Hepatitis B Virus Large Surface Antigen Promotes Tumorigenesis of Human Hepatocellular Carcinoma Cells through Activating Src/PI3K/Akt Pathway

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Abstract

There are three hepatitis B virus (HBV) envelope glycoproteins collectively known as hepatitis B virus surface antigen (HBsAg), including the large (LHBs), middle (MHBs), and small (SHBs) surface proteins. Among them, LHBs has been retained in a large proportion of liver tissues in HBV-associated hepatocellular carcinoma (HCC) patients and shown oncogenic function in transgenic mice. However, the concrete functions of LHBs in carcinogenesis of HBV-associated HCC remain elusive. In this study, we investigated the molecular and functional roles of LHBs in HBV-associated hepatocarcinogenesis. Tumor xenograft experimental data in nude mice indicated that LHBs increased tumor formation of hepatoma cells. Moreover, LHBs expression, but neither MHBs nor SHBs expression could promote colony formation and cellular proliferation of hepatoma and hepatic cells in vitro. These effects of LHBs on hepatoma cells were due to Src/PI3K/Akt signal instigation mediated by PKCα/Raf1 activation. Furthermore, stable LHBs expression could promote cellular proliferation, elevate colony formation, induce G1-S cell cycle progression, and confer apoptosis resistance via Src tyrosine kinase activation in hepatoma cells. Specific Src inhibitor Saracatinib administration could reverse cellular proliferation instigation and tumor formation promotion conferred by LHBs expression in vitro and in vivo. Finally, the positive correlations between increased staining of pSrc(Y416), pAkt(S473), and proliferative marker Ki67 with LHBs expression were observed in hepatoma tissues from HCC patients. In conclusion, our results propose that LHBs could promote tumorigenesis of hepatoma cells dependent on PKCα/Raf1/Src/PI3K/Akt signal activation, which reveals a novel insight into the underlying mechanisms for HBV-associated hepatocarcinogenesis.
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) (1, 2). Although chronic HBV infection has been noted associated with the development of HCC soon after the discovery of hepatitis B virus surface antigen (HBsAg), the oncogenic functions of HBV are not completely known, partly due to 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 due to 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 hepatitis B virus surface antigen (LHBs), C-terminally truncated middle hepatitis B virus surface antigen (MHBs), hepatitis B virus X protein (HBx), and a novel spliced transcript of HBV, referred to as hepatitis B spliced protein (HBSP) (6).

The viral genome of HBV encodes for four 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 three viral surface antigens by differential initiation of translation at each of three in-frame initiation codons (7). Initiation at the nearest start codon (S) produces the small hepatitis B virus surface antigen (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 et al. has shown that HBV transgenic mice that overproduce LHBs and accumulate toxic quantities of SHBs within the hepatocyte 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 down-regulated during neoplastic transformation, just as the morphological and biochemical phenotypes of foci of altered hepatocytes, hepatic adenoma and hepatocellular carcinoma 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, MAPK, NF-κB pathways and alter gene expression resulting in hepatoma formation due to 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 HCC patients with chronic HBV infection.
Materials and Methods

**Cell culture and human HCC samples.** One immortalized hepatic cell line (L02) and two 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 DMEM supplemented with 10% FBS at 37°C in a humidified 5% CO₂ incubator. The cell lines have been characterized at the bank by DNA fingerprinting analysis using STR (short tandem repeat) markers. All cell lines were placed under cryostage after they were obtained from the bank and used within 6 months of thawing fresh vials. Thirty seven pairs of frozen fresh tumor liver tissues and their peripheral nontumor tissues after surgical resection were collected from HCC patients who had received neither chemotherapy nor radiotherapy before surgical resection in Nantong Tumor Hospital (Jiangsu, China) with informed consent and Institutional Review Board approval between 2004 and 2008.

**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 based on 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 (RNAi). Transient and stable transfections with various plasmids were performed as previously described (23). Two siRNAs against SRC gene Src siRNA (h), two siRNAs against AKT1/2 gene Akt1/2 siRNA (h), two siRNAs against PKCa gene PKCα siRNA (h), two siRNAs against RAF1 gene Raf1 siRNA (h), and corresponding control siRNA-A (Santa Cruz Biotechnology, Santa Cruz, CA) were transfected into Huh7 and SK-Hep1 cells in 6-well plates using X-tremeGENE siRNA Transfection Reagent (Roche Applied Science, Mannheim, Germany) according to the manufacturer’s instructions. Gene silencing effect was confirmed by western blot and RT-PCR at 72 hours post transfection.

Western blotting. Protein extraction from cultured cells or tumor tissues and western blotting analysis were performed as previously described (23). Primary antibodies used included those against LHBs, GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA), Akt, pAkt(S473), Src, pSrc(Y416), Raf1, pRaf1(S338), PKCα, cyclin D1, cyclin D3, CDK4, CDK6, cleaved caspase-3 (Cell Signaling Technology, Danvers, MA), and PCNA (BD Biosciences, Rockville, MD).

Tumor xenograft experiments and Saracatinib (AZD0530) treatment. Tumor xenograft experiments in nude mice were performed as previously described (23). The specific Src inhibitor Saracatinib (Selleck, Houston, TX) was dissolved with DMSO for 5mg/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 per kg body weight in vehicle corn oil via oral gavage.
for four weeks since hepatoma cells subcutaneous injection.

**Histology, Immunohistochemistry Analysis and Evaluation.** Tumor sections from subcutaneous tumor xenografted nude mice and HCC patients were H&E stained and immunohistochemistry analyzed as described previously (24, 25). Primary antibodies used included those against LHBs, Ki67 (Millipore, Billerica, MA), pAkt(S473), and pSrc(Y416). The intensity of immunohistochemistry staining in the tumor cells was scored independently by two pathologists using the semiquantitative IRS (immunoreactive score) scale according to Remmele and Stegner (26).

**Colony formation assay, cell proliferation assay, 5-Bromo-2’-deoxyuridine (BrdU) incorporation assay, cell cycle analysis, and Annexin V/PI staining.** Colony formation assay, cell proliferation assay, 5-Bromo-2’-deoxyuridine (BrdU) incorporation assay, and cell cycle analysis were performed as previously described (23). Annexin V/PI staining was performed by using Annexin V–FITC Apoptosis Detection Kit (BD Biosciences, Rockville, MD) according to the manufacturer’s instructions.

**Statistical analysis.** Experimental data were presented as mean ± SD or SEM of at least three independent replicates through analyzing with GraphPad Prism 5 (GraphPad Software, La Jolla, CA) and assessing comparisons between different groups by the Student’s t-test, one-way ANOVA. The association between LHBs and clinicopathological characteristics was assessed using Fisher’s exact test. The correlation between LHBs with Ki67, pSrc(Y416), and pAkt(S473) staining obtained by immunohistochemistry was determined using 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 performed in nude mice with Huh7 cells stably transfected with LHBs. As shown in Figure 1A, stable LHBs expression in clone 1 and 2 compared with control Huh7 cells was confirmed by western blot, and Huh7-LHBs clone 2 was selected for further investigation due to higher stable LHBs expression. Tumor xenograftation experiments showed that LHBs expression significantly accelerated overall tumor growth compared with the control group as assessed by the tumor volume (Figure 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 (Figure 1C, D). H&E staining showed more aggressive tumor growth gained by LHBs stably transfected Huh7 cells compared with control cells in nude mice (Figure 1E). Since 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 performed 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 (Figure 2A-C) and cellular proliferation (Figure 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.
**LHBs expression activates PKCa/Raf1/Src/PI3K/Akt signal pathway in hepatoma cells.** In order to characterize the mechanistic signaling pathway underlying instigated tumor formation conferred by LHBs expression in hepatoma cells, cell proliferation assay was performed in LHBs expressing Huh7 and SK-Hep1 cells after treatment with small molecular inhibitors against PI3K (LY294002, Wortmannin), ERK (PD98059), MEK (U0126), p38/MAPK (SB203580), NF-κB (PDTC, BAY117082), Src (PP2), and mTOR (Rapamycin). As shown in Figure 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 (Figure 2E-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 (Figure 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 three intersected tumor tissues generated by Huh7-LHBs cells compared with control Huh7 cells from xenografted nude mice (Figure 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 performed in Huh7 cells after Src and PI3K/Akt signal
inhibition. To assess RNAi knockdown effect on Src and Akt signals activation, two siRNAs against Src and two siRNAs against Akt1/2 were prepared and transfected into Huh7 cells to evaluate their knockdown efficiency by using RT-PCR and western blot (Figure S2A, B). 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 (Figure 3A, 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 (Figure 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 (Figure 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 (Figure 3E, F). These results demonstrate that LHBs expression could induce PI3K/Akt activation via instigating Src tyrosine kinase activity in hepatoma cells.

Since previous study has proved that PreS2 activators including LHBs and C-terminally truncated MHBs triggered PKCa/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 Figure 4A, significant decreased Src (Y416) and Raf1 (S338) phosphorylation levels were observed after Go6976 (PKCa/β inhibitor), Safingol (PKCa 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 PKCa/Raf1 signal activity is required for LHBs-induced Src kinase activation. To verify the crucial role of PKCa/Raf1 signal in LHBs-induced Src kinase activation in hepatoma cells, Src (Y416) phosphorylation levels were assessed after endogenous PKCa or Raf1 activity inhibitions in LHBs-expressing Huh7 and SK-Hep1 cells. Consistent with abovementioned result, both inhibition of endogenous PKCa activity with PKCa siRNA cotransfection (Figure 4B) or competitive PKC pseudosubstrate treatment (Figure 4C), and prohibition of endogenous Raf1 activity with dominant negative Raf1 S621A mutant cotransfection (Figure 4D) or Raf1 siRNA cotransfection (Figure 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 PKCa/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 supression in LHBs-Huh7 cells. Western blot analysis for the proliferative cell marker, namely proliferating cell nuclear antigen (PCNA) expression (Figure 5A), cell proliferation assay (Figure 5B), BrdU incorporation assay (Figure 5C, Figure S3A),
and soft agar colony formation assay (Figure 5D, Figure S3B) showed that increased cellular proliferation of LHBs-Huh7 cells was reversed by endogeneous Src kinase inhibition with kinase dead mutant Src K295M cotransfection. Furthermore, flow cytometry analysis of cell cycle status revealed that induced G1-S cell cycle progression of LHBs-Huh7 cells was also reversed by Src kinase inhibition with Src K295M cotransfection (Figure 5E, 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 (Figure S3C). Moreover, Annexin □/PI staining assay (Figure 5F, Figure S3D) and western blot analysis for cleaved caspase-3 (Figure 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 promotive hepatoma cellular proliferation conferred by stable LHBs expression in Huh7 and SK-Hep1 cells (Figure 6A, 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 (Figure 6C-F). These results demonstrate 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 HCC patients.

**LHBs expression positively correlates with increased Ki67, pSrc(Y416), and pAkt(S473) staining in tumor tissues from HCC patients.** To ascertain the correlation between LHBs expression with Src/Akt signal activation and cellular proliferative status during HBV-associated hepatocarcinogenesis in vivo, immunohistochemistry was performed to analyze the staining patterns of proliferative marker Ki67, pSrc(Y416), and pAkt and Src in tumor tissues from 37 HCC patients. Based on the immunohistochemical results of LHBs staining, all 37 HCC patients were divided into two 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 clinicopathological parameters (Table S2). Immunohistochemistry 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 (Figure 7A-D, 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 hepatitis B virus 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 due to 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 due to its potential of activating transcription factors such as activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) to trigger cellular proliferation, both of which could play essential roles during inflammation-related tumorigenesis (27, 33, 34). Our current study demonstrates 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.

Since two 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 firstly investigated the contribution of these two 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 two 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 pro-neoplastic 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-κB activation conferred by LHBs expression, and the mechanistic link between NF-κB 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 PIK3CA 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 due to 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 demonstrated 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 based on 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 PKCα/β-dependent Raf1 activation (27), our present investigation further revealed a mechanistic link between Src/Akt signal activation and LHBs expression through PKCα/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 G1-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 HCC patients 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 HBV-associated HCC patients to assess its feasible therapeutic efficiency.

Based on our current results, we propose a schematic model illustrating a possible molecular mechanism and functional basis for HBV-associated hepatocarcinogenesis conferred by LHBs expression (Figure S4). In conclusion, our results presented here reveal a novel association between LHBs expression and PKCα/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 PKCα/Raf1/Src/PI3K/Akt signal suppression treatment.
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Reference


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Figure legends

Figure 1. LHBs expression promotes tumor formation of hepatoma cells in nude mice. (A) Western blot analysis was performed to confirm stable LHBs expression in Huh7 cells (Clone 1 and 2). (B) In vivo subcutaneous tumor growth curves of Huh7-LHBs (Clone 2) and Huh7-Control cells (n=6). *, P<0.05 compared with Huh7-Control. (C) Photos of all harvested six subcutaneous tumors from three nude mice of each group were shown. (D) Total tumor weight from each group of mice was calculated and shown. *, P=0.008. (E) H&E staining images of representative subcutaneous tumors. (Tu: tumor tissue; Black scale bar: 50μm).

Figure 2. LHBs expression rather than MHBs or SHBs expression exerts oncogenic promotion in hepatic cells. (A-C) (Upper) 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, F) Cell proliferation assay for Huh7 cells (E) and SK-Hep1 cells (F) transiently transfected with empty vector (Control) and LHBs plasmid (LHBs) respectively after treatment for 24h with DMSO, LY294002 (50μM), Wortmannin (10nM), PD098059 (50μM), U0126 (20μM), SB203580 (10μM), PDTC (10μM), BAY117082 (10μM), PP2 (10μM), or Rapamycin (10nM). *, P<0.05.
Figure 3. LHBs expression activates Src/PI3K/Akt signal pathway in hepatoma cells. (A) Western blot analysis for Huh7 cells after transient LHBs expression under treatment for 24h with PP2 (10µM). (B) Western blot analysis for Huh7 cells transiently transfected with empty vector, LHBs plasmid, or cotransfected LHBs plasmid with Src WT plasmid or Src K295M plasmid together. (C) Western blot analysis for Huh7 cells transiently transfected with empty vector, LHBs plasmid, or cotransfected LHBs plasmid with NS siRNA, Src siRNA#1, or Src siRNA#2 together. (D) Western blot analysis for Huh7 cells after transient LHBs expression under treatment for 24h with LY294002 (50µM). (E) Western blot analysis for Huh7 cells transiently transfected with empty vector, LHBs plasmid, or cotransfected LHBs plasmid with Akt WT plasmid or Akt K179M plasmid together. (F) Western blot analysis for Huh7 cells transiently transfected with empty vector, LHBs plasmid, or cotransfected LHBs plasmid with NS siRNA, Akt siRNA#1, or Akt siRNA#2 together. *, P<0.05 compared with the left lane.

Figure 4. LHBs expression induces Src activation dependent on PKCα/Raf1 signal pathway. (A) Western blot analysis for Huh7 and SK-Hep1 cells after transient LHBs expression under treatment for 24h with Go6976 (100nM), Safingol (10µM), LY333531 (10nM), CGP53353 (2µM), or GW5047 (1µM). (B) Western blot analysis for Huh7 and SK-Hep1 cells transiently transfected with empty vector, LHBs plasmid, or cotransfected LHBs plasmid with NS siRNA, PKCα siRNA#1, or PKCα siRNA#2 together. (C) Western blot analysis for Huh7 and SK-Hep1 cells after transient LHBs expression under treatment for 24h with PKC pseudosubstrate (50µM) or its vehicle. (D) Western blot analysis for Huh7 and SK-Hep1 cells transiently transfected with empty vector, LHBs plasmid, or cotransfected LHBs plasmid with Raf1 WT or Raf1 S621A together. (E) Western blot analysis for Huh7 and SK-Hep1 cells
transiently transfected with empty vector, LHBs plasmid, or cotransfected LHBs plasmid with NS siRNA, Raf1 siRNA#1, or Raf1 siRNA#2 together. *, $P<0.05$ compared with the left lane.

Figure 5. LHBs expression promotes cellular proliferation, G1-S cell cycle progression and apoptosis resistance via Src activation in hepatoma cells. (A) Western blot analysis for Huh7 cells after transfected with empty vector, LHBs plasmid, or cotransfected LHBs plasmid with Src WT plasmid or Src K295M plasmid together. *, $P<0.05$ compared with the left lane. (B) Cell proliferation assay for abovementioned Huh7 cells. *, $P<0.05$. (C) BrdU incorporation assay and following flow cytometry analysis for abovementioned Huh7 cells. *, $P<0.05$. (D) Colony formation assay for abovementioned Huh7 cells. Quantification of colonies. *, $P<0.05$. (E) Flow cytometry analysis for cell cycle status of abovementioned Huh7 cells. (F) Annexin V/PI staining assay for cellular apoptosis analysis of abovementioned Huh7 cells cultured in serum deprivation medium for 24 hours. *, $P<0.05$.

Figure 6. Saracatinib (AZD0530) administration alleviates provocative tumor formation conferred by LHBs expression. (A-B) Cell proliferation assay for Huh7 cells (A) and SK-Hep1 cells (B) after stable LHBs expression under treatment with Saracatinib (1μM). *, $P<0.05$. (C-D) In vivo subcutaneous tumor growth curves (C) and tumor weight quantification of intersected subcutaneous tumor tissues (D) of Huh7 cells after stable LHBs expression under Saracatinib treatment (25mg kg⁻¹ body weight daily for four weeks) (n=18). *, $P<0.05$. (E-F) In vivo subcutaneous tumor growth curves (E) and tumor weight quantification of intersected subcutaneous tumor tissues (F) of SK-Hep1 cells after stable LHBs expression under Saracatinib treatment (25mg kg⁻¹ body weight daily for four weeks) (n=18). *, $P<0.05$. 
Figure 7. LHBs expression positively correlates with increased Ki67, pSrc(Y416), and pAkt(S473) staining in tumor tissues from HCC patients. (A) Representative positive (Case 1) and negative (Case 2) immunohistochemistry staining with LHBs, Ki67, pSrc(Y416), and pAkt(S473) in human hepatoma tissues. (Black scale bar: 50μm). (B-D) Correlation analysis between LHBs with Ki67 (B), LHBs with pSrc(Y416) (C), and LHBs with pAkt(S473) (D) in human hepatoma tissues.
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