Hepatitis B Virus Large Surface Antigen Promotes Liver Carcinogenesis by Activating the Src/PI3K/Akt Pathway

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Abstract

Of the three envelope glycoproteins encoded by hepatitis B virus (HBV) that are collectively referred to as HBV surface antigen (HBsAg), the large HBsAg (LHBs) glycoprotein is expressed preferentially in HBV-associated hepatocellular carcinoma. LHBs can act as an oncogene in transgenic mice, but how it contributes functionally to hepatocarcinogenesis remains unclear. In this study, we determined the molecular and functional roles of LHBs during HBV-associated hepatocarcinogenesis. LHBs increased tumor formation of hepatoma cells. Moreover, expression of LHBs but not other HBV envelope glycoproteins specifically promoted proliferation of hepatoma and hepatic cells in vitro. Mechanistic investigations revealed that these effects were caused by activation of the Src/PI3K/Akt pathway through proximal stimulation of PKCθ/Raf1 signaling by LHBs. Proliferation induced by stable LHBs expression was associated with increased G1–S cell-cycle progression and apoptosis resistance mediated by Src kinase activation, as established in hepatocellular carcinoma clinical specimens. Importantly, LHBs-induced cellular proliferation and tumor formation were reversed by administration of the Src inhibitor saracatinib. Together, our findings suggest that LHBs promotes tumorigenesis of hepatoma cells by triggering a PKCθ/Raf1 to Src/PI3K/Akt signaling pathway, revealing novel insights into the underlying mechanisms of HBV-associated hepatocarcinogenesis. Cancer Res; 71(24); 7547–57. ©2011 AACR.

Introduction

More than 350 million people worldwide are chronically infected with hepatitis B virus (HBV), which is a leading cause for chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC; refs. 1, 2). Although chronic HBV infection has been noted to be associated with the development of HCC soon after the discovery of HBV surface antigen (HBsAg), the oncogenic functions of HBV are not completely known, partly because of its noncytopathic characteristic (3). Progression from chronic hepatitis B to HCC involves direct effects of the virus protein on the cellular function of infected hepatocytes, as well as indirect effects through the process of inflammation, regeneration, and cirrhosis because of HBV infection (4,5). Regarding the effect of viral proteins expression on hepatocellular malignant transformation, several HBV proteins have been found in infected tissues more frequently than others, including large HBsAg (LHBs), C-terminally truncated middle HBsAg (MHBs), HBV X protein (HBx), and a novel spliced transcript of HBV, referred to as hepatitis B–spliced protein (HBSP; ref. 6).

The viral genome of HBV encodes for 4 overlapping open reading frames, which include preS1/preS2/S, preC/C, X, and P (2). The preS1/preS2/S region of the virus genome encodes the 3 viral surface antigens by differential initiation of translation at each of 3 in-frame initiation codons (7). Initiation at the nearest start codon (S) produces the small HBsAg (SHBs), which is the most abundant protein in hepatitis B patients. Initiation at the more upstream start codon (preS2) generates the MHBs, the function of which is still unknown. Initiation at the most upstream start codon (preS1) yields the LHBs, which is thought to play pivotal roles in binding of the virus to host cell receptors and in the assembly of the virion and its release from the cell (7,8).

Previous study has shown that HBV sequences introduced into transgenic mice are able to confer only a tissue-specific expression of HBsAg rather than any other HBV proteins (9). Chisari and colleagues have shown that HBV transgenic mice that overproduce LHBs and accumulate toxic quantities of SHBs within the hepatocytes develop severe, prolonged hepatocellular injury that initiates a programmed response with the liver, characterized by inflammation, regenerative hyperplasia, transcriptional deregulation, aneuploidy, and eventually progresses to neoplasia (10). Another study has also proved that
HBsAg-positive ground-glass hepatocytes emerged throughout the liver parenchyma in nearly all HBV transgenic mice, but the extensive expression of HBsAg is gradually downregulated during neoplastic transformation, just as the morphologic and biochemical phenotypes of foci of altered hepatocytes, hepatic adenoma, and HCC in transgenic mice, resembling those described in chemical hepatocarcinogenesis (11). Although the critical indirect roles of LHBs during malignant transformation in transgenic mice have been observed previously, the direct effects of LHBs on hepatocellular function remain poorly understood.

Extensive studies over past years have identified aberrant activation of major signaling cascades such as PI3K/Akt/mTOR pathway, Raf/MEK/ERK pathway, WNT/β-catenin pathway, and HGF/c-MET pathway involved in pathogenesis of HCC (12–15). Abrupt alterations that occur in liver tissues with HBV infection cause significant changes in several cellular signaling including WNT/β-catenin, p53, pRb, mitogen—activated protein kinase (MAPK), NF-κB pathways and alter gene expression resulting in hepatoma formation because of increased proliferation, cell-cycle progression, and apoptosis resistance (5). As a dominant nonreceptor tyrosine kinase activated in HCC carcinogenesis, abnormal Src signaling activation conferred by HBx expression has been reported to participate in HBV-associated hepatocarcinogenesis (16–18). However, the interaction between Src signal activation with LHbs expression during HBV infection has not been characterized.

In this study, we sought to determine the direct oncogenic function of LHbs expression in HBV-associated hepatocarcinogenesis. Our present investigation reveals that LHbs expression promotes tumorigenesis of hepatoma cells dependent on PKCα/Raf1/Src/PI3K/Akt signal activation in vitro and in vivo, which may shed a new light into the molecular mechanisms underlying HBV-associated hepatocarcinogenesis and provide a promising therapeutic target for patients with HCC with chronic HBV infection.

Materials and Methods

Cell culture and human HCC samples

One immortalized hepatic cell line (L02) and 2 hepatoma cell lines (Huh7 and SK-Hep1), in addition to an African green monkey kidney epithelial cell line (Vero), were obtained directly from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco’s Modified Eagle’s Medium supplemented with 10% FBS at 37°C in a humidified 5% CO2 incubator. The cell lines have been characterized at the time of cell bank by DNA fingerprinting analysis using STR (short tandem repeat) markers. All cell lines were placed under cryostage after being frozen with dimethyl sulfoxide (DMSO) for 5 mg/mL additive stock solution. Saracatinib gavage solution or vehicle control gavage solution was prepared freshly by combination saracatinib additive stock solution or DMSO with corn oil at a ratio of 95% corn oil: 5% DMSO. Subcutaneous tumor xenografted nude mice were fed with saracatinib gavage solution or vehicle control gavage solution daily at a dose of 25 mg/kg body weight in vehicle corn oil via oral gavage for 4 weeks as hepatoma cells subcutaneous injection.

Histology, immunohistochemical analysis, and evaluation

Tumor sections from subcutaneous tumor xenografted nude mice and patients with HCC were stained and immunohistochemically analyzed as described previously (24, 25). Primary antibodies used included those against LHbs, Ki67 (Millipore), pAkt(S473), and pSrc

Construction of plasmids

The plasmids containing LHbs (pcDNA3-LHBs-flag), MHbs (pcDNA3-MHbs-flag), SHbs (pcDNA3-SHbs-flag), Akt (pcDNA3.1-Akt), and Raf1 (pcDNA3.1-Raf1) were generated as previously described (19–22). The dominant-negative mutant Raf1-S621A containing a serine-to-alanine mutation at amino acid 621 was constructed on the basis of aforementioned Raf1 expression plasmid. The plasmid containing Src (pcDNA3.1-Src) was a generous gift from Dr. Jianguo Gu (Tohoku Pharmaceutical University, Miyagi, Japan). The kinase dead mutant Src-K295M plasmid and dominant-negative mutant Akt-K179M plasmid were kindly provided by Dr. Mien-Chie Hung (MD Anderson Cancer Center, Houston, TX). All plasmid constructs were confirmed by DNA sequencing.

Plasmids transfection and RNA interference

Transient and stable transfections with various plasmids were carried out as previously described (23). Two siRNAs against SRC gene Src siRNA (h), 2 siRNAs against AKT1/2 gene Akt1/2 siRNA (h), 2 siRNAs against PKCζ gene PKCζ siRNA (h), 2 siRNAs against RAF1 gene Raf1 siRNA (h), and corresponding control siRNA-A (Santa Cruz Biotechnology) were transfected into Huh7 and SK-Hep1 cells in 6-well plates using the X-tremeGENE siRNA Transfection Reagent (Roche Applied Science) according to the manufacturer’s instructions. Gene silencing effect was confirmed by Western blot analysis and RT-PCR at 72 hours posttransfection.

Western blotting

Protein extraction from cultured cells or tumor tissues and Western blot analysis were carried out as previously described (23). Primary antibodies used included those against LHbs, GAPDH (Santa Cruz Biotechnology), Akt, pAkt(S473), Src, pSrc (Y416), Raf1, pRaf1(S338), PKCζ, cyclin D1, cyclin D3, CDK4, CDK6, cleaved caspase-3 (Cell Signaling Technology), and proliferating cell nuclear antigen (PCNA; BD Biosciences).

Tumor xenograft experiments and saracatinib (AZD0530) treatment

Tumor xenograft experiments in nude mice were carried out as previously described (23). The specific Src inhibitor saracatinib (Selleck) was dissolved with dimethyl sulfoxide (DMSO) for 5 mg/mL additive stock solution. Saracatinib gavage solution or vehicle control gavage solution was prepared freshly by combination saracatinib additive stock solution or DMSO with corn oil at a ratio of 95% corn oil: 5% DMSO. Subcutaneous tumor xenografted nude mice were fed with saracatinib gavage solution or vehicle control gavage solution daily at a dose of 25 mg/kg body weight in vehicle corn oil via oral gavage for 4 weeks as hepatoma cells subcutaneous injection.
The intensity of immunohistochemical staining in the tumor cells was scored independently by 2 pathologists using the semiquantitative IRS (immunoreactive score) scale according to Remmele and Stegner (26).

Colony formation assay, cell proliferation assay, bromodeoxyuridine incorporation assay, cell-cycle analysis, and Annexin V/PI staining

Colony formation assay, cell proliferation assay, bromodeoxyuridine (BrdUrd) incorporation assay, and cell-cycle analysis were carried out as previously described (23). Annexin V/propidium iodide (PI) staining was carried out by using the Annexin V–FITC Apoptosis Detection Kit (BD Biosciences) according to the manufacturer's instructions.

Statistical analysis

Experimental data were presented as mean ± SD or SEM of at least 3 independent replicates through analyzing with GraphPad Prism 5 (GraphPad Software) and assessing comparisons between different groups by the Student t test, one-way ANOVA. The association between LHBs and clinicopathologic characteristics was assessed using the Fisher exact test. The correlation between LHBs with Ki67, pSrc(Y416), and pAkt (S473) staining obtained by immunohistochemistry was determined using the Spearman correlation test. Differences were considered significant at values of $P < 0.05$.

Results

LHBs expression promotes tumor formation of hepatoma cells in vivo and in vitro

To elucidate the effect of LHBs expression on tumor formation in vivo, tumor xenograft experiments were carried out in nude mice with Huh7 cells stably transfected with LHBs. As shown in Fig. 1A, stable LHBs expression in clones 1 and 2 compared with control Huh7 cells was confirmed by Western blot analysis, and Huh7-LHBs clone 2 was selected for further investigation because of higher stable LHBs expression. Tumor xenograft experiments showed that LHBs expression significantly accelerated overall tumor growth compared with the control group as assessed by the tumor volume (Fig. 1B). Four weeks after tumor xenograft, nude mice were sacrificed and subcutaneous tumor tissues were examined. Notably, LHBs stably expressing Huh7 cells showed increased tumor growth compared with control cells as determined through tumor photography and weight measurement (Fig. 1C and D). H&E staining showed more aggressive tumor growth gained by LHBs stably transfected Huh7 cells than control cells in nude mice (Fig. 1E). Because the initiating ATG codons for the MHBs and SHBs have been mutated to ACG in the plasmid containing LHBs, aforementioned provocative tumor formation should be attributed to LHBs expression but not MHBs or SHBs expression. To
further assess the oncogenic effect of LHBs, MHBs, and SHBs expression on hepatocarcinogenesis, colony formation assay and cell proliferation assay were carried out in Huh7, SK-Hep1, and L02 cells after transiently transfection with LHBs, MHBs, and SHBs. LHBs expression, but not MHBs or SHBs expression were found to significantly promote both colony formation (Fig. 2A–C) and cellular proliferation (Fig. 2D) of Huh7, SK-Hep1, and L02 cells. In contrast, Vero cells undergoing identical experiments did not show increased colony formation despite LHBs expression (data not shown). Taken together, these data show that LHBs expression could promote tumor formation of hepatoma cells in vivo and in vitro.

Figure 2. LHBs expression rather than MHBs or SHBs expression exerts oncogenic promotion in hepatic cells. A–C, top, colony formation assay for Huh7 cells (A), SK-Hep1 cells (B), and L02 cells (C) transiently transfected with empty vector (control), LHBs plasmid (LHBs), MHBs plasmid (MHBs), and SHBs plasmid (SHBs), respectively. Bottom, quantification of colonies. ∗, P < 0.05 compared with control. NS, not significant. D, cell proliferation assay for Huh7 cells (left), SK-Hep1 cells (middle), and L02 cells (right) transiently transfected with empty vector (control), LHBs plasmid (LHBs), MHBs plasmid (MHBs), and SHBs plasmid (SHBs), respectively. ∗, P < 0.05 compared with control. E and F, cell proliferation assay for Huh7 (E) and SK-Hep1 cells (F) transiently transfected with empty vector (control) and LHBs plasmid (LHBs), respectively, after treatment for 24 hours with DMSO, LY294002 (50 μmol/L), Wortmannin (10 nmol/L), PD098059 (50 μmol/L), U0126 (20 μmol/L), SB203580 (10 μmol/L), PDTC (10 μmol/L), BAY117082 (10 μmol/L), PP2 (10 μmol/L), or rapamycin (10 nmol/L). ∗, P < 0.05.
Moreover, Western blot analysis showed that the phosphorylation in LHBs-Huh7 and LHBs-SK-Hep1 cells (Fig. 2E and F). Rapamycin, and PP2 treatment compared with DMSO treatment in LHBs-SK-Hep1 cells. However, a considerable inhibition of the phosphorylation compared with DMSO treatment in LHBs-Huh7 and after PD98059, U0126, SB203580, PDTC, and BAY117082 treatment.

p38/MAPK (SB203580), NF-κB (IκBα), JNK (C3), extracellular signal regulated kinase (ERK; PD98059), MAP/ERK kinase (MEK; U0126), protein kinase B (Akt; Akt1), and glycogen synthase kinase 3β (GSK3β) were measured. Akt signals in hepatoma cells. Consistent with aforementioned phenomenon in vitro, Western blot analysis of subcutaneous tumor tissues also showed increased pSrc(Y416) and pAkt(S473) levels in 3 intersected tumor tissues generated by Huh7-LHBs cells compared with control Huh7 cells from xenografted nude mice (Supplementary Fig. S1). All these data might involve in instigated tumor formation conferred by LHBs expression.

LHBs expression activates PKCα/ Raf1/Src/PI3K/Akt signal pathway in hepatoma cells

To characterize the mechanistic signaling pathway underlying instigated tumor formation conferred by LHBs expression in hepatoma cells, cell proliferation assay was carried out in LHBs expressing Huh7 and SK-Hep1 cells after treatment with small molecular inhibitors against phosphoinositide 3-kinase (PI3K; LY294002, Wortmannin), extracellular signal-regulated kinase (ERK; PD98059), MAP/ERK kinase (MEK; U0126), p38/MAPK (SB203580), NF-κB (PDTC, BAY117082), Src (PP2), and mTOR (rapamycin). As shown in Fig. 2E and F, no significant change of the proliferation ratio was observed after PD98059, U0126, SB203580, PDTC, and BAY117082 treatment compared with DMSO treatment in LHBs-Huh7 and LHBs-SK-Hep1 cells. However, a considerable inhibition of the proliferation ratio was noted after LY294002, Wortmannin, rapamycin, and PP2 treatment compared with DMSO treatment in LHBs-Huh7 and LHBs-SK-Hep1 cells (Fig. 2E and F). Moreover, Western blot analysis showed that the phosphorylation level of Src(Y416) and Akt(S473) were much higher in LHBs-Huh7 cells with respect to the control Huh7 cells (Fig. 3A), indicating LHBs expression could activate Src and Akt signals in hepatoma cells. Consistent with aforementioned phenomenon in vitro, Western blot analysis of subcutaneous tumor tissues also showed increased pSrc(Y416) and pAkt(S473) levels in 3 intersected tumor tissues generated by Huh7-LHBs cells compared with control Huh7 cells from xenografted nude mice (Supplementary Fig. S1). All these data indicate that both PI3K/Akt/mTOR and Src signaling pathways might involve in instigated tumor formation conferred by LHBs expression.

To further illuminate the regulatory relationship between Src and PI3K/Akt signals activation induced by LHBs expression, Western blot analysis was carried out in Huh7 cells after Src and PI3K/Akt signal inhibition. To assess RNA interference knockdown effect on Src and Akt signals activation, 2 siRNAs against Src and 2 siRNAs against Akt1/2 were prepared and transfected into Huh7 cells to evaluate their knockdown
efficiency by using RT-PCR and Western blot analysis (Supplementary Fig. S2A and S2B). Interestingly, specific Src inhibitor PP2 treatment or Src siRNA cotransfection reversed increased phosphorylation level of Akt(S473) by LHBs transfection in Huh7 cells, suggesting LHBs might promote PI3K/Akt activation through Src signal pathway (Fig. 3A and C). To clarify the functional role of Src tyrosine kinase activation in LHBs-induced Akt activation, kinase dead mutant Src K295M plasmid cotransfection was used to competing endogenous Src tyrosine kinase activity. Inhibition of Src tyrosine kinase activation mediated by Src K295M plasmid transfection could dramatically reverse upregulated phosphorylation level of Akt(S473) by LHBs expression in Huh7 cells (Fig. 3B). However, blockade PI3K activity with its specific inhibitor LY294002, which could significantly downregulate the increased Akt(S473) phosphorylation level, had no effect on enhanced Src(Y416) phosphorylation level induced by LHBs expression (Fig. 3D). Consistent with the aforementioned phenomenon, both inhibition of endogenic Akt expression with Akt siRNA transfection and prohibition endogenic Akt kinase activity with dominant-negative mutant Akt K179M plasmid transfection could decrease Akt(S473) phosphorylation level without effect on Src(Y416) phosphorylation level in LHBs expressing Huh7 cells (Fig. 3E and F). These results show that LHBs expression could induce PI3K/Akt activation via instigating Src tyrosine kinase activity in hepatoma cells.

Because previous study has proved that PreS2 activators including LHBs and C-terminally truncated MHBs triggered PKCα/β-dependent activation of Raf1/Erk2 signaling, resulting in an increased hepatocyte proliferation rate in transgenic mice (27), we hypothesized that PKC-dependent Raf1 activation might establish a mechanistic link between LHBs expression with Src kinase activation. As shown in Fig. 4A, significant decreased Src(Y416) and Raf1 (S338) phosphorylation levels
were observed after Go6976 (PKCα/β inhibitor), safinogol (PKCα inhibitor), and GW5047 (Raf1 inhibitor) treatment, despite no significant change of Src(Y416) phosphorylation levels after LY333531 (PKCβ inhibitor) and CGP53353 (PKCβII inhibitor) treatment, compared with DMSO treatment in LHBs-expressing Huh7 and SK-Hep1 cells, suggesting PKCα/Raf1 signal activity is required for LHBs-induced Src kinase activation. To verify the crucial role of PKCα/Raf1 signal in LHBs-induced Src kinase activation in hepatoma cells, Src(Y416) phosphorylation levels were assessed after endogenous PKCα or Raf1 activity inhibitions in LHBs-expressing Huh7 and SK-Hep1 cells. Consistent with the above mentioned result, both inhibition of endogenous PKCα activity with PKCα siRNA cotransfection (Fig. 4B) or competitive PKC pseudosubstrate treatment (Fig. 4C), and prohibition of endogenous Raf1 activity with dominant-negative Raf1 S621A mutant cotransfection (Fig. 4D) or Raf1 siRNA cotransfection (Fig. 4E) decreased Src(Y416) phosphorylation levels in LHBs-transfected Huh7 and SK-Hep1 cells. Taken together, these data reveal that LHBs expression induces Src/PI3K/Akt signal activation dependent on PKCα/Raf1 pathway in hepatoma cells.

**LHBs expression promotes hepatoma cellular proliferation, G1→S cell-cycle progression, and apoptosis resistance through Src activation**

To further characterize the potential oncogenic role of LHBs-induced Src kinase activation in hepatoma cells, we examined cellular proliferation rate, cell-cycle status, and cellular apoptosis after Src signal suppression in LHBs-Huh7 cells. Western blot analysis for the proliferative cell marker, namely PCNA expression (Fig. 5A), cell proliferation assay (Fig. 5B), BrdUrd incorporation assay and following flow cytometric analysis for above mentioned Huh7 cells (Fig. 5C), cell-cycle status (Fig. 5D), and colony formation assay for above mentioned Huh7 cells (Fig. 5E) showed that increased cellular proliferation of LHBs-Huh7 cells was reversed by endogenous Src kinase inhibition with kinase dead mutant Src K295M cotransfection. Furthermore, flow cytometric analysis of cell-cycle status revealed that induced G1→S cell-cycle progression of LHBs expression.
LHBs-Huh7 cells was also reversed by Src kinase inhibition with Src K295M cotransfection (Fig. 5F; Supplementary Table S1). Western blot analysis for G1–S cell-cycle regulators such as cyclin D1, cyclin D3, CDK4, and CDK6 showed that despite no alteration with cyclin D3 and CDK6 protein levels after LHBs expression, increased cyclin D1 and CDK4 protein levels was reversed by cotransfection with Src K295M in LHBs-Huh7 cells (Supplementary Fig. S3C). Moreover, Annexin V/PI staining assay (Fig. 5F; Supplementary Fig. S3D) and Western blot analysis for cleaved caspase-3 (Supplementary Fig. S3E) in LHBs-Huh7 cells under serum starvation indicated that apoptosis resistance conferred by LHBs expression was reversed by Src kinase inhibition with Src K295M cotransfection. Taken together, these results indicate that LHBs expression could promote cellular proliferation, G1–S cell-cycle progression, and apoptosis resistance in hepatoma cells.

Saracatinib administration alleviates provocative tumor formation conferred by LHBs expression

To further determine whether Src signal activation conferred by LHBs expression could be used as a novel molecular therapeutic target in HBV-associated hepatocarcinogenesis, cellular proliferative rate, and tumor formation were assessed in LHBs stably expressing Huh7 and SK-Hep1 cells after a specific Src inhibitor saracatinib treatment, which was used to suppress Src kinase activity. Results of cell proliferation assay showed that saracatinib treatment could significantly attenuate promote hepatoma cellular proliferation conferred by stable LHBs expression in Huh7 and SK-Hep1 cells (Fig. 6A and B). More importantly, tumor xenograft experiments in nude mice showed that orally saracatinib administration could also alleviate instigative tumor formation induced by stable LHBs expression in Huh7 and SK-Hep1 cells (Fig. 6C–F). These results show saracatinib administration could suppress cellular proliferation in vitro and tumor formation in vivo through intervening Src signal activation conferred by LHBs expression, which might be used as a potential therapeutic target for HBV-associated patients with HCC.

LHBs expression positively correlates with increased Ki67, pSrc(Y416), and pAkt(S473) staining in tumor tissues from patients with HCC

To ascertain the correlation between LHBs expression with Src/Akt signal activation and cellular proliferative status...
LHBs Promotes Hepatocarcinogenesis via Src/PI3K/Akt Signal

Figure 7. LHBs expression positively correlates with increased Ki67, pSrc(Y416), and pAkt(S473) staining in tumor tissues from patients with HCC. A, representative positive (case 1) and negative (case 2) immunohistochemical staining with LHBs, Ki67, pSrc(Y416), and pAkt (S473) in human hepatoma tissues (black scale bar, 50 μm). B–D, correlation analysis between LHBs with Ki67 (B), LHBs with pSrc(Y416) (C), and LHBs with pAkt(S473) (D) in human hepatoma tissues.

during HBV-associated hepatocarcinogenesis in vivo, immunohistochemistry was carried out to analyze the staining patterns of proliferative marker Ki67, pSrc(Y416), and pAkt and Src in tumor tissues from 37 patients with HCC. On the basis of the immunohistochemical results of LHBs staining, all 37 patients with HCC were divided into 2 groups: LHBs negative group (n = 18) and LHBs positive group (n = 19). LHBs expression in immunohistochemistry correlated positively with the tumor grade (P = 0.004) but did not show association with any other clinicopathologic parameters (Supplementary Table S2). Immunohistochemical analysis showed that LHBs expression was positively correlated with Ki67 (n = 37, r = 0.502, P = 0.001), pSrc(Y416) (n = 37, r = 0.373, P = 0.023), and pAkt(S473) (n = 37, r = 0.444, P = 0.005) staining in HCC tumor tissues (Fig. 7A–D; Supplementary Table S3). These results suggest that Src/Akt signal activation and increased cellular proliferation positively correlate with LHBs expression during the development of HBV-associated HCC.

Discussion

Although chronic HBV infection has been linked epidemiologically to the development of HCC for more than 30 years, the molecular mechanisms underlying viral-induced hepatocarcinogenesis remain largely controversial (3, 28). While significant advances have been noted in understanding of indirect roles of chronic HBV infection proposed on the molecular basis of HBV-associated HCC, including virus persistence, genetic alterations conferred by HBV DNA integration, and hepatic cell clone expansion because of chronic inflammation and fibrosis, increasing studies indicate that expression of HBV proteins such as HBx, PreS2 activators, and HBSP could modulate hepatic malignant transformation (5, 6, 29–32). In the absence of a dominant oncogene encoded by HBV genome, molecular pathways engaged in growth signal transduction being hijacking by viral proteins for malignant transformation in hepatic cells served as underlying mechanisms for HBV-associated tumorigenesis (5). Among all putative oncopromotive proteins encoded by HBV genome, LHBs addressed our attentions because of its potential of activating transcription factors such as activator protein-1 (AP-1) and NF-κB to trigger cellular proliferation, both of which could play essential roles during inflammation-related tumorigenesis (27, 33, 34). Our current study shows the provocative effect of LHBs on tumor formation of hepatoma cells in vivo and in vitro, which provides further evidence on the oncogenic function of LHBs during HBV-related HCC development.

Because 2 signal transduction cascades including insulin/IGF/IRS-1/MAPK and WNT/Frizzled/β-catenin pathways are activated early in over 90% of HCC tumors (35), we first investigated the contribution of these 2 pathways to tumor formation instigation conferred by LHBs expression. MAPK/MEK/ERK signal inhibition with specific inhibitors PD98059, U0126, and SB203580 could not alleviate increased hepatoma cellular proliferation induced by LHBs expression, and LHBs expression could not alter protein expression or nuclear localization of β-catenin (data not shown), which excluded the possibilities of these 2 pathways involved in this phenomenon. Perturbation of hepatic NF-κB signal activity provides a mechanistic link between inflammation and cancer in hepatic inflammation–fibrosis–cancer axis, and NF-κB is a major factor controlling both proneoplastic and malignant cells to resist apoptosis-based tumor surveillance mechanisms (36, 37). But NF-κB activity inhibition with specific inhibitors PDTC and BAY117082 in our experiments could not attenuate provocative hepatoma cellular proliferation conferred by LHBs expression, which excluded the dedication of NF-κB activity on this phenomenon and conflicted with previous finding that LHBs could activate NF-κB to trigger cellular proliferation (27). However, our current investigation could not exclude the
potential NF-xB activation conferred by LHBs expression, and the mechanistic link between NF-xB activity and hepatic cellular proliferation remains largely debated and awaits further investigation (38).

Genetic approaches and microarray technologies for analyzing gene expression profiles have revealed strikingly distinctive molecular mechanisms operate in HBV-related HCC, which includes a high copy number of HBV, mutations in PI3KCA and TP53, and specific activation of the PI3K/Akt/mTOR pathway (5, 39). Moreover, the PI3K/Akt/mTOR signaling pathway could be overactivated by enhanced stimulation of receptor tyrosine kinases, particularly the IGF receptor and EGFR (40). Expression of both IGF and IGF receptor is upregulated in HCC and human cirrhotic liver, resulting in stimulation of the PI3K/Akt/mTOR signaling pathway (5). Evidence also suggested that anomalies in PTEN function may lead to overactivation of the PI3K/AKT/mTOR pathway in HCC (41). Interestingly, our present study confirmed that elevated hepatoma cellular proliferation ratio because of LHBs expression was dependent on PI3K/Akt/mTOR signal activation by using PI3K/Akt/mTOR pathway specific inhibitors LY294002, Wortmannin, and rapamycin treatment, which further showed the fundamental effect of PI3K/Akt/mTOR pathway activation on HBV-related hepatocarcinogenesis. Moreover, mounting evidences indicated the substantial role of Src signaling in the process of HCC development. Previous studies reported that increased Src tyrosine kinase activity was observed in HCC specimens on the basis of in vitro Src kinase assays, in comparison with liver tissue from normal subjects and chronic hepatitis (16, 17). In another report, stimulation of hepatocytes with stromal cell-derived factor-1 (SDF-1) led to the activation of the Src kinases, which in turn stimulates the Akt signal (42). Our results presented here also indicated the crucial role of Src tyrosine kinase activation in promotive hepatoma cellular proliferation conferred by LHBs expression by using specific Src inhibitor PP2 treatment. More importantly, we also showed that LHBs-induced Akt signal activation was dependent on Src tyrosine kinase activity by using specific inhibitor treatment, kinase dead mutant cotransfection, and specific siRNA cotransfection. Consistent with previous study indicating PreS2 activators triggered PKC<sup>α</sup>/β-dependent Raf1 activation (27), our present investigation further revealed a mechanistic link between Src/Akt signal activation and LHBs expression through PKC<sup>α</sup>/Raf1 pathway, which could be potential molecular basis for HBV-related hepatocarcinogenesis.

In addition to cellular proliferative promotion, our current study found that LHBs expression could also accelerate G<sub>1</sub>-S cell-cycle progression and endue with apoptosis resistance through Src activation in hepatoma cells, all of which constitute the oncogenic function of LHBs expression in HBV-associated tumorigenesis. Moreover, correlative analysis among Src/Akt signal activation, cellular proliferative status, and LHBs expression in our investigation here also revealed that LHBs expression was positively correlated with Src/Akt activation and cellular proliferation in HCC tumor tissues. Our present demonstrations of the increased pSrc(Y416) staining in HCC tissues and the potent inhibitory effect of Src kinase dead mutant on oncogenic functions conferred by LHBs expression in hepatoma cells in vitro provide a strong rationale that specific Src inhibitor might be developed as an anticancer agent in patients with HCC with chronic HBV infection. Our current study also elucidates the potential therapeutic effect of an oral Src specific inhibitor saracatinib on hepatoma cell xenografted tumor in nude mice, which merits further clinical investigation on patients with HBV-associated HCC to assess its feasible therapeutic efficiency.

On the basis of our current results, we propose a schematic model illustrating a possible molecular mechanism and functional basis for HBV-associated hepatocarcinogenesis conferred by LHBs expression (Supplementary Fig. S4). In conclusion, our results presented here reveal a novel association between LHBs expression and PKC<sup>α</sup>/Raf1/Src/PI3K/Akt signal activation in the development of HBV-associated HCC, thus revealing a putative molecular mechanism for the development and progression of HBV-associated HCC. These results shed a new light for potential therapeutic intervention to prevent hepatocarcinogenesis in the high-risk group of chronic hepatitis B patients with PKC<sup>α</sup>/Raf1/Src/PI3K/Akt signal suppression treatment.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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