

Review

Pre-S2 Mutant-Induced Mammalian Target of Rapamycin Signal Pathways as Potential Therapeutic Targets for Hepatitis B Virus-Associated Hepatocellular Carcinoma

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Chronic hepatitis B virus (HBV) infection is a major risk factor for hepatocellular carcinoma (HCC). Pre-S2 mutant represents an HBV oncoprotein that is accumulated in the endoplasmic reticulum (ER) and manifests as type II ground glass hepatocytes (GGHs). Pre-S2 mutant can induce ER stress and initiate multiple ER stress-dependent or -independent cellular signal pathways, leading to growth advantage of type II GGH. Importantly, the mammalian target of rapamycin (mTOR) signal pathways are consistently activated throughout the liver tumorigenesis in pre-S2 mutant transgenic mice and in human HCC tissues, leading to hepatocyte proliferation, metabolic disorders, and HCC tumorigenesis. In this review, we summarize the pre-S2 mutant-induced mTOR signal pathways and its implications in HBV-related HCC tumorigenesis. Clinically, the presence of pre-S2 mutant exhibits a high resistance to antiviral treatment and carries a high risk of HCC development in patients with chronic HBV infection. Targeting at pre-S2 mutant-induced mTOR signal pathways may thus provide potential strategies for the prevention or therapy of HBV-associated HCC.

Key words: Hepatitis B virus (HBV); Hepatocellular carcinoma (HCC); Ground glass hepatocytes (GGHs); Pre-S2 mutant; Mammalian target of rapamycin (mTOR)

INTRODUCTION

Hepatocellular carcinoma (HCC) is the leading cause of cancer-related deaths worldwide, and a continued increase in incident rate is predicted^{1,2}. Identification of novel therapeutic targets for HCC is thus urgently needed.

Chronic hepatitis B virus (HBV) infection is a major risk factor for the development of HCC^{3,4}. Several hypotheses are proposed to explain the mechanisms of HBV-related tumorigenesis, such as insertional mutagenesis of HBV genome, inflammation, regeneration, and transactivating functions of HBV gene products, such as X protein (HBx) and truncated middle surface protein^{5–7}.

Studies from our group recognize the pre-S2 mutant, which harbors deletion mutation (nucleotide 4–57 deletion) in the pre-S2 region of HBV large surface antigen (LHBs), as a viral oncoprotein that is accumulated in the endoplasmic reticulum (ER) and manifests as type II ground glass hepatocytes (GGHs)^{8,9}. The retention of pre-S2 mutant protein in ER can induce ER stress and initiate an ER stress-dependent nuclear factor- κ B/cyclooxygenase-2 signal pathway to protect hepatocytes from apoptosis^{9,10}. Additionally, pre-S2 mutant can induce an ER stress-independent c-Jun activation domain-binding protein (1/p27/retinoblastoma/cyclin A) signal pathway

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to promote cell cycle progression^{11,12}. The pre-S2 mutant-induced ER stress can also cause DNA damage, centrosome overduplication, and genomic instability^{13–15}. The transforming ability of pre-S2 mutant has been investigated in an immortalized human hepatocyte line HH4¹¹. In addition, transgenic mice carrying pre-S2 mutant can develop HCC¹⁶. These studies support that combined effects of the pre-S2 mutant-induced signal pathways lead to growth advantage of type II GGHs and eventually HCC development^{17,18}.

Our recent studies further reveal that the mammalian target of rapamycin (mTOR) signal pathway plays a critical role in pre-S2 mutant-driven tumorigenesis. Activation of mTOR signal pathways is consistently observed throughout the liver tumorigenesis in pre-S2 mutant transgenic mice and in human HCC tissues, leading to hepatocyte proliferation, metabolic disorders, and HCC tumorigenesis^{19–21}. This review summarizes our works focused on the pre-S2 mutant-induced mTOR signal pathways and its implications in HBV-related HCC tumorigenesis. We also propose potential therapeutic strategies targeted at pre-S2 mutant-induced mTOR signal pathways for HCC.

PRE-S2 MUTANT-INDUCED VEGF-A/VEGFR-2/ AKT/mTOR SIGNAL PATHWAY PROMOTES HEPATOCYTE PROLIFERATION

HBV infection can induce chronic inflammation and contribute to HCC formation through the expression of cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α)^{22,23}. HBx has been shown to alter cytokine expression to modulate the immune response and proliferation of hepatocytes^{24,25}. In transgenic mice of large surface antigen, the overproduction of large surface antigen can cause inflammation and regenerative hyperplasia to induce HCC development^{26,27}. Therefore, inflammatory cytokines or growth factors may play a role in the disease progression from a precursor lesion to HCC through activation of growth factor/receptor signaling involving phosphatidylinositol 3-kinase/protein kinase B (Akt)/mTOR or Ras/Raf-1/extracellular signal-regulated kinase (ERK)^{28,29}.

As expected, a human cytokine/growth factor antibody array reveals that the expression of pre-S2 mutant in HuH-7 hepatocytes enhances the secretion of several growth factors, including vascular endothelial growth factor (VEGF)-A and -D, transforming growth factor (TGF)- β 1 and - β 3, fibroblast growth factor (FGF)-7 and -9, and hepatocyte growth factor¹⁹. VEGF-A is selected for detailed study because of its angiogenesis and role in growth in the early stage lesions of human carcinogenesis^{30,31}. The increased level of transcription, protein expression, and secretion of VEGF-A in hepatocytes expressing pre-S2 mutant is confirmed by real-time polymerase chain reaction (RT-PCR), Western blotting, and

ELISA¹⁹, respectively. The expression of VEGF-A can be reduced by treatment with an ER stress inhibitor vomitoxin in hepatocytes expressing pre-S2 mutant¹⁹, indicating that pre-S2 mutant upregulates VEGF-A through ER stress. In addition, the enhanced proliferation of hepatocytes expressing pre-S2 mutant is significantly suppressed by the addition of neutralizing VEGF antibody to the culture supernatant¹⁹. The culture supernatant of VEGF-A from hepatocytes expressing pre-S2 mutant can increase human umbilical vein endothelial cell (HUVEC) proliferation¹⁹, suggesting that the pre-S2 mutant-upregulated VEGF-A promotes cell growth via an autocrine/paracrine loop.

To further study the mechanism of VEGF-A-induced hepatocyte proliferation, we first assess the expression of VEGF receptor (VEGFR)-1 and -2, which are reported to function in the regulation of VEGF-A signaling. The expression of VEGFR-2 mRNA and protein is upregulated in hepatocytes expressing pre-S2 mutant; however, the expression of VEGFR-1 is not increased¹⁹. Subsequently, we demonstrate an enhanced expression of the VEGF-A downstream signal molecules Akt, mTOR, and its phosphorylated (p) form (p-Akt, p-mTOR) in hepatocytes expressing pre-S2 mutant, which can be attenuated by VEGF-A neutralization¹⁹. Unexpectedly, another VEGF-A/VEGFR signal pathway, Raf-1 and ERK, shows no enhanced expression or activation. Collectively, these results demonstrate that pre-S2 mutant can activate an ER stress-dependent VEGF-A/VEGFR-2/Akt/mTOR signal pathway to promote hepatocyte proliferation (Fig. 1).

In order to evaluate the role of pre-S2 mutant-induced VEGF-A/Akt/mTOR signal pathway in HCC tumorigenesis, we established transgenic mice overexpressing pre-S2 mutant in their livers. The extent of expression of each biomarker is then evaluated by Western blotting and normalized with age-matched nontransgenic liver tissues. Upregulation of VEGF-A, p-Akt, and p-mTOR signals is detected at as early as 1 month of age; the upregulated VEGF-A signal then subsides at 6 months, followed by p-Akt1/2/3 at 12 months³². The p-mTOR signal is consistently upregulated at all time points³², indicating that mTOR is a key regulator of pre-S2 mutant-driven hepatocarcinogenesis. Unlike the sequential activation of the VEGF-A/Akt/mTOR signal cascade, no clear pattern is observed for Raf-1/ERK signals³². To validate the signal activation in transgenic mice, 58 pairs of HBV-related nontumorous and HCC tissues are studied for signal activation. In consonance with the data in the transgenic mouse model, p-mTOR signal is significantly upregulated in tumor tissues³², supporting the important role of mTOR signal in HBV tumorigenesis.

A previous study demonstrated for the first time that type II GGHs exhibit enhanced VEGF-A expression through ER stress induced by the accumulation of pre-S2

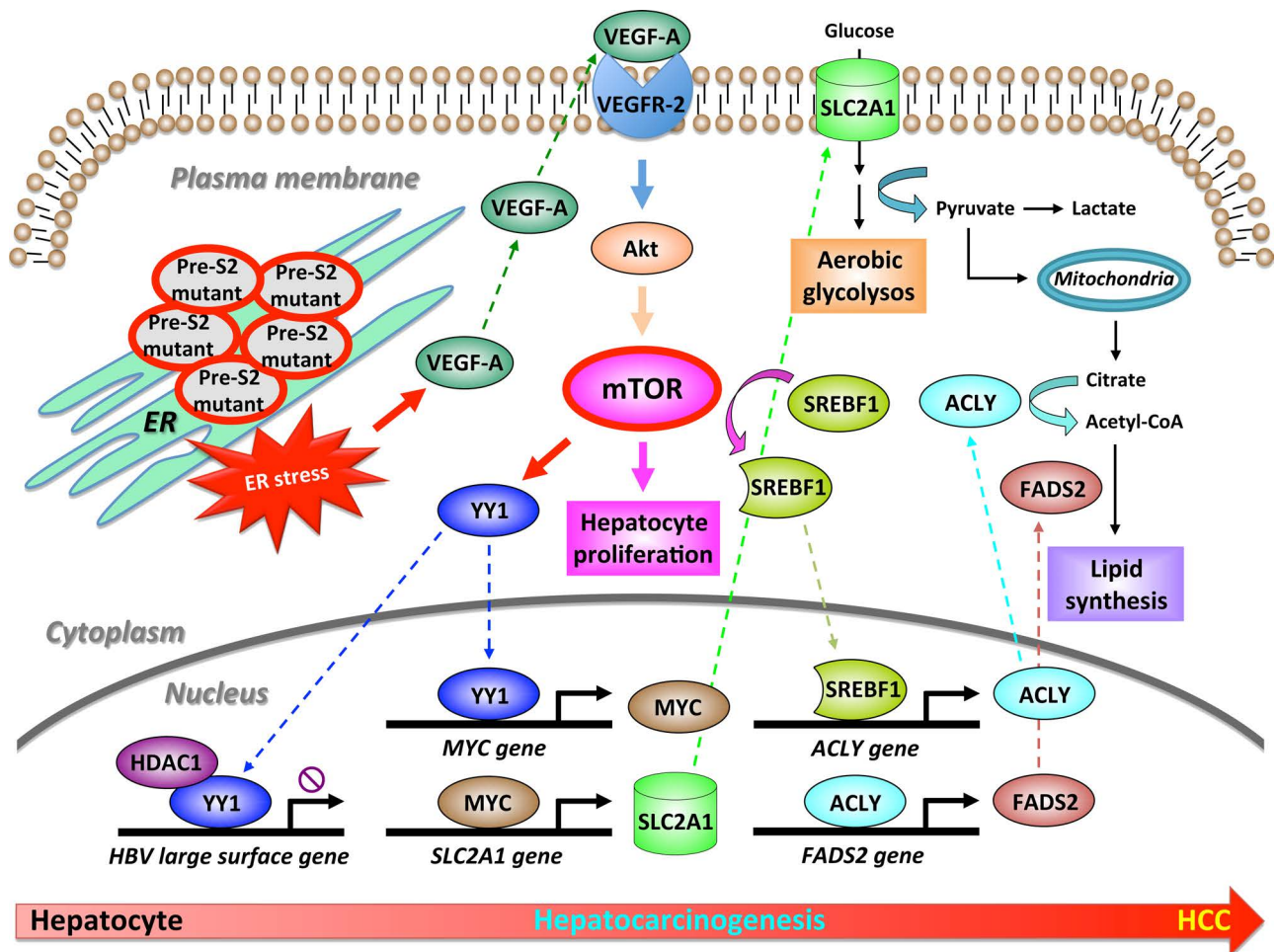


Figure 1. Schematic overview of pre-S2 mutant-induced mTOR signal pathways. In chronic HBV infection, pre-S2 mutant accumulates in the ER of hepatocytes and induces ER stress. Through the induction of ER stress, the pre-S2 mutant can upregulate VEGF-A. The upregulated VEGF-A then functions in an autocrine (or paracrine) manner mediated by VEGFR-2 to activate Akt and mTOR signals. The activated mTOR signal can promote hepatocyte proliferation directly or indirectly by initiating two metabolic pathways, one involving YY1/MYC/SLC2A1 to stimulate aerobic glycolysis and another involving SREBF1/ACLY/FADS2 to promote lipid biosynthesis. The pre-S2 mutant-induced mTOR signal can additionally feedback suppress HBV large surface gene expression through YY1 recruitment of HDAC1-mediated transcriptional repression. HBV, hepatitis B virus; ER, endoplasmic reticulum; VEGF-A, vascular endothelial growth factor-A; VEGFR-2, VEGF receptor-2; Akt, protein kinase B; mTOR, mammalian target of rapamycin; YY1, Yin Yang 1; SLC2A1, solute carrier family 2 (facilitated glucose transporter) member 1; SREBF1, sterol regulatory element-binding transcription factor 1; ACLY, adenosine triphosphate (ATP) citrate lyase; FADS2, fatty acid desaturase 2; HDAC1, histone deacetylase 1.

mutant in ER¹⁹. The enhanced expression and secretion of VEGF-A can activate Akt/mTOR signaling to promote HBV-related hepatocarcinogenesis through VEGFR-2, thereby providing a potential mechanism for the progression from a benign precursor lesion of GGHs to HCC, providing a potential target for chemoprevention in the high-risk group of patients with chronic HBV infection. In addition, HBV is shown to play a crucial role in modulating the accumulation and activation of both cellular components of the tumor microenvironment, such as immune cells and fibroblasts, and noncellular components of the tumor microenvironment, such as cytokines and growth factors, markedly influencing disease

progression and prognosis of HCC³³. The autocrine/paracrine effects of pre-S2 mutant-upregulated VEGF-A on hepatocyte and/or endothelial cell proliferation may provide a novel insight into the role of pre-S2 mutant in the tumor microenvironment of HCC.

PRE-S2 MUTANT-INDUCED mTOR/YY1/HDAC1 SIGNAL PATHWAY FEEDBACK INHIBITS LARGE SURFACE ANTIGEN SYNTHESIS

One intriguing observation in chronic HBV infection is the low detection rate of HBV surface antigen (HBsAg), usually below 20% of cases in human HCC tissues, whereas HBsAg can be detected in almost 100%

of cases in paired nontumorous livers³⁴. The same finding is observed in HBsAg-expressing transgenic mice, which are accompanied by a decreased or absent expression of HBsAg in transgenic HCCs³⁵. These observations indicate that the decreased HBsAg expression is a consistent phenomenon during the process of HBV-associated tumorigenesis. Although the levels of HBV DNA and HBsAg usually decline along with the natural course of chronic HBV infection^{36,37}, there exists such a possibility that host cell factors may become activated to inhibit HBsAg expression or HBV replication during HBV-related tumorigenesis.

This speculation gains support from our clinical observation that shows a significantly inverse relationship between decreased LHBs and enhanced p-mTOR expressions in 13 of 20 paired human HBV-related HCC tissues³⁸. A similar phenomenon is observed in HuH-7 hepatocytes expressing pre-S2 mutant, showing that the pre-S2 mutant-induced mTOR activation occurs at 48 h with concurrently decreased LHB RNA expression, followed by the decrease of LHB protein expression at 72 h after transfection³⁸. Blockage of mTOR activation by the mTOR inhibitor rapamycin or mTOR-specific RNA interference can restore both RNA and protein expression levels of LHBs in hepatocytes expressing pre-S2 mutant³⁸. Importantly, secreted LHBs in culture supernatant show the same patterns³⁸, implying that serum HBsAg level may be concurrently decreased when mTOR becomes activated during HBV tumorigenesis. Together, these results demonstrate that the pre-S2 mutant-induced mTOR activation feedback suppresses LHB expression and secretion.

To elucidate the mechanism of LHB suppression by mTOR activation, we first perform a luciferase reporter assay in hepatocytes expressing pre-S2 mutant to detect the luciferase activity driven by the pre-S1 promoter, which controls the transcription of the LHB gene. The pre-S1 promoter-driven luciferase activity is decreased in hepatocytes expressing pre-S2 mutant and can be restored by mTOR inhibitor or RNAi treatment³⁸, suggesting that mTOR activation represses pre-S1 promoter activity. Further assays with various deletion mutants of pre-S1 promoter reporter plasmids show that nucleotides 2,812–2,816 of the pre-S1 promoter is the minimal region for mediating mTOR signal-induced transcriptional repression³⁸. Subsequently, the 2,812–2,816 site of the pre-S1 promoter is confirmed as the binding site of the transcription factor Yin Yang 1 (YY1) by electrophoretic mobility shift assay and DNA affinity precipitation assay³⁸. Pre-S2 mutant-induced mTOR activation can enhance YY1 protein expression and nuclear localization³⁸. Accumulating evidence indicates that YY1 can execute transcriptional repression by complexing with corepressors, among which histone deacetylase 1 and 2 (HDAC1 and HDAC2) are the most relevant^{39,40}. Selective knockdown of HDAC1, but not HDAC2,

protects the pre-S1 promoter from repression by pre-S2 mutant-induced mTOR activation in hepatocytes. Moreover, the YY1 antibody can coimmunoprecipitate higher levels of HDAC1 from pre-S2 mutant-expressed cells than control cells in an mTOR-dependent manner³⁸, suggesting that HDAC1 is physically associated with YY1 and contributes to mTOR activation-induced transcriptional repression of LHBs (Fig. 1).

A previous study, for the first time, demonstrated one interesting negative feedback regulation of surface antigen synthesis by the activation of the mTOR signal during the progression of HBV tumorigenesis³⁸. The decreased levels of HBsAg and HBV DNA in serum or hepatocytes, therefore, may not necessarily represent a good sign of disease improvement during the natural course of HCC development, but instead it may indicate a disease progression toward tumorigenesis, especially at the advanced stage of the diseases. This finding, together with the detection of pre-S mutations in serum^{41–43}, should provide an additional hallmark to predict disease progression in the follow-up of patients with chronic HBV infection. In addition, several mTOR inhibitors have been developed at various phases of clinical trials⁴⁴. According to our findings in a previous study³⁸, targeting mTOR signaling for HBV-related HCC may potentially lead to HBV reactivation. There are increasing reports on the reactivation of HBV replication and hepatitis flare-up in HBV-related HCC patients receiving anticancer treatments^{45,46}. Our results provide an explanation for the untoward effect of mTOR inhibitors on HCC patients with chronic HBV infection and emphasize the necessity of combining antiviral agents when mTOR inhibitors are used for HBV-related HCC therapy.

PRE-S2 MUTANT-INDUCED mTOR/YY1/MYC/SLC2A1 SIGNAL PATHWAY INDUCES AEROBIC GLYCOLYSIS IN HEPATOCYTES

Metabolic changes are common features in the development of many types of human cancers⁴⁷. It has been reported that cancer cells frequently display high rates of aerobic glycolysis in comparison to their nontransformed counterparts, a phenomenon known as the “Warburg effect,” to support the increased demand of macromolecules for cell growth and proliferation⁴⁸. Recently, several reports have uncovered multiple metabolic changes in HCC, including elevated glycolysis, which is one of the principal changes linked to highly proliferative malignant phenotype^{49–51}. A previous study based on HBV transgenic mice has also consistently revealed a metabolic alteration of hepatocytes from the glycogen storage (glycogenotic) state toward an increase in glycolysis (the glycogen-poor state) during neoplastic transformation⁵². However, the underlying mechanism of HBV in the regulation of aerobic glycolysis in HCC development remains unclear.

mTOR is a highly conserved serine/threonine kinase that controls cell growth and proliferation⁵³. In addition to its better known functions in promoting protein synthesis, mTOR is now emerging as a key regulator of cellular metabolism and cancer⁵⁴. Research has documented that mTOR activation is sufficient to stimulate specific metabolic pathways, including aerobic glycolysis⁵⁵. Our studies have demonstrated that the pre-S2 mutant can activate mTOR through the induction of ER stress-dependent VEGF-A/VEGFR-2/Akt signaling in GGHs to promote tumorigenesis¹⁹. The activated mTOR signal can further upregulate YY1³⁸, a transcription factor involved in cell proliferation and regulation of oncogenes⁵⁶. We thus propose that pre-S2 mutant-induced mTOR activation may regulate aerobic glycolysis through YY1 signal cascade in HBV-related tumorigenesis.

In supporting our notion, the pre-S2 mutant transgenic mice exhibit glycogen depletion in HCC tissues as determined by periodic acid–Schiff staining and colorimetric-based assay²⁰. The cDNA microarray data of pre-S2 mutant transgenic livers were adopted to identify the YY1-activated oncogenes. RT-PCR was performed to confirm the selected genes' transcription levels. The transcription factor MYC was identified as the only gene showing significant upregulation (≥ 1.5 -fold) in transgenic HCCs relative to the nontransgenic livers²⁰. By further analysis of the microarray data for potential MYC-activated glycolytic genes, the expression of the solute carrier family 2 (facilitated glucose transporter) member 1 (SLC2A1) was found significantly increased in transgenic HCCs²⁰. These observations led us to test whether the pre-S2 mutant may regulate tumor glycolysis through activation of the mTOR/YY1/MYC/SLC2A1 signal cascade. Indeed, Western blot analysis of pre-S2 mutant transgenic mice livers showed that the expression levels of p-mTOR and YY1 were significantly elevated at as early as 1 month of age and persistently activated throughout the study period, while MYC and SLC2A1 expression was upregulated only upon tumor formation²⁰. Moreover, the mTOR/YY1/MYC/SLC2A1 signal cascade was induced in HuH-7 and HepG2 hepatocytes expressing the pre-S2 mutant; selective knockdown of the upstream activators by RNA interference can sequentially diminish the activation of downstream targets²⁰. The pre-S2 mutant-induced mTOR signal cascade can promote SLC2A1 translocation to the cell surface, where it functions in mediating cellular glucose uptake, resulting in stimulation of aerobic glycolysis and ultimately hepatocyte proliferation²⁰ (Fig. 1).

To ascertain the association of the mTOR/YY1/MYC/SLC2A1 signal pathway with human HBV-related hepatocarcinogenesis, Western blot analysis was performed on 30 pairs of HBV-related HCCs and adjacent nontumorous livers for the expression of each biomarker. The p-mTOR, YY1, MYC, and SLC2A1 signals were consistently

and significantly expressed at higher levels in HCCs than in the paired nontumorous livers in 18 of 30 tissue pairs²⁰, supporting the essential role of the mTOR/YY1/MYC/SLC2A1 signal pathway in HBV tumorigenesis. Furthermore, in the model of chemopreventive study in transgenic mice harboring both pre-S2 mutant and HBx, the combined resveratrol and silymarin product can significantly decrease the transgenic tumor size compared with the untreated group⁵⁷. In a previous study conducted in our laboratory, we observed a lower expression level of the mTOR/YY1/MYC/SLC2A1 signaling pathway in the treated group than in the untreated group²⁰, suggesting that the mTOR/YY1/MYC/SLC2A1 signaling pathway mediates the chemopreventive effect of combined resveratrol and silymarin product on tumor growth.

This study, for the first time, demonstrates the contributing role of pre-S2 mutant in metabolic disturbances of HBV-related HCC development. The pre-S2 mutant can stimulate aerobic glycolysis through the activation of the mTOR/YY1/MYC signaling to upregulate SLC2A1. Upon the activation of SLC2A1 at the advanced stage of tumorigenesis, hepatocytes undergo a metabolic switch from the glycogen-storage state toward increased aerobic glycolysis. The increased SLC2A1 expression in HCC not only indicates an increased utilization of energy but can also directly cause tumorigenesis⁵⁸. Therefore, our findings suggest that the pre-S2 mutant may promote tumorigenesis by sustaining high activation rates of aerobic glycolysis through the mTOR signal cascade.

PRE-S2 MUTANT-INDUCED mTOR/SREBF1/ACLY/FADS2 SIGNAL PATHWAY DISTURBS LIPID METABOLISM IN HEPATOCYTES

Growing evidence indicates that cancer cells show specific alterations in lipid metabolisms that are important for cell growth and proliferation^{59,60}. HCC has also been linked to nonalcoholic fatty liver, obesity, and related metabolic disorders⁶¹. Numerous reports uncover aberrant lipidomic profiles in human HCCs and mouse HCC models^{62,63}. Furthermore, aberrations of lipid metabolism often are seen in chronic HBV infection⁶⁴. However, the contributing role of disturbed lipid biosynthesis in HBV tumorigenesis remains to be clarified.

To elucidate the role of lipid metabolism in HBV tumorigenesis, we investigated the dynamic pattern of lipid metabolism in pre-S2 mutant-induced tumorigenesis. We first analyzed lipid profiles in transgenic mice harboring pre-S2 mutant. By Oil red O staining, mild and diffused fatty change in hepatocytes was observed in 1-month-old transgenic mice compared to the age-matched nontransgenic mice²¹. The staining intensity weakens as the disease progresses and eventually disappears in the later stages (6 and 12 months)²¹. Remarkably, large amounts of enlarged lipid droplets were found accumulated

in transgenic tumors rather than the surrounding non-tumors²¹. Furthermore, the levels of triglycerides and cholesterol in transgenic livers were measured by colorimetric-based assays and showed consistency with the accumulation pattern found in the Oil red O staining²¹. These results indicate that lipid metabolism is disturbed in pre-S2 mutant-induced tumorigenesis.

It is now becoming clear that mTOR promotes *de novo* lipogenesis by inducing the cleavage of the sterol regulatory element-binding transcription factor 1 (SREBF1), which then translocates to the nucleus and induces the expression of many lipogenesis-related genes^{65,66}. Considering that the pre-S2 mutant can induce reprogramming of glucose metabolism for hepatocyte proliferation through activation of mTOR signal pathway²⁰, it is reasonable to investigate whether the pre-S2 mutant may also promote lipid biosynthesis through mTOR activation during the process of HBV tumorigenesis. The cDNA microarray data of pre-S2 mutant transgenic livers were adopted to identify the candidates of SREBF1 target lipogenic genes. The adenosine triphosphate (ATP) citrate lyase (ACLY) was the only gene that showed significant upregulation (≥ 1.5 -fold) in transgenic HCCs compared with the non-transgenic livers²¹. This finding leads us to hypothesize that the pre-S2 mutant may regulate lipid metabolism through the activation of the mTOR/SREBF1/ACLY signal cascade. To test this hypothesis, Western blot analysis was performed to examine the expression of the signal molecules in different stages of pre-S2 mutant transgenic livers. As previously observed, the expression of p-mTOR was significantly elevated throughout the study period²¹. Interestingly, the precursor form of SREBF1 was upregulated in the middle to the late stage, 6 and 12 months, while the cleaved nuclear form of SREBF1 was found increased only upon tumor formation²¹. Both total and phosphorylated ACLY exhibit biphasic overexpression at the early and tumor stages²¹. Moreover, we demonstrate that the pre-S2 mutant can activate ACLY through mTOR/SREBF1 signaling to promote *de novo* lipogenesis and cell proliferation in HuH-7 and HepG2 hepatocytes²¹ (Fig. 1). The pre-S2 mutant can additionally upregulate the lipogenic enzyme fatty acid desaturase 2 (FADS2) through ACLY-dependent histone acetylation in hepatocytes²¹. FADS2 promoter-driven luciferase activities are increased in hepatocytes expressing the pre-S2 mutant; this upregulation can be abrogated by RNA interference-mediated ACLY inhibition and restored by treatment with the histone deacetylase inhibitor trichostatin A²¹. The essential role of the mTOR/SREBF1/ACLY/FADS2 signal pathway in human HBV-related tumorigenesis is further validated in 30 chronic HBV-infected HCC patients, showing a significantly increased expression of the signal pathway in 20 of 30 HCC tissues compared to the paired nontumorous liver tissues²¹.

This study, together with the role of the pre-S2 mutant in regulation of glucose metabolism, proposes a novel molecular mechanism to explain the pre-S2 mutant-induced metabolic disturbances in HBV-related tumorigenesis²¹. In chronic HBV infection, the pre-S2 mutant can stimulate aerobic glycolysis through an ER stress-dependent mTOR/YY1/MYC/SLC2A1 signal pathway. The aerobic glycolysis by-product, citrate, can be converted into acetyl-CoA, the raw material of triglycerides and cholesterol, by catalysis of ACLY, which is upregulated by another pre-S2 mutant-induced mTOR/SREBF1 signal pathway. Thus, ACLY plays an important role in linking the glucose metabolism to the endogenous biosynthesis of triglycerides and cholesterol at the advanced stage of tumorigenesis. Our findings suggest that the pre-S2 mutant plays a role in HBV tumorigenesis by disturbing normal metabolism, including elevating glycolysis and promoting lipid biosynthesis through the mTOR signal cascade. Targeting the glycolytic mTOR/YY1/MYC/SLC2A1 and the lipogenic mTOR/SREBF1/ACLY/FADS2 signal pathways may be a promising therapeutic strategy for HCC therapy.

PRE-S2 MUTANT-INDUCED mTOR SIGNAL PATHWAYS REPRESENT POTENTIAL THERAPEUTIC TARGETS FOR HCC

Oral nucleos(t)ide analogs (NAs), including lamivudine, adefovir, entecavir, telbivudine, and tenofovir, are currently used to treat chronic HBV infection⁶⁷. Early observations reveal that NA treatment not only significantly reduces the incidence of HCC⁶⁸ but also lowers HCC recurrence rate⁶⁹. Recently, a study revealed the resistance of pre-S2 mutant to NAs, providing an explanation for the high proportion of patients who still suffer from HCC recurrence after surgery despite anti-HBV therapy⁷⁰. This highlights the importance of target therapies against pre-S2 mutant-induced signaling pathways that should be taken into consideration.

Resveratrol and silymarin are naturally occurring compounds that have anticarcinogenic effects on various types of cancers^{71,72}. In the model of a chemopreventive study in transgenic mice harboring both pre-S2 mutant and HBx, the combined resveratrol and silymarin product has been shown to significantly ameliorate fatty liver and inhibit HCC growth⁵⁷. Western blot analysis performed on the expression profiles of the mTOR signal cascade in the tumor adjacent tissues reveals that the treated group expresses a lower level of mTOR/YY1/MYC/SLC2A1 signaling than the untreated group²⁰. We further examine the *in vitro* effect of resveratrol and silymarin on mTOR/YY1/MYC/SLC2A1 signaling cascade in HBV tumorigenesis. Consistent with pre-S2 mutant alone, HuH-7 and HepG2 hepatocytes expressing both pre-S2 mutant and HBx show activation of the mTOR signal cascade,

which can be inhibited by the combined resveratrol and silymarin treatment²⁰. These results indicate that the combined resveratrol and silymarin product may represent a potential agent for the prevention or therapy of HCC in high-risk chronic HBV carriers.

The bi-aryl urea sorafenib is an oral multikinase inhibitor that inhibits cell surface tyrosine kinase receptors including VEGFR and downstream intracellular serine/threonine kinases such as Raf-1⁷³. Sorafenib is the only approved therapy for advanced HCC⁷⁴. The principal mechanism of sorafenib in action is that it competitively inhibits ATP binding to the catalytic domains of the respective kinases⁷⁵. Several VEGF/VEGFR-based therapies are developed on the basis of different strategies including small molecular inhibition (e.g., sunitinib, Zactima, vatalanib) and antiligand targeting (e.g., bevacizumab and VEGF trap)²⁹. As the name indicates, the primary target of VEGF/VEGFR-based therapy is focusing on the antiangiogenesis effects. The pre-S2 mutant induced ER stress-dependent VEGF-A/VEGFR-2/Akt/mTOR signal pathway, and the downstream mTOR signaling involved in hepatocarcinogenesis could also be neutralized by severing the source of VEGF-A/VEGFR signal cascade under sorafenib treatment.

Given mTOR involvement in many forms of cancers, it is a favorable target in cancer therapy^{76,77}. Everolimus is a rapamycin analog, an oral mTOR inhibitor that binds to the FK506-binding protein 12 and directly interacts with mTOR complex 1 (mTORC1), inhibiting its downstream signaling⁷⁸. However, mTOR inhibition induces insulin receptor substrate-1 expression and abrogates feedback inhibition of the pathway, resulting in Akt activation, which weakens its therapeutic effects⁷⁹. One report suggests that targeting Akt and ERK signaling combined with rapamycin-based therapeutic approaches may be a new strategy for enhancing the treatment efficacy and inhibition of mTORC1 results in activation of Akt and ERK, but inhibiting mTORC2 leads to Akt and ERK suppression⁸⁰. Moreover, a study shows that inhibition of mTORC2 induces glycogen synthase kinase 3-dependent and F-box and WD repeat domain-containing 7-mediated proteasomal degradation of SREBF1 and suppresses lipogenesis in cancer cells⁸¹. These results indicate that an mTOR inhibitor that inhibits both mTORC1 and mTORC2 could be the next generation of mTOR signaling-based target therapy.

The pre-S2 mutant induces mTOR activation and recruits the YY1-HDAC1 complex to feedback suppress transcription from the pre-S1 promoter³⁸. Inhibition of mTOR can result in HBV reactivation, and it is demonstrated in a phase I clinical trial on everolimus that HBV reactivation is observed in four HBsAg-seropositive patients and indicates that prophylactic antiviral therapy should be mandatory for such patients⁸². One of The

European Association for the Study of the Liver (EASL) 2012 guidelines recommend that HBV⁺ candidates for chemotherapy and immunosuppressive therapy should be tested for HBV DNA levels and should receive pre-emptive NA administration during therapy (regardless of HBV DNA levels) and for 12 months after cessation of therapy (A1).

In addition, several groups have demonstrated that HBsAg gene-modified dendritic cell-based vaccine can induce a specific immune response and strong antitumor effects in vitro or in mouse model^{83–85}. Considering that hepatocytes harboring pre-S2 mutant exhibit stronger growth advantage than hepatocytes expressing HBsAg and that HBsAg expression frequently decreases in HCCs possibly due to the negative regulation by the pre-S2 mutant, it is reasonable to speculate that the pre-S2 mutant may be a more powerful antigen than HBsAg for developing a dendritic cell-based vaccine for HCC therapy.

It is a critical issue that viral oncoproteins can be continuously produced in the liver for years under NA treatment, presumably using cccDNA or integrated HBV genome as the template^{86,87}. Distinct from oral NA, pegylated interferon (Peg-IFN) has been shown to effectively suppress cccDNA and reduce serum HBsAg level^{87,88}. Moreover, lymphotoxin- β receptor (LT- β R) and downstream APOBEC3 cytidine deaminase pathway has also been proposed to be an alternative mechanism to specifically degrade cccDNA⁸⁹. Therefore, combining NAs and drugs that degrade cccDNA, such as Peg-IFN and LT β R activator, to accelerate pre-S2 mutant elimination and reduce HCC development or recurrence is worth further investigation. In addition, label-retaining cancer cells are recently described as a novel subpopulation of HCC-derived cancer stem cells and display a high resistance to sorafenib treatment⁹⁰. This phenomenon also highlights the importance of the development of novel cancer therapeutic drugs and/or combined therapeutic strategies for HCC.

CONCLUSION

Clinically, the prevalence of pre-S2 mutant is 37% in chronic HBV carriers and as high as 60% in HBV-related HCC patients¹⁷. The presence of pre-S2 mutant exhibits a high resistance to antiviral treatment⁷⁰ and carries a high risk of HCC development^{41,42} in patients with chronic HBV infection. In this review, we highlight that mTOR dysregulation occurs with liver tumorigenesis in pre-S2 mutant transgenic mice and in human HCC tissues and represents the most important molecular mechanism initiated by pre-S2 mutant. Targeting at pre-S2 mutant-induced mTOR signal pathways may thus provide potential strategies for the prevention or therapy of HBV-associated HCC.

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