

REVIEW

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The emerging role of hepatitis B virus Pre-S2 deletion mutant proteins in HBV tumorigenesis

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Abstract

Chronic hepatitis B virus (HBV) infection can cause hepatocellular carcinoma (HCC). Several hypotheses have been proposed to explain the mechanisms of HBV tumorigenesis, including inflammation and liver regeneration associated with cytotoxic immune injuries and transcriptional activators of mutant HBV gene products. The mutant viral oncoprotein-driven tumorigenesis is prevailed at the advanced stage or anti-HBe-positive phase of chronic HBV infection. Besides HBx, the pre-S2 (deletion) mutant protein represents a newly recognized oncoprotein that is accumulated in the endoplasmic reticulum (ER) and manifests as type II ground glass hepatocytes (GGH). The retention of pre-S2 mutant protein in ER can induce ER stress and initiate an ER stress-dependent VEGF/Akt/mTOR and NFκB/COX-2 signal pathway. Additionally, the pre-S2 mutant large surface protein can induce an ER stress-independent pathway to transactivate JAB-1/p27/RB/cyclin A,D pathway, leading to growth advantage of type II GGH. The pre-S2 mutant protein-induced ER stress can also cause DNA damage, centrosome overduplication, and genomic instability. In 5-10% of type II GGHs, there is co-expression of pre-S2 mutant protein and HBx antigen which exhibited enhanced oncogenic effects in transgenic mice. The mTOR signal cascade is consistently activated throughout the course of pre-S2 mutant transgenic livers and in human HCC tissues, leading to metabolic disorders and HCC tumorigenesis. Clinically, the presence of pre-S2 deletion mutants in sera frequently develop resistance to nucleoside analogues anti-virals and predict HCC development. The pre-S2 deletion mutants and type II GGHs therefore represent novel biomarkers of HBV-related HCCs. A versatile DNA array chip has been developed to detect pre-S2 mutants in serum. Overall, the presence of pre-S2 mutants in serum has implications for anti-viral treatment and can predict HCC development. Targeting at pre-S2 mutant protein-induced, ER stress-dependent, mTOR signal cascade and metabolic disorders may offer potential strategy for chemoprevention or therapy in high risk chronic HBV carriers.

Keywords: Ground glass hepatocytes, Pre-S mutants, Endoplasmic reticulum stress, Chronic HBV infection, Hepatocellular carcinoma

Introduction

Hepatitis B virus (HBV) has been well established to cause hepatocellular carcinoma (HCCs) [1]. Several hypotheses have been proposed to explain the mechanism of HBV tumorigenesis, including inflammation and liver regeneration associated with cytotoxic immune injuries, HBV DNA insertional mutagenesis, and viral oncoproteins-driven tumorigenesis [2-4]. Although HCC can occur at any stage of chronic HBV infection, the majority of cases occur at the

advanced stage or anti-HBe-positive phase with the peak incidence at sixth decade [4]. The development of HCC related to the inflammation and liver regeneration is likely due to the cytotoxic T cell immune injuries toward HBV antigen-expressing hepatocytes which may result in bridging hepatic necrosis and fibrosis [3,5]. In the anti-HBe-positive phase, however, the mutant viral oncoproteins may play an important or driving role in HCC development.

In the early 1970s when the Australia antigen was found to be associated with hepatitis B virus (HBV) infection, Hadziyannis and Popper first recognized the surface antigen in the "ground glass" hepatocytes (GGH) of HBV carriers [6,7]. Under electron microscopy, GGHs are characterized by an abundance of endoplasmic reticulum (ER), among

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which particles of surface antigens accumulate [8]. It is believed that the overloaded ER makes the cytoplasm of GGHs become “foggy” or “glassy”. After the introduction of immunohistochemistry, a series of studies demonstrated that different types of GGHs correlated to the expression patterns of HBV surface/core antigens and the replicative stages of chronic HBV infection [9,10]. Two types of GGHs were later designated by us as type I and II GGHs [11]. 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 diseases, frequently co-expressed with a nuclear or cytoplasmic core antigen [9,12,13]. Type II GGHs, however, express a unique expression pattern of surface antigens at the cell margin (Figure 1A). Most interestingly and 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). Conversion from type I GGH to type II GGH could be demonstrated in the serial biopsies from the same individual, frequently associated with hepatitis B e antigen (HBeAg) seroconversion [14]. 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 clonally-proliferated or preneoplastic lesions of HCC [15]. The distinct marginal expression pattern of surface antigens in type II GGHs suggests that there may exist a unique form of mutant surface proteins which exhibit growth advantage to promote the clustering distribution of hepatocytes.

The above-mentioned observation of the unique biologic and pathologic features of type II GGHs drives us to explore in details the underlying molecular and biologic mechanism of type II GGHs and its potential significance in HBV tumorigenesis. In the past decade, we clarified the biologic and molecular significance of type II GGHs which

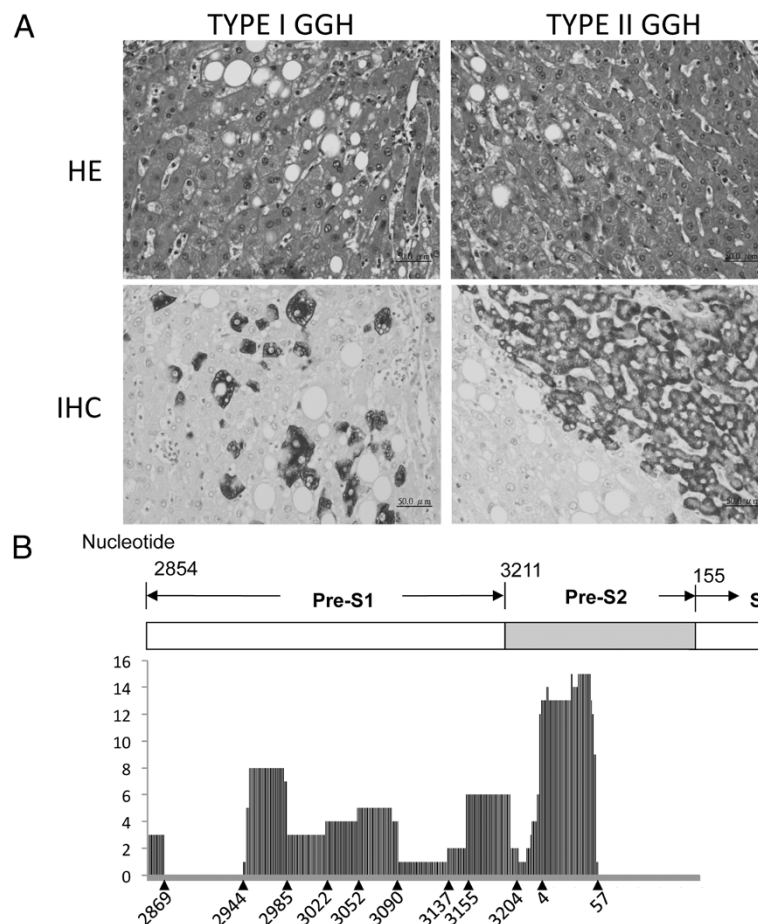


Figure 1 Two types of ground glass hepatocytes and the corresponding deletions at pre-S regions of large surface protein. Ground glass hepatocytes (GGH) and the expression patterns of hepatitis B surface antigens. Type I GGH are usually scattered singly and express an inclusion-like pattern of the surface antigen, while type II GGH consistently cluster in nodules and express a marginal pattern of the surface antigen (A). 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 (B). Hematoxylin–Eosin stain, HE; Immunohistochemical stain, IHC.

contain a unique form of pre-S2 deletion mutant large surface protein (pre-S2 mutant). The significance of pre-S2 mutant proteins in HCC development, the signal pathway initiated by pre-S2 mutant proteins, and the transgenic mice model of pre-S2 mutant tumorigenesis were studied in detail. Importantly, we developed a DNA array chip to detect pre-S2 mutant gene as the predictive marker of HCC in chronic HBV carriers. Potential chemoprevention or therapy targeting at mTOR signal cascade and metabolic pathway was proposed in this review.

Review

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 containing type II GGHs, 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 middle surface gene in the pre-S2 region. 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 [11,15]. It is also interesting to note that 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 [16,17]. This hypothesis is supported by the pathologic observation that the hepatic lobules, which contain type II GGH, usually show no inflammatory activities or lymphocyte infiltration [6,14,18]. 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 deletion sites at the pre-S1 region may interfere with the transcriptional activities of the pre-S2/S promoter and affect the regulation of HBV replication and synthesis of small surface antigens [19,20]. 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 [21,22]. The pre-S regions that remain in cytoplasmic orientation can bind to the cytosolic heat shock protein Hsc70 [23] and presumably interact with the core particle during virion assembly [24]. The cytoplasmic orientation of the pre-S region is required for the transcriptional activator function of LHB and middle surface protein *in vitro* [25,26]. The luminal orientation of the pre-S domain, after virus maturation and secretion, is exposed on the surface of the virion and is involved in virus attachment and recognition [27,28]. Mutants with various types of in-frame deletion in pre-S1 region were found to be replication competent *in vitro* [17,20,29] and can be the predominant strain *in vivo* [30–32]. In HCC patients, the pre-S mutants

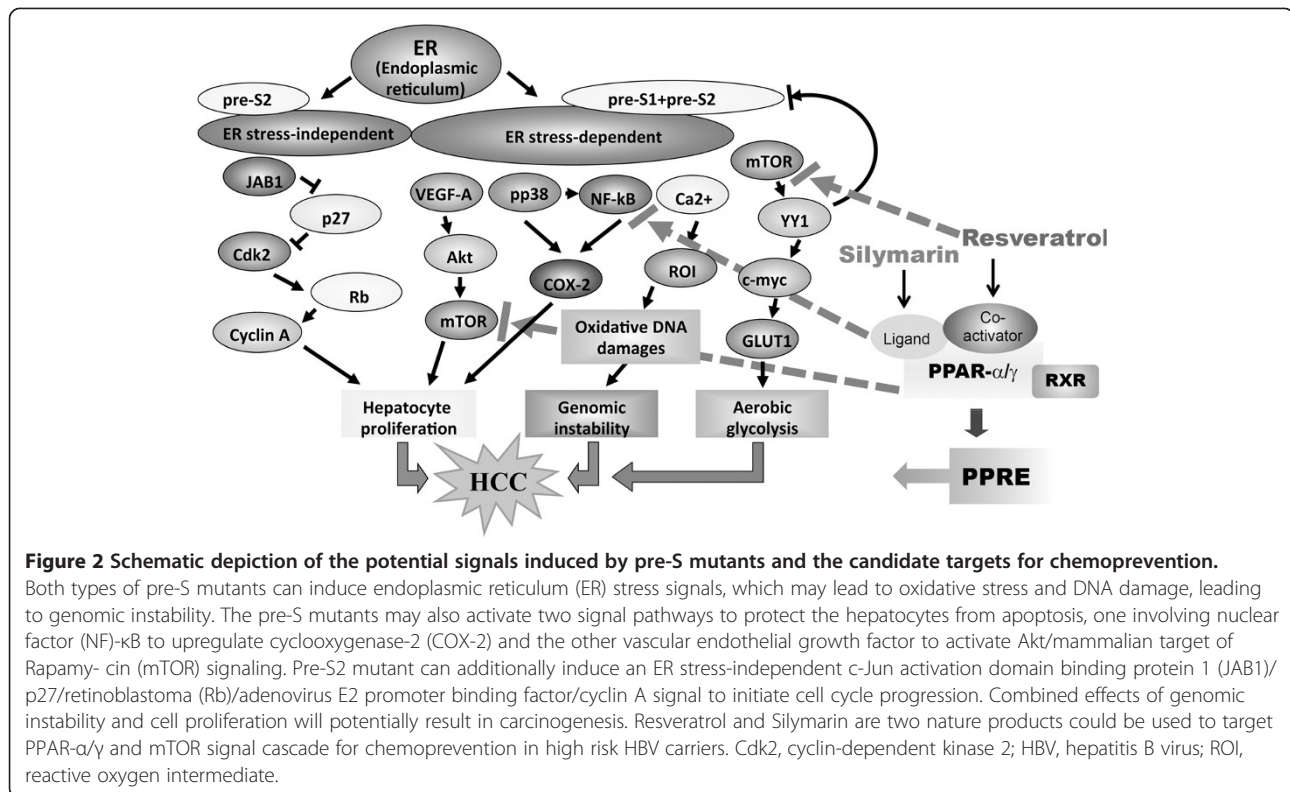
are prevalent for up to 63%, including mutants with combinations of deletions over the pre-S1 and pre-S2 regions (Figure 1B) [33,34].

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 [35]. The activation of ER stress has been implicated in a variety of human diseases including neurodegenerative disease, inflammation, and cancer [36]. It has been demonstrated that the secretion of surface proteins was compromised by pre-S deletions, especially pre-S2 mutants [11]. 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) [11]. These results indicate that both pre-S1 and pre-S2 mutant surface proteins induce ER stress signals in hepatocytes. In consistence with this assumption, elevated Grp78 expression was detected in both type I and type II GGHs in the liver [11]. 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 [11,37,38]. The pre-S2 mutants, albeit inducing a weaker level of ER stress signal, exhibited higher levels of mutation frequency and transforming capabilities in primary hepatocyte cell line HH4 [39].

One remarkable biological phenomenon conferred by pre-S1 and pre-S2 mutant proteins is the distinct subcellular localizations of these two proteins in the hepatocytes. Accumulation of pre-S1 mutant proteins frequently displayed an inclusion-like configuration in type I GGHs (Figure 1). In contrast, peripheral or marginal distribution of pre-S2 mutant proteins was evident in type II GGHs [11,40]. It is plausible that marginal distribution of pre-S2 mutant proteins is a result of an active recruitment process of pre-S2 mutant proteins, by other ER components, towards the cell periphery. The triggering factor of this recruitment and the patho-biological implication of this process will shed light on the pathogenesis of GGHs as the pre-neoplastic lesions for the development of HCC.

ER stress-dependent and-independent pathways induced by pre-S2 mutants, leading 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), NF κ B activation and cyclo-oxygenase-2 (COX-2) expression and thereby induces oxidative DNA damages (Figure 2) [35–37]. Treatments with antioxidants reduced ER stress and improved protein folding [41]. As expected, the expression of pre-S mutant



proteins induced oxidative DNA damages, as demonstrated by the increase in 8-hydroxyguanine on the DNA lesion and increased levels of 8-oxoguanine glycosylase 1 (ogg1) and X-ray cross-complementation 1 (xrcc1) [38]. These studies suggest the presence of genomic instability in GGHS [42]. 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 [39]. 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 [43]. 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 in Huh-7 cells and sequentially activated at the early, middle, and advanced stages of transgenic livers harboring pre-S2 deletion mutant (Figure 2) [44,45]. 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 [39]. In addition, transgenic mice carrying pre-S2 mutant developed HCC [40]. These studies further support the role of pre-S2 mutants and GGHS in HBV hepatocarcinogenesis [14,46].

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 [11]. 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 [42]. Through its binding to JAB1, the pre-S2 mutant protein induces JAB1 nuclear translocation, which activates p27/retinoblastoma/Cdk2/cyclin A, D pathways and leads to cell cycle progression and centrosome over-duplication [39,43]. 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 direct or indirect effects in HBV hepatocarcinogenesis [47]. Other than the molecular mechanisms of pre-S2 deletion mutants in hepatocarcinogenesis as mentioned above, HBx also has been shown to mediate the activation

of multiple signal pathways including the mTOR signal cascade [48-52]. In our laboratory, we observed a strong HBx expression in the cytosolic fraction of GGHs in 5 of 20 HBsAg-positive human livers. Interestingly, HBx was consistently co-expressed with HBsAg, but not *vice versa*, in GGHs. The expression of both oncoproteins, however, was only rarely detected in HCC tissues [45]. Transgenic mice harboring the HBx, pre-S2 mutant, and a double (HBx plus pre-S2 mutant) construct have been established in our laboratory. We observed that the transgenic livers harboring double construct plasmids developed HCCs at an average of 15.1 months, earlier than that of HBx (16.9 months) or pre-S2 mutant (24.5 months) alone. Interestingly, the oncogenic signals of VEGF-A, p-Akt1/2/3, mitogen-activated protein kinases (MAPK) signaling, and p-mTOR were sequentially and differentially activated at different stages in the progression of tumorigenesis [45]. The combined expression of HBx and pre-S2 mutant can exhibit enhanced oncogenic effects in HBV tumorigenesis. The exact role of HBx and pre-S2 mutant protein, either alone or in combination, in human HCC development remains to be clarified.

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 [32,53]. The frequencies of pre-S mutation increased successively in different stage of chronic HBV infection. In a meta-analysis study [54], the summarized results showed that pre-S mutants were 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 [32,34,55]. 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 [34]. Therefore, pre-S2 mutants represent a significant proportion of virus in advanced stage patients [56,57].

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 [58], the frequencies of pre-S mutants have been reported to be increased after antiviral therapy by nucleos(t)ide analogues which is closely associated with the drug resistance and predict the high risk development of HCC [59,60]. 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 [59]. The presence of

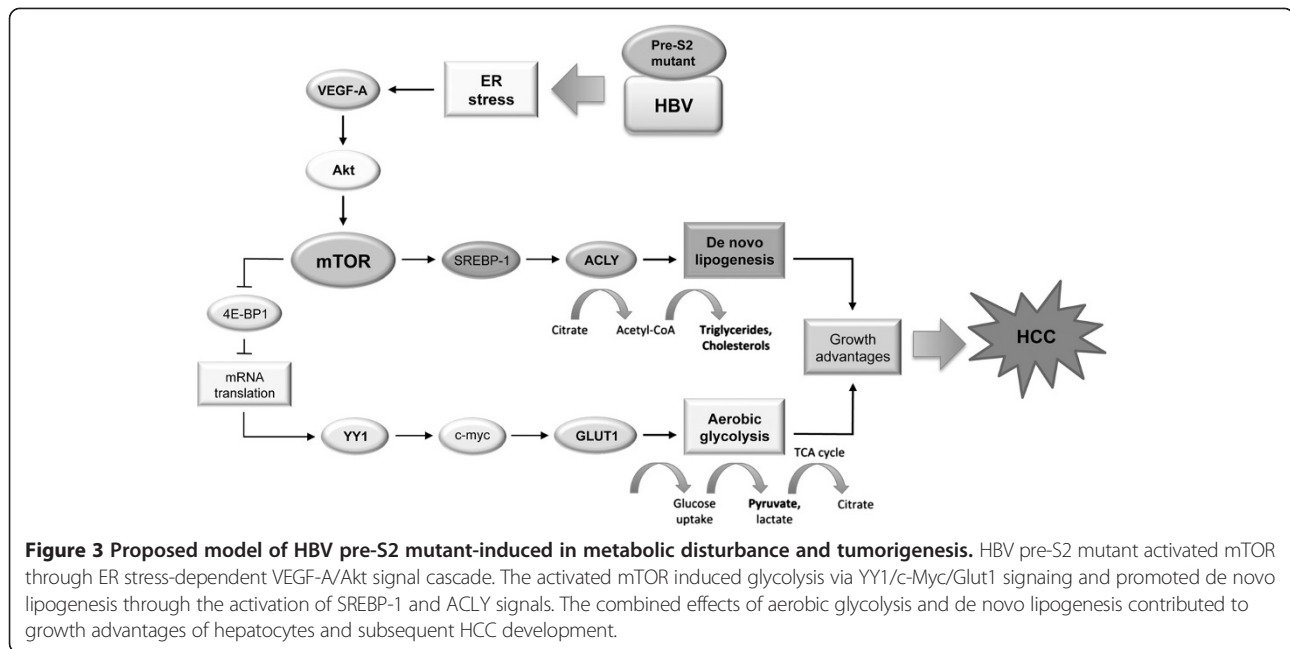
pre-S mutants, especially pre-S2 mutant, has been found to be significantly associated with the risk of HCC development [59-64]. Pre-S deletion mutants detected in serum has also been reported to increase the risk of post-operative recurrence of HCC [65]. Of particular note, pre-S mutation could occur early in age and significantly associated with HCC in pediatric patients [66,67]. Pre-S2 deletion mutants in sera can be detected in nearly half of children with HCC, in contrast to none in children with chronic HBV infection [66]. By using tissue samples, pre-S2 deletion mutants can be detected in about 80% of pediatric HCC [67]. 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 [68], type II GGHs were found to be the independent variables associated with late recurrence and the overall survival. However, a prospective cohort study is needed to test the specificity and sensitivity of pre-S2 deletion mutants and ground glass hepatocytes in the predictive value of HCCs.

Development of DNA chip 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 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) [69]. 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 [69]. 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.

Potential chemoprevention or therapy of HBV-related HCCs targeting at ER stress-induced signal cascade and metabolic disorders

Recently, metabolic disorders have been shown to be associated with cancer development. In addition to its better-known functions in promoting protein synthesis and suppressing autophagy, mTOR is now emerging as a key regulator of cellular metabolism and cancer [70]. By analysis of metabolic gene expression profiles in transgenic mice livers and *in vitro* culture system harboring pre-S2



mutant protein, we observed that mTOR exhibited a significant role in the metabolic switch toward increased glycolysis and lipid accumulation in HCC tissues (Figure 3). We demonstrated that pre-S2 mutant could activate two mTOR-induced metabolic pathways, one involving Yin Yang 1 (YY1), c-myc and, glucose transporter 1 (GLUT1) to upregulate aerobic glycolysis, and the other involving sterol regulatory element-binding protein-1 (SREBP-1) and acyl-CoA lyase (ACLY) to promote de novo lipid biosynthesis (Teng C.F. et al., unpublished data). The activation of mTOR-dependent metabolic signaling cascades was further validated in human HBV-related HCC tissues. To protect HBV carriers from developing HCC and preventing recurrence after HCC resection, it is important to develop chemopreventive agents for the high risk patients targeting at the specific signal pathway, with the combination of antivirals. Among the natural products, silymarin and resveratrol may represent potential candidate products because of their popular and long-term usage in human communities [71]. Silymarin, the active component-silibinin, has been evaluated clinically in the treatment of hepatitis and liver damage because of its anti-inflammation and anti-oxidant effects [71,72]. Resveratrol has been verified to be effective to prevent cancer development at various stages of carcinogenesis including initiation, promotion, tumor invasion, and metastasis [73-75]. Importantly, resveratrol is also a promising product for metabolic syndrome mediated through mTOR inhibition and upregulation of PPARs and PGC-1 α [76,77]. The combination of silymarin and resveratrol could target on the major signal pathways induced by pre-S2 mutants (Figure 2). Preliminary studies in our laboratory revealed a remarkable effect of this combined

product on reducing lipid metabolism and decreasing the incidence of HCC development in transgenic mice harboring HBx and pre-S2 mutant [78]. Further studies or clinical trials are needed to validate the effect of the natural products, with or without the combination of antivirals in chemoprevention or therapy of HBV-related HCCs.

Conclusion

In this review, we provide a comprehensive overview to provide evidence on the emerging role of HBV pre-S2 deletion mutant protein in HBV tumorigenesis. 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 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 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.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

IHS conceived of the study and participated in its design and coordination. LHW carried out the pre-S2 mutants identification and their signaling pathways. WCH carried out chemoprevention on transgenic mice. HCW carried out

immunohistochemical staining. CFT identified the impact of pre-S mutants in mTOR signaling cascade regulation. HWT identified GGHS patterns and performed the statistical analysis. WH participated in study design and the development of pre-S DNA chip. All authors contributed to manuscript drafting. All authors read and approved the final manuscript.

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