**Abstract**

Although chronic hepatitis B virus (HBV) infection has been well-established to cause hepatocellular carcinoma (HCC), the precise molecular mechanism of HBV tumor genesis remains to be elucidated. Current views of HBV tumorogenesis include inflammation and liver regeneration associated with cytotoxic immune injuries at the early stage, the random dysregulation of cellular growth control genes by genetic abnormalities, and the transcriptional activators of mutant HBV gene products that accumulate in the endoplasmic reticulum (ER) and manifest as type II ground glass hepatocytes (GGH). The retention of pre-S2 mutant protein in GGHs can induce ER stress-dependent and –independent pathways to initiate VEGF/Akt/mTOR, NFκB/COX-2 and JAB-1/p27/RB/cyclin A, D pathway, leading to growth advantage of type II GGH. The pre-S2 mutant protein-induced ER stress response can also cause DNA damage, centrosome overduplication, and genomic instability. Type II GGHs can co-express pre-S2 mutant protein and HBx antigen which together exhibit an enhanced oncogenic effect in transgenic mice. Distinct from type II GGHs, type I GGHs usually distribute sporadically and express stronger ER stress which will lead to apoptosis. Overall, the current evidence suggests that the presence of GGHs, particularly type II, and the detection of pre-S2 mutants in serum has implications for anti-viral treatment and can predict HCC development in patients with chronic HBV infection. A well-designed cohort study and clinical trial should be conducted to confirm that type II GGHs represent precursor lesions of HBV-related HCCs.

**INTRODUCTION**

Although chronic Hepatitis B virus (HBV) has been well established to cause hepatocellular carcinoma (HCCs) [1], the exact mechanism leading to HBV tumorigenesis remains to be elucidated. The prevailing views of HBV tumorigenesis include inflammation and liver regeneration associated with cytotoxic immune (CTL) injuries, HBV DNA insertional mutagenesis, and viral oncoproteins-driven tumorigenesis [2-4]. HCC can occur at the early stage of chronic HBV infection which has active HBV replication and CTL response [3,5], while the majority of HCC cases occur at the advanced stage or anti-HBe-positive phase at the sixth decade of age [4]. In the advanced or anti-HBe-positive phase, it is believed that the mutant viral oncoproteins which can escape CTL attack play a major or driving role in the subsequent HCC development.

Hadziyiannis and Popper first recognized the HBV surface antigens in the “ground glass” hepatocytes (GGH) of chronic HBV carriers [6,7]. Under electron microscopy, GGHs are...
characterized by an abundance of endoplasmic reticulum (ER), among which particles of surface antigens accumulate [8]. It is believed that the overloaded ER makes the cytoplasm of GGHs become “foggy” or “glassy”. Chisari, et al were the first group to demonstrate the formation of GGHs in transgenic mice which express large amount of wild type large surface antigen [9]. Two types of GGHs were later designated as type I and II GGHs [10]. Type I GGHs are usually scattered singly in the hepatic lobules with the expression of “inclusion-like” pattern of surface antigens (Figure 1A). This type of GGHs usually occurs at the early stage or in patients with active replicative diseases [11-13]. Type II GGHs, however, express a unique expression pattern of surface antigens at the cell margin (Figure 1A). Most importantly, type II GGHs consistently cluster in nodules and usually occur at the advanced stages or anti-HBe-positive phase [14], and are frequently associated with cirrhosis or hepatocellular carcinoma (HCC). The consistent clustering distribution of type II GGHs, especially in the non-tumorous liver tissues of patients with HCC receiving surgery, drives us to hypothesize that type II GGHs may represent clonal preneoplastic or focal adenomatous lesions of HCC [15]. GGHs have been proposed to progress from focal adenomatous hyperplasia, adenoma, and HCC in transgenic mice harboring wild type HBV genome [16]. The exact pathogenesis and molecular mechanism of GGHs in HBV tumor genesis, however, remains uncharacterized, although chronic liver injury, necroinflammation, and regenerative hyperplasia have been proposed to explain the disease progression in different transgenic mice model [17].

In the past decade, we studied in detail the biologic and molecular significance of type II GGHs which contain a unique form of pre-S2 deletion mutant protein. The significance of pre-S2 mutant proteins in HCC development, the signal pathway initiated by pre-S2 mutant proteins, the clinical significance of pre-S mutants in serum, and the transgenic mice model of pre-S2 mutant tumorigenesis were studied. Importantly, we developed

![Figure 1](image_url)

**Figure 1.** A. Ground glass hepatocytes (GGH) and the expression patterns of hepatitis B surface antigens. Type I GGH are usually scattered singly and express an inclusion-like pattern of the surface antigen (B), while type II GGH consistently cluster in nodules and express a marginal pattern of the surface antigen (D). Black arrows indicate hepatocytes containing ground glass substances in both type I (A) and type II GGH (C). B. The profile of deletions over the pre-S regions in sera of HBV-related HCC patients. A complex combination of deletions at pre-S1 and pre-S2 regions may occur in patients of chronic HBV carriers. Hematoxylin–Eosin stain, HE; Immunohistochemical stain, IHC.
a combined DNA array chip to detect pre-S2 mutant gene and an ELISA kit to detect pre-S2 mutant protein in serum as the predictive marker of HCC in chronic HBV carriers.

**Ground glass hepatocytes contain pre-S deletion mutant proteins which accumulate in endoplasmic reticulum (ER) and induce ER stress signals**

By dissecting the cirrhotic nodules in liver tissues of chronic HBV hepatitis patients, we are surprised to find that type II GGHs consistently harbored pre-S2 mutants with in-frame deletions over the pre-S2 regions (predominantly, nt. 2–55 or 4–57) with or without point mutations at the start codon (ATG–ATA) of the S promoter. These mutations lead to a decreased synthesis of middle and small surface antigens and result in a defective secretion of the mutant large surface antigens which then accumulate in ER, leading to the GGH formation in chronic HBV infection [10,15]. The deletion site at the pre-S2 region coincides with the epitope of cytotoxic T-cell and B-cell neutralization responses and suggests that the pre-S2 deletion mutants represent an immune escape mutant [18,19]. In consistence with the immune escape epitope of the pre-S2 deletion site, the liver tissues containing type II GGHs are usually devoid of inflammatory infiltrate. Distinct from type II GGHs, the singly distributed type I GGHs contain entirely different pre-S mutants with variable deletions over the pre-S1 regions. The liver lobules containing the type I GGHs usually show presence of inflammatory infiltrate, distinct from that in type II GGHs. The deletion sites at the pre-S1 region may interfere with the transcriptional activities of the S promoter and affect the regulation of HBV replication and synthesis of small surface antigens [20,21]. The pre-S1 containing large surface antigen (LHB) exhibits a dual topology, and only half of the LHB translocate post-translationally into the ER lumen [22,23].

The accumulation of mutant or unfolded proteins causes stress in ER that is sensed by the glucose-regulated protein 78 (Grp78). Unfolded proteins will sequester Grp78 and dissociate from three ER transmembrane transducers leading to their activation [28]. It has been demonstrated that the secretion of surface proteins was compromised by pre-S deletions, especially pre-S2 mutants [10]. In addition, ectopic expression of pre-S mutant proteins in Huh-7 cells increased the levels of ER chaperones (Grp78 and 94) and activated PERK and C-jun N-terminal kinase (JNK) [10]. Northern and Western blot analyses revealed that the pre-S1 mutant induced stronger levels of ER chaperone (Grp78 and 94) response, calcium release, cyclooxygenase-2 (COX-2) and inflammatory cytokines, and oxidative stress intermediates, which tend to result in apoptosis [10,29,30]. The pre-S2 mutants, albeit inducing a weaker level of ER stress signal, exhibited a higher levels of mutation frequency and transforming capabilities in primary hepatocyte cell line HH4 [31].

**ER stress-induced by pre-S2 mutants leads to oxidative DNA damage, genomic instability, and transforming capabilities in transgenic mice model**

The induction of ER stress has been shown to increase the level of reactive oxygen species (ROS), NFκB activation and cyclooxygenase-2 (COX-2) expression and thereby induces oxidative DNA damages (Figure 2) [28,29,32]. Treatments with antioxidants reduced ER stress and improved protein folding [33]. As expected, the expression of pre-S mutant proteins induced oxidative DNA damages, as demonstrated by the increase in 8-hydroxyguanalone on the DNA lesion and increased levels of 8-oxoguanine glycosylase 1 (OGG1) and X-ray cross-complementation 1 (XRCC1) [30]. These studies suggest the presence of genomic instability in GGHs [34]. Notably, as a promising gene transactivator and an ER stress inducer, pre-S2 mutant protein also promotes centrosome instability through two independent mechanisms. First, pre-S2 mutant protein could upregulate cyclin A and sustained cyclin D1 and cyclin-dependent kinase-4 via gene transactivation. This event subsequently promoted cell cycle progression even in the presence of ER stress and resulted in nodular proliferation in transgenic mice livers [31]. Second, ER stress facilitates the release of calcium from the ER and thereby activates calcium-dependent calpain proteases. Notably, cyclin A is a substrate of calpain and the proteolysis results in cytoplasmic redistribution of cyclin A and thereby stimulates centrosome overduplication [35]. These studies demonstrate that pre-S2 mutant protein is a direct driver of genomic instability through the induction of DNA damages and centrosome abnormality.

The most important molecular mechanism initiated by pre-S2 mutant is the VEGF/Akt/mTOR signal pathway which is activated at the early, middle, and advanced stages of transgenic livers harboring pre-S2 deletion mutant (Figure 2) [36,37]. The mTOR signaling is commonly activated in human HCC tissues and represents a candidate target for therapy. The transforming ability of pre-S2 mutant proteins has been investigated in an immortalized human hepatocyte line HH4 which is immortalized by human papilloma virus [31]. In addition, the transgenic mice carrying pre-S2 mutant developed HCC [38]. These studies further support the role of pre-S2 mutants and GGHs in HBV hepatocarcinogenesis [14,39].

Aside from the ER stress-dependent signaling pathways, a distinct ER stress-independent response has been found specifically for pre-S2 deletion mutant protein and is significant for their biologic and carcinogenic preferences [10]. The pre-S2 mutant protein specifically interacts with c-Jun activation domain binding protein 1 (JAB1), which enhances activator protein-1 transcriptional activity and cell proliferation [34]. Through its binding to JAB1, the pre-S2 mutant protein induces JAB1 nuclear translocation, which activates p27/retinoblastoma/Cdk2/cyclin A pathways and leads to cell cycle progression and centrosome overduplication [31,35]. These findings have provided clear mechanisms for the growth advantage induced by the pre-S2 mutant protein.

Two HBV viral proteins, the X protein (HBx) and pre-S2 deletion mutant protein, have been considered to have either
Figure 2 Schematic depiction of the potential signals induced by pre-S mutants and the candidate targets for chemoprevention. Both types of pre-S mutants can induce endoplasmic reticulum (ER) stress signals, which may lead to oxidative stress and DNA damage, leading to genomic instability. The pre-S mutants may also activate two signal pathways to protect the hepatocytes from apoptosis, one involving nuclear factor (NF)-κB to upregulate cyclooxygenase-2 (COX-2) and the other vascular endothelial growth factor to activate Akt/mammalian target of Rapamycin (mTOR) signaling. Pre-S2 mutant can additionally induce 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. Combined effects of genomic instability and cell proliferation will potentially result in carcinogenesis. Resveratrol and Silymarin are two nature products could be used to target PPAR-α/γ and mTOR signal cascade for chemoprevention in high risk HBV carriers. Cdk2, cyclin-dependent kinase 2; HBV, hepatitis B virus; ROI, reactive oxygen intermediate.

Based on our studies cited above, we propose that GGHs, especially type II which harbored the pre-S2 deletion mutants, may represent the precursor lesions of HCC in the advanced stage of chronic HBV infection. As mentioned above, the mechanism of HBV tumorigenesis is complicated and several factors may cooperate to jointly promote the tumorigenesis. In patients who developed HCC at early stage of life, chronic liver injuries and subsequent liver regeneration may play an important role as inferred from the transgenic animal models, mainly from the group of Chisari, et al [9,17]. Distinct from the concept that type II GGHs plays a direct role for HBV tumorigenesis, they proposed that GGHs overexpressing large surface antigens will undergo cell death and the regenerative hyperplasia of other undefined hepatocytes may progress inexorably and indirectly contribute to HBV tumorigenesis [16,46]. Whether chronic liver injury is mandatory for HCC development is still controversial [47]. In the human liver tissues in which type II GGHs located, there is usually no inflammatory infiltrate observed, distinct from that in type I GGHs or liver tissues of chronic HBV exacerbation [14,26]. Since pre-S2 deletion mutant is an immune escape variant due to the deletion of B and T cell epitope [18,19], the liver tissues harboring type II GGHs can be devoid of chronic necroinflammatory change. Therefore, the mechanism of tumorigenesis exhibited by type II GGHs in human may be different from that of GGHs proposed...
Development of DNA Chip and ELISA kit to detect pre-S2 mutants in serum as the predictive hallmark of HCC

To efficiently detect pre-S deletion mutants in serum, we have successfully developed the oligonucleotide Pre-S Gene Chip and the ELISA system to detect the pre-S deletion mutants in sera. The Pre-S Gene Chip contains 42 DNA probes that target the pre-S region of the LHBS gene, offering a highly sensitive and specific method for pre-S deletion detection with short turnaround time (<3 days) [65]. Screening the pre-S deletion mutants revealed interesting findings that the detection rate of pre-S mutants were relatively low (7%) in the sera of patients with acute exacerbation of chronic HBV infection but gradually increased in later periods of chronic HBV infection, as they were 37% in advanced stage of chronic HBV carriers, and as high as 60% in HCC patients [65]. Combined detection of pre-S mutants and other markers of HBV replication such as HBeAg and viral loads is believed to offer a reliable predictive method for predicting HCC risks in chronic HBV carriers.

CONCLUSION

In this review, we provide an overview to provide evidence on the emerging role of HBV pre-S2 deletion mutant protein in HBV tumorigenesis, although cohort studies and genetic studies are needed to clarify whether type II GGHs represent the pre-neoplastic or adenomatous lesions and directly contribute to HCC development. The HBV pre-S2 deletion mutant proteins are retained in the ER and induce ER stress response. Series of ER stress-dependent and -independent growth signals are then activated. Among the diverse pathways, mTOR-mediated signal cascade represent a major mechanism for the disturbed metabolism, genomic instability, and growth advantage, which can potentially drive the type II GGHs toward the pre-neoplastic and neoplastic lesions. To identify the patients at high risk for HCC development represents the major task in combating chronic HBV infection in the coming decades. The development of a DNA chip and ELISA kit for detecting pre-S2 deletion mutant will meet this demand. Chemopreventive or therapeutic agents can then be provided to these high risk HBV carriers to prevent from HCC development.

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REFERENCES


by Chisari, et al in transgenic mice which harbor wild type large surface antigen.

Presence of pre-S2 mutants in serum predicts the resistance of nucleoside analogues anti-virals and a higher risk of HCC development.

The emergence of pre-S deletion mutants occurs during the natural course of HBV infection, possibly due to selective pressure by the immune system [48,49]. The frequencies of pre-S mutation increased successively in different stage of chronic HBV infection. In a meta-analysis study [50], the summarized results showed that pre-S mutation was detected in around 10% of asymptomatic HBsAg carriers, 20% of patients with chronic hepatitis B, 35% of patients with liver cirrhosis and 50% patients with HCC. The pre-S2 deletion mutants are more frequently detected in anti-HBeAg-positive patients and in patients with HCC than in HBeAg-positive patients [27,48,51]. The ratio of pre-S mutant clones related to wild type in serum also accumulates, as it was 6.4% at high replicative phase, 13% at intermediate, and 37.5% at low or nonreplicative phases [27]. Therefore, pre-S2 mutants represent a significant proportion of virus in advanced stage patients [52,53].

Although anti-viral nucleoside analogues therapy has been associated with a lower risk of HCC development or recurrence after liver resection in chronic HBV carriers [54], the frequencies of pre-S mutation have been reported to be increased after antiviral therapy by nucleotide analogues which is closely associated with the drug resistance and predict the high risk development of HCC [55,56]. Interestingly, in the control group treated with alpha-interferon, the pre-S2 mutants were significantly reduced or absent, suggesting that alpha-interferon may degrade or inhibit the synthesis of pre-S2 mutant proteins [55]. The presence of pre-S mutants, especially pre-S2 mutant, has been found to be significantly associated with the risk of HCC development [55-60]. Pre-S deletion mutants detected in serum has also been reported to increase the risk of post-operative recurrence of HCC [61]. Of particular note, pre-S mutation could occur early in age and significantly associated with HCC in pediatric patients [62,63]. Pre-S2 deletion in sera can be detected in nearly half of children with HCC, in contrast to none in children with chronic HBV infection [62]. By using tissue samples, pre-S2 deletion mutants can be detected in about 80% of pediatric HCC [63]. Overall, the combined effects of cell cycle progression, genomic instabilities, and survival advantage exhibited by pre-S2 mutant proteins strongly suggest that type II GGHs are potential preneoplastic loci for HCC development and de novo recurrence after surgical resection. In a cohort of 82 patients with HBV-related HCC who received curative surgery [64], type II GGHs were found to be the independent variables associated with late recurrence and the overall survival. In the transgenic mice model using whole HBV genome, Chisari’s group demonstrated a sequential evolution from GGHs to focal hyperplasia, adenomatous hyperplasia or dysplasia, and finally the development of HCC [16]. Therefore, they hypothesized that GGHs represent the precursor lesions of human HCC development.


33. Malikova JD, Miao H, Zhang K, Wolfsön A, Pennathur S, Pipe SW.


41. Benn J, Schneider RJ. Hepatitis B virus HBx protein activates Ras-GTP complex formation and establishes a Ras, Raf, MAP kinase signaling cascade. See comment in PubMed Commons below Proc Natl Acad Sci U S A. 1994; 91: 10350-10354.


63. Abe K, Thang SN, Wu HC, Tran TT, Le Hoang P, Truong KD. Pre-S2 deletion mutants of hepatitis B virus could have an important role in hepatocarcinogenesis in Asian children. See comment in PubMed Commons below Cancer Sci. 2009; 100: 2249-2254.
