascade in pre-S2 mutant-mediated hepatocarcinogenesis was inhibited by the combined treatment of resveratrol and silymarin\textsuperscript{[79]}. However, these findings require further validation. Glycyrrhizin acid (GA) has also been reported to suppress ER stress in acute liver injury via several functions, including effective hepatoprotection and the reduction of elevated transaminases\textsuperscript{[92]}. Long-term treatment with glycyrrhizin prevented HCC development in chronic hepatitis C infection\textsuperscript{[89]}. Together, these strategies for prevention and treatment of HBV-related HCC should be further investigated.

DECLARATIONS

Authors’ contributions
Drafted the manuscript: Zheng Y
Revised the manuscript: Qian YY
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Conflicts of interest
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Not applicable.

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