SH3GL1 Promotes Liver Tumorigenesis by Activating β-Catenin Signaling

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Research

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Abstract

Hepatocellular carcinoma (HCC) is the most prevalent subtype of primary liver cancer and one of the leading causes of cancer-related death in the world. Liver cancer stem cells (CSCs) have been well established to be responsible for liver tumorigenesis, metastasis, drug resistance and tumor relapse. Yet the underlying mechanisms of CSCs self-renewal and maintenance are poorly understood. Here, we first report that SH3GL1 was dramatically upregulated in liver cancer and higher SH3GL1 expression was significantly correlates with worse clinical outcomes of liver cancer patients. Loss of function experiments demonstrate that SH3GL1 functions as a novel oncogene to potentiate liver cancer cell proliferation and liver CSCs self-renewal maintenance *in vitro* as well as xenograft tumor grow *in vivo*. Mechanistically, our study reveals that SH3GL1 facilitates liver CSCs by physically interacting with and activating β-catenin signaling. Our study characterizes SH3GL1 as a new regulator of oncogenic β-catenin signaling and defines newly therapeutic target for liver cancer patients.

Introduction

Liver cancer is one of the most serious cancer and one of the leading causes of cancer-related death worldwide [1]. Hepatocellular carcinoma (HCC) represents the vast majority histological subtype, accounts for 70%-80% of incidents of primary liver cancer [2, 3]. However, the 5-year survival rate of HCC patients remains worse, and more than 751, 000 patients with HCC die each year in the world. Heterogeneity is a major characteristic of HCC, suggesting that developing clinical therapy specifically for patients with liver cancer is difficulty. Emerging evidence have been reported that heterogeneity is the consequence of the hierarchical tissue of tumor cells by a subset of cells with stem cell-like property, which is also known as cancer stem cells (CSCs) [4]. CSCs hypothesis indicates that a small subset cells within liver tumor responsible for liver tumorigenesis, therapeutic resistance and tumor relapse [4]. Therefore, it is of clinical significance to identify new molecules or signaling pathways that specifically implicates in regulating CSCs self-renewal as clinically targeted therapeutics.

The canonical Wnt signaling pathway is a widely established signaling pathway that have been documented to be essentially required in both stem cells and cancer cells [5, 6]. Aberrant activation of β-catenin signaling and the dysregulation of Wnt/β-catenin components have been reported to be implemented in a variety of human cancers tumorigenesis, including HCC. [7, 8]. Upon ligands including Wnts binding to cell membrane receptors, including a Frizzled (Fz) protein and LDL receptor-related protein 6 (LRP6), thereby initiating a signaling transduction cascade resulting in the stabilization of the transcription factors β-catenin, which then enters into nucleus to implement the formation of a protein complex with T cell factor (TCF) or lymphoid enhancer factor (LEF) to transcriptionally activate the expression of Wnt downstream genes, such as c-Myc and cyclin D1. Wnt/β-catenin signaling pathway has been widely established to play central roles in cancer initiation and development, including regulation of transformation, uncontrolled cell growth, and invasion as well as sustaining CSCs stemness. However, the underlying mechanisms of Wnt/β-catenin signaling regulate CSCs stemness maintenance remains poor understood. Thus, it is of importance to elucidate the elaborate principles or

upstream regulators that involved in Wnt/β-catenin signaling regulation as novel clinically applicable therapeutic targets.

SRC homology 3 (SH3) domain is a domain contains 50 amino acids, which has been widely accepted to be highly evolutionary conserved in a variety of species and plays fundamental roles in transmitting signaling [9-11] as well as multiple disease initiation and progression, particularly in tumorigenesis [12, 13]. SH3-domain GRB2-like 1 (SH3GL1) is also known as endophilin A1 and interacted with proteins containing SH3 domain [12]. SH3GL1 consisting of a BAR domain, a variable domain and an SH3 domain. SH3GL3 has been reported to play repressive roles in lung cancer [11], glioblastoma [14] by regressing tumor cell proliferation and migration. SH3GL1 has been characterized as a disease candidate gene of adolescent idiopathic scoliosis [15]. Recent advance reported that SH3GL1 was highly expressed in colorectal cancer and activates EGFR signaling to resistant to chemotherapeutics [16]. Following report further revealed that knocking down SH3GL1 significantly inhibits medulloblastoma cell growth *in vitro* [17]. However, not much is currently known about the biological function of SH3GL1 in HCC progression and the underlying molecular mechanisms. Thus, our enthusiasm was on to investigate the precise functions and roles of SH3GL1 in HCC tumorigenesis and liver CSC self-renewal.

Materials And Methods

Cell culture and transfections

Liver cancer cell lines, including Hep3B, HepG2, Huh-7 were purchased from the American Type Culture Collection (Manassas, VA), These cells were grown in DMEM or RPMI 1640 medium with 10% fetal bovine serum. For depletion of SH3GL1 in liver cancer cells, the SH3GL1 shRNA was cloned into the expression vector pLKO.1. For SH3GL1 knockdown cell line establishment, pLKO.1 plasmid together with packaging vectors psPAX2 and pMD2.G (Addgene) were co-transfected into HEK-293T cells for lentivirus production. Indicated cells were plated into 6-well plate at a density of 3×10^5 before lentivirus infection. Stable cell lines were selected by puromycin (1 mg/mL) at 37 °C for 7-10 days.

Quantitative real-time PCR

Total RNAs were isolated using the Trizol Reagent (Invitrogen, USA). Briefly, 1 mg of total RNA of each cells sample was transcribed reversely into cDNA. Quantitative Real-Time PCR was performed using Trans Start Top Green qPCR Super Mix (Transgene, China). The relative mRNA expression levels were determined by the $2^{(-\Delta\Delta Ct)}$ method. The GAPDH mRNA was used as an internal control. The sequences of primers used in this study listed as follows. SH3GL1_F: GGGCAAGATCCCCGATGAG. SH3GL1_R: CACCTGCTCGATGTCAGTCTC. GAPDH_F: GGAGCGAGATCCCTCCAAAAT. GAPDH_R: GGCTGTTGTCATACTTCTCATGG. CCND1_F: GCTGCGAAGTGGAAACCATC. CCND1_R: CCTCCTTCTGCACACATTTGAA. c-Myc_F: GGCTCCTGGCAAAAGGTCA. c-Myc_R: CTGCGTAGTTGTGCTGATGT.

Immunohistochemistry

Immunohistochemistry assay was performed according to the previous report with minor modification [18]. Briefly, clinical liver cancer patients' specimens were deparaffinized and rehydrated through a descending alcohol series, following by antigen retrieval with sodium citrate buffer. Each section was blocked with 10% normal goat serum plus 0.1% Triton X-100 in PBS for 20 min at room temperature, and then incubated with primary antibodies specifically against SH3GL1 at 4 °C for overnight. Subsequently, each section was incubated with second antibody conjugates HRP using diaminobenzidine detection. The staining signal was scored according published paper [19].

Cell proliferation assay

Cell proliferation assay was performed as a previous study [20].

TOP-flash assay

TOP-flash assay was performed as a previous study [20].

Tumor sphere enrichment and *in vitro* limiting dilution assay

Tumor sphere initiation and *in vitro* limiting dilution assay were implemented as a previously study [21]. Briefly, single indicated liver cancer cells were seeded into six-well ultra-low attachment plates (Corning, Life Sciences) at a density of 3.5×10^4 cells/well and maintained in DMEM/F12 medium supplemented with 20 ng/ml epidermal growth factor (EGF), 20 ng/ml basal fibroblast growth factor (bFGF), 2% B-27 (Invitrogen). The spheres were then photographed and recorded. For *in vitro* limiting dilution assay, indicated single cells were seeded at the density of 20, 50, 100 cells with above serum-free medium and the spheres were recorded.

Western blot

The protein in indicated liver cancer cells were harvested using RIPA buffer supplemented with a protease inhibitor cocktail. Equal mass of protein samples was subjected to SDS-PAGE electrophoresis separation. The protein samples in gel were then transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA) and followed by incubating with primary antibodies overnight at 4 °C and visualized by horseradish peroxidase conjugated secondary antibodies.

Colony formation assay

Huh-7 and HepG2 cells transfected with control shRNA or SH3GL1 shRNA were seeded to 6-well plates at a density of 2, 000 cells per well. Indicated cells were grown for 3 weeks to enrich the colony. Thereafter, the colony were stained with 0.01% crystal violet for 2 hours at room temperature. After washed with PBS for three times, the colony were imaged and colony were visualized in naked eyes were counted. The experiment was performed at least for three independent times.

BrdU incorporation assay

Indicated single liver cancer cells were seeded into 24-well plates at a density of 50, 000 cells per well. After culturing for 36 h, cells were cultured with 100 mM BrdU (5-bromo-2'-deoxyuridine). Subsequently, the cells were fixed and incubated with primary antibody specifically against BrdU antibody. More than six random fields were determined for BrdU positive cells in indicated cell samples.

Xenograft tumor formation *in vivo*

Xenograft tumor formation *in vivo* was implemented as a previous study [22].

Statistical analysis

All data are presented as the mean ± standard deviation (SD). All experiments were implemented in triplicate. The statistical analyses were conducted using GraphPad prism 6.0 software. Two-tailed Student's t-tests were used for statistical analysis. $P < 0.05$ was considered to be statistically significant. The Kaplan-Meier curve was used for survival analysis, and the difference between the different groups was analyzed using a log-rank test. Spearman's correlation analysis was employed to determine the correlations between the SH3GL1 mRNA expression sand c-Myc or Cyclin D1 in the TCGA data set. *, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.0001$, respectively.

Results

SH3GL1 expression is increased in liver cancer and liver CSCs

Firstly, public database TIMER [23] (http://cistrome.org/TIMER/) was employed to identify the expression levels of SH3GL1 in a variety of tumor types. The results showed in (Fig 1A) revealed that SH3GL1 was markedly overexpressed in liver, lung, and breast cancer, suggesting that SH3GL1 may execute its function as a novel oncogenic driver in those cancer tumorigeneses. We further validated this finding using TCGA dataset and the analysis showed that SH3GL1 expression was significantly increased in liver cancer (Fig 1B). To further examine the relationship of SH3GL1 expression and liver cancer patients' survival, we performed Kaplan-Meier survival analysis in TCGA dataset and the results revealed a statistically significant worse prognosis for liver cancer patients with high SH3GL1 expression levels compared with those with low levels roles of SH3GL1 (Fig 1C). Additionally, we next assessed expression pattern of SH3GL1 in clinical specimens of liver patients. Immunohistochemistry (IHC) staining assays were performed in a total of 60 liver tissue specimens and its corresponding normal tissues. As shown in (Fig 1D-E), SH3GL1 staining was negative or weak in para-tumor tissues and SH3GL1 expression level was significant high in the liver cancer. Importantly, we detected the expression of SH3GL1 in several HCC cell lines and normal hepatic cells LO2. As shown in (Fig 1F-G), comparing to LO2, both mRNA and protein expression of SH3GL1 were markedly overexpressed in HCC. Given that CD15 and CD133 have been widely accepted as liver CSCs biomarkers, CD15 and CD133 double positive cells could initiates more tumor spheres in HCC cell lines or HCC primary cells [20]. We then sorted $CD15^{+}CD133^{+}$ cells, namely liver CSCs, using flow cytometry to initiate liver CSCs from Huh-7 and HepG2 cells. Actually, we found that the $CD15^{+}CD133^{+}$ cells form more tumor spheres than the $CD15^{-}CD133^{-}$ (none CSCs) cells

(data not shown). To further elucidate the biological role of SH3GL1 in CSCs maintenance, we compared the expression of SH3GL1 in liver CSCs and none CSCs and observed that SH3GL1 expression was markedly higher in liver CSCs than none CSCs (Fig 1H), implying that increased SH3GL1 expression may facilitate liver tumorigenesis. These data strongly support that the expression of SH3GL1 was substantially upregulated in liver cancer and associated with poor clinical outcomes of liver cancer patients.

SH3GL1 is essential for sustaining liver CSCs stemness and growth

To achieve a new avenue for the potential significance of SH3GL1 in liver tumorigenesis, we used short hairpin RNAs (shRNA) approach to directed ablate SH3GL1 expression in Huh-7 and HepG2 cells, which represent the highest expression of SH3GL1 (Fig 1F-G). The shRNA knockdown efficiency was examined (Fig 3C). The shRNA mediated depletion of SH3GL1 did not affect SH3GL2 and SH3GL3 expression (data not shown). Actually, SH3GL1 depletion remarkably impaired Huh-7 and HepG2 cell growth (Fig 2A). This finding was further confirmed by BrdU incorporation analysis. As shown in (Fig 2B-C), silencing SH3GL1 decreased Huh-7 and HepG2 proliferation rate, as reflected by the reduced proliferative cells in SH3GL1 silencing cells. Consistently, knocking down SH3GL1 inhibited Huh-7 and HepG2 colony formation ability (Fig 2D-E). As colony formation ability represents liver CSCs self-renewal maintenance capability, implying that SH3GL1 might executes crucial roles in sustaining liver CSCs stemness.

Tumor sphere formation assay was performed to investigate the functional role of SH3GL1 in liver CSCs maintenance. We observed that Huh-7 and HepG2 CSCs tumor sphere enrich ability was inhibited following silencing SH3GL1, as indicated by decreased tumor spheres number derived from liver CSCs following silencing SH3GL1 (Fig 2F-G). This observation was further confirmed by *in vitro* limiting dilution assay. As shown in (Fig 2H), targeting SH3GL1 inhibits liver CSCs self-renewal ability. All together, these data indicate that SH3GL1 plays critical roles in liver cancer cell proliferation and sustaining liver CSCs self-renewal.

SH3GL1 activates Wnt/b-catenin signaling

To explore the underlying mechanisms of SH3GL1 potentiates liver CSCs self-renewal, we detected two key downstream genes expression profiles of four key signaling pathways implicated in liver CSCs self-renewal, including Wnt/β-catenin [24], Notch [25], STAT3 [26], Hedgehog [27] signaling pathways. We found that c-Myc and CCND1, which are β-catenin downstream genes, were dramatically decreased following silencing SH3GL1 in Huh-7 and HepG2 cells (Fig 3A-B). On the contrary, silencing SH3GL1 did not affect the expression of Hedgehog, STAT3 and Notch downstream genes (Fig 3A-B), implying that SH3GL1 primes liver tumorigenesis through activating β-catenin signaling. Consequently, SH3GL1 disruption dramatically repressed the expression of β-catenin, c-Myc and CCND1 comparing with scrambled control shRNA (shCtrl) cells in liver CSCs SH3GL1 (Fig 3C). Notably, we explored the expression profile of SH3GL1 and c-Myc and CCND1 in the TCGA dataset and found that SH3GL1 was remarkably associated with c-Myc and CCND1 expression (Fig 3D).

To further investigate the underlying mechanisms that SH3GL1 upregulates β-catenin expression thereby promoting liver tumorigenesis, immunofluorescence assay was employed to determine whether there has any physically interaction of SH3GL1 and β-catenin in two independent HCC specimens. As shown in (Fig 3E), SH3GL1 was co-localized with β-catenin in two clinical HCC specimens (Fig 3F). This finding was further verified by Co-IP assay. As shown in (Fig 3G), SH3GL1 was found to interact with β-catenin in HepG2 CSCs. Immunofluorescence staining for β-catenin in HepG2 CSCs to characterize the biological significance of interaction between SH3GL1 and β-catenin. As shown in (Fig 3H), silencing SH3GL1 decreased total and nuclear part of β-catenin expression in HepG2 CSCs, suggesting that SH3GL1 promotes β-catenin signaling may specifically decrease β-catenin expression. Additionally, TOP-Flash reporter assay was employed to ascertain whether knockdown SH3GL1 affect β-catenin transcriptional activity. As shown in (Fig 3J), disruption SH3GL1 substantially repressed the activation of the TOP-Flash reporter in liver CSCs under the activation of Wnt3A condition. Consistently, knocking down SH3GL1 significantly decreased β-catenin expression (Fig 3K). Taken together, these data demonstrate that SH3GL1 directly and physically interacts with β-catenin, thereby activating β-catenin signaling to potentiate liver tumorigenesis.

Targeting SH3GL1 induces regression on HCC cell growth *in vivo*

We examined the effects of SH3GL1 silencing on the HCC growth *in vivo* using xenograft tumor growth model. A total of 2 X 10^6 Huh-7 cells expressing with SH3GL1 control shRNA or SH3GL1 shRNA were injected into the groin of mice. As expected, xenograft tumors derived from SH3GL1 shRNA cells grow significantly slower than control shRNA tumors (Fig. 4A-B) and tumor weight was also smaller than control tumors (Fig. 4C). Additionally, IHC staining was employed to examine the biological function of SH3GL1 on β-catenin activation. As shown in (Fig. 4D-E), β-catenin staining signal was dramatically decreased in xenograft tumors derived from SH3GL1 shRNA cells than control shRNA tumors. These data strongly support that β-catenin could be activated by SH3GL1 and SH3GL1 plays vital roles in liver tumorigenesis.

Discussion

Tumor initiation and development rely on aberrant activation of oncogenes and inactivation of tumor suppressor genes. Cancer stem cells (CSCs) hypothesis suggest that CSCs are widely accepted to account for tumorigenesis, therapeutic resistance, and tumor recurrence [28]. Several signaling pathways, including, Wnt/b-catenin [24], Notch [25], STAT3 [26], Hedgehog [27], have been deeply investigated to regulate liver CSCs self-renewal or stemness maintenance. The progress in CSCs targeting therapeutic strategies could give us a new approach to human liver cancer treatment [28]. Wnt/β-catenin signaling pathway has been well documented to play essential roles in the development of vertebrate. Thus, it is not clinically applicable to directly target to β-catenin as clinical therapy for liver cancer patients. Highlighting that it is of valuable significance to characterize how it is activated or new downstream effecters of Wnt/β-catenin signaling as promising clinical treatments for liver cancer patients.

Protein with SH3 domain have been well defined to function as key players in cancer initiation and progression[18, 28–31]. Lin *et al.* reported that SH3GL3 expression was dramatically decreased in lung cancer and acts as a novel tumor suppressor in lung tumorigenesis [30]. Another study further revealed that SH3GL3 was downregulated in glioblastoma and potent represses glioblastoma progression by inactivating STAT3 signaling [29]. On the contrary, we first performed analysis on publicly available genomic databases and identified that SH3GL1 expression was convincingly higher in HCC tissues and cells, and functions as a putative oncogene involved in stimulating HCC tumorigenesis and maintain Liver CSCs self-renewal. SH3GL3 was been reported to be downregulated in glioblastoma and epigenetically silenced by EZH2 [29]. Regrettably, no report reveals the underlying principles of SH3GL1 upregulation in HCC and the detail molecular mechanisms of overexpression of SH3GL1 needs to be elaborately studied.

This investigation reveals our novel study on the important functions of SH3GL1 in facilitating liver tumorigenesis and the underlying mechanism. Silencing SH3GL1 attenuates HCC cell growth as well as liver CSC stemness, which is consistent with the functional role in osteosarcoma [32], medulloblastoma [17]. Our study first evaluates SH3GL1 value as a novel molecular therapeutic target. The important significance of SH3GL1 in HCC is evident by the fact that the expression of SH3GL1 is associated with the prognosis of patients with HCC. In this study, we provide evidence that targeting SH3GL1 decreases β-catenin expression and thereby inhibiting β-catenin signaling pathway. We observe that SH3GL1 directly and physically interact with β-catenin, suggesting that SH3GL1 is an upstream regulator of β-catenin signaling. It is therefore to further characterize the binding partners of SH3GL1 to deeply unravel the functional roles of SH3GL1 in liver tumorigenesis. We will attempt to define the potential binding partners to further elaborate the function and identify compounds that specifically and directly decrease SH3GL1/β-catenin signaling axis as potential clinical therapies for liver cancer patients. In summary, our novel findings uncover a new mechanism for dysregulation for canonical Wnt/b-catenin signaling, suggesting SH3GL1 holds potential as a key therapeutic target in liver cancer.

Declarations

Author contribution

C.Z designed the study and wrote the manuscript. L.N and C.Z performed all the experiments and data analysis. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare that they have no potential competing financial interests or personal interest that could have appeared to influence the work reported in this paper.

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Figures

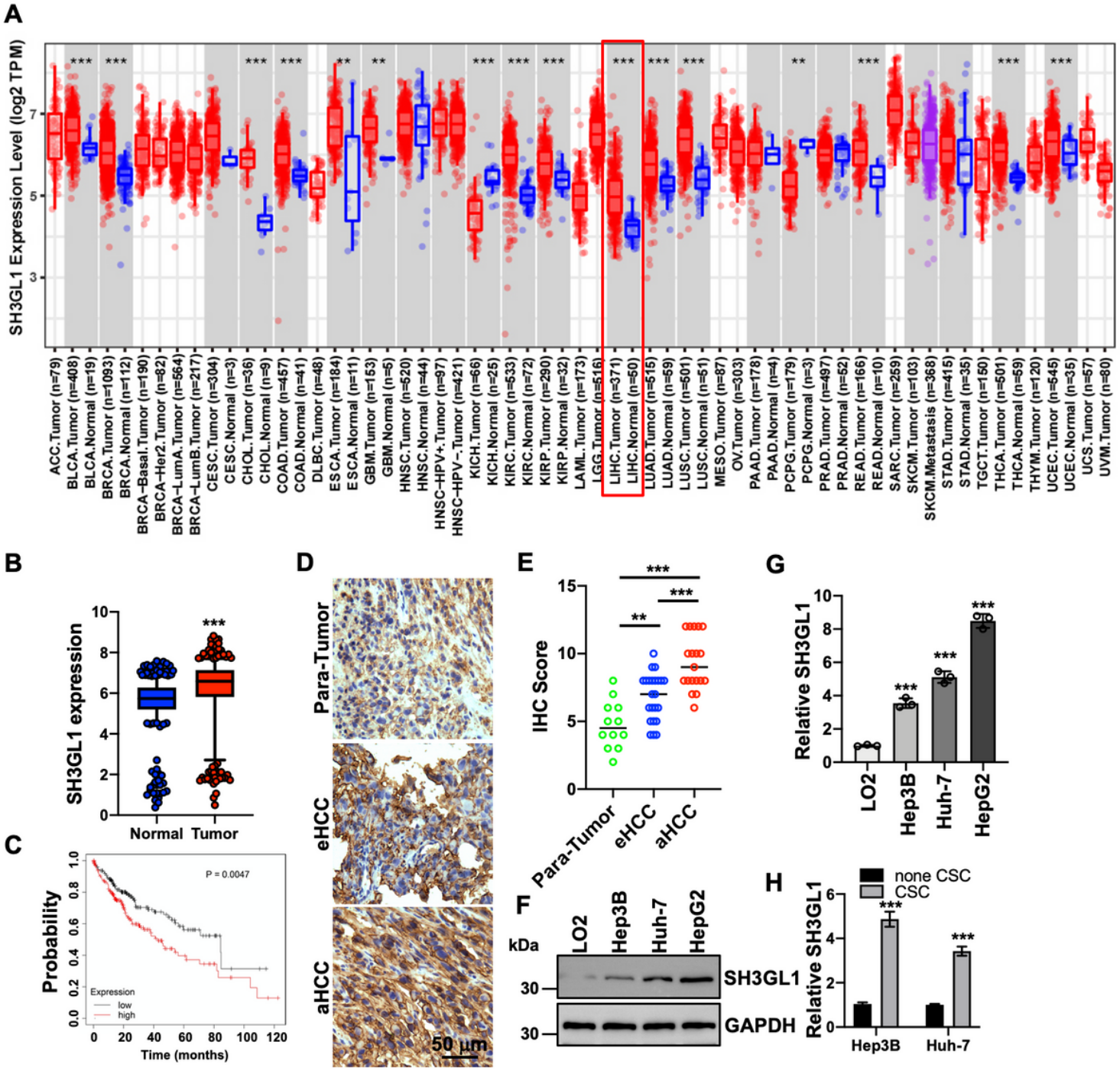

Figure 1

SH3GL1 expression was high in liver cancer. A. The expression pattern of SH3GL1 in various solid tumors. B. SH3GL1 expression was dramatically higher in liver cancer than normal tissues. C. Higher SH3GL1 expression was inversely related with the clinical outcomes of patients with liver cancer. D. The representative images of immunohistochemical staining with SH3GL1 in 60 paired liver cancer and their corresponding normal tissues. Scale bar=50 μm. E. The quantitation results of panel D. F-G. Both mRNA

(panel G) and protein (panel F) expression level of SH3GL1 was dramatically higher in liver cancer cell lines. H. SH3GL1 expression was significantly highly expressed in CSCs. *** indicate p < 0.0001.

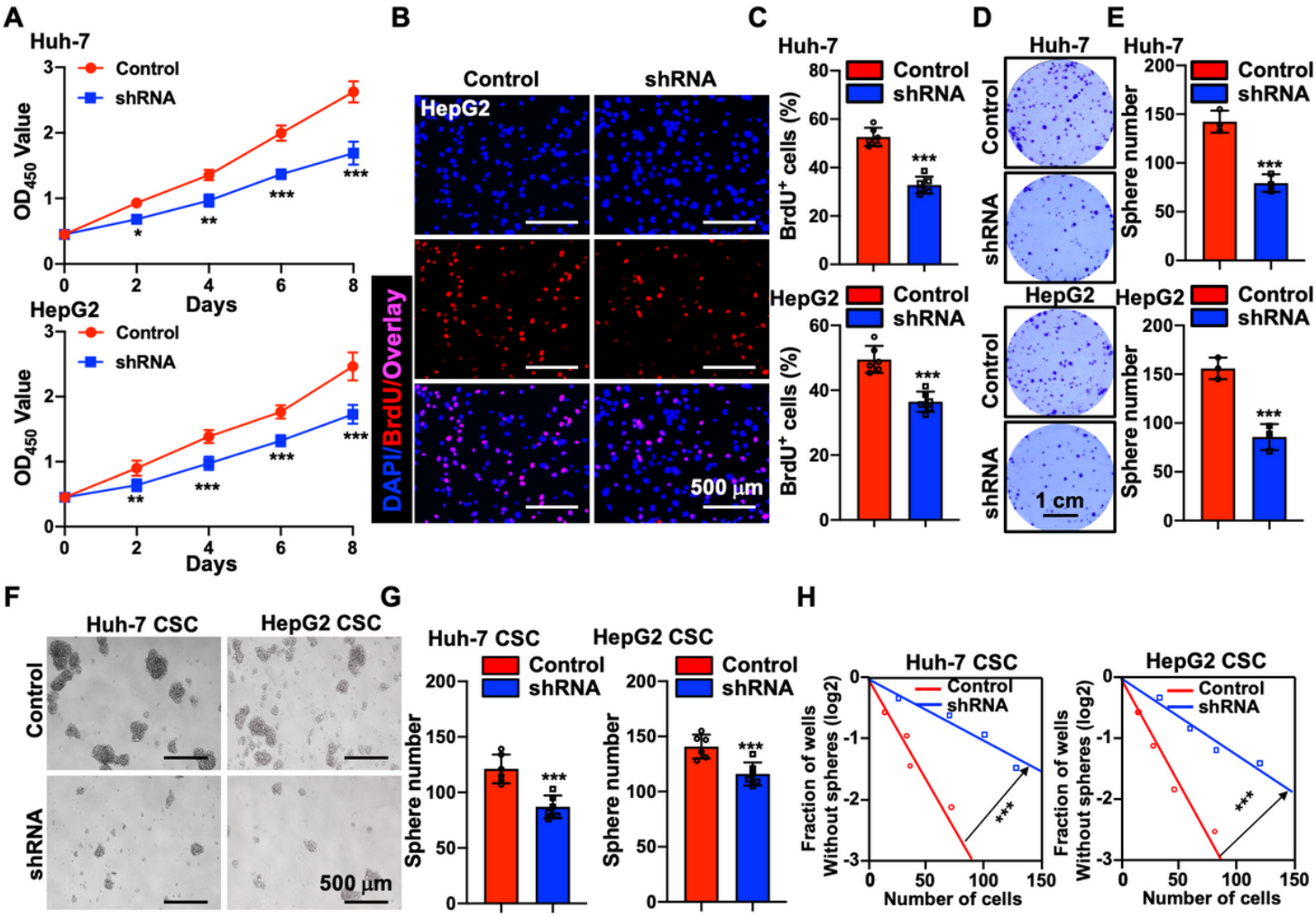

Figure 2

SH3GL1 is essential for sustaining liver CSC stemness. A. Silencing SH3GL1 represses Huh-7 (top panel) and HepG2 (down panel) cells proliferation. B. Silencing SH3GL1 decreases proliferative rate of Huh-7 (top panel) and HepG2 (down panel) cells. Scale bar=500 μm. C. The quantitation data of panel B. D. Silencing SH3GL1 inhibits Huh-7 (top panel) and HepG2 (down panel) colony formation ability. Scale bar=1 cm. E. The quantitation data of panel D. F. Targeting SH3GL1 suppresses liver CSCs self-renewal ability. Scale bar=500 μm. G. The quantitative data of panel F. H. Silencing SH3GL1 represses liver CSCs self-renewal ability assessed by in vitro limiting dilution assay and the quantification results was shown. *, ** and *** indicate p < 0.05, p < 0.01 and p < 0.0001, respectively.

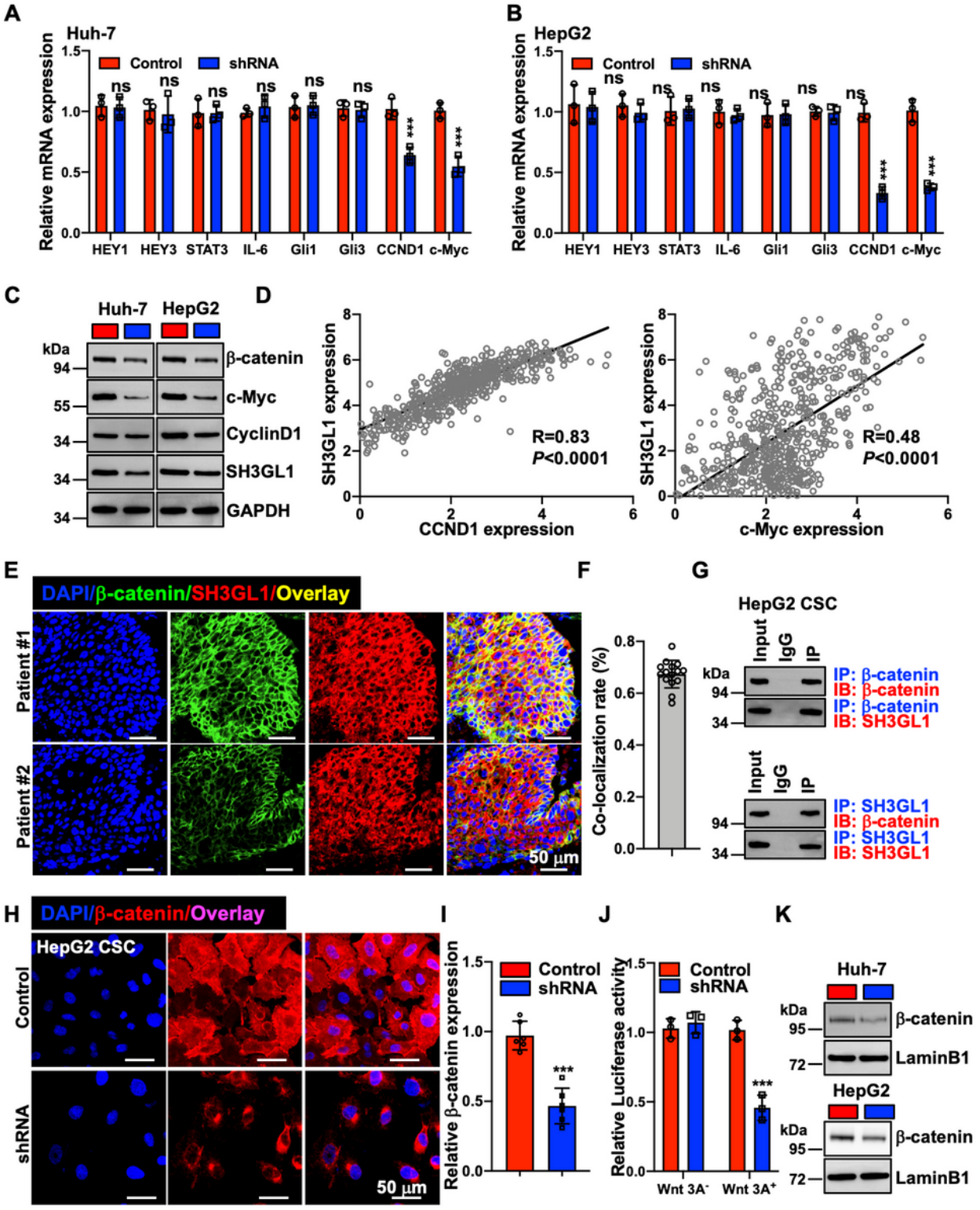

Figure 3

Wnt/β-catenin signaling was blocked by silencing SH3GL1. A-B. Wnt/β-catenin signaling was inhibited following silencing SH3GL1. C. Silencing SH3GL1 suppresses Wnt/β-catenin downstream genes expression. D. SH3GL1 transcript expression was positively correlated with Wnt/β-catenin downstream genes mRNA expression. E. Representative images of immunofluorescence staining for β-catenin and SH3GL1 in HCC clinical specimens. Scale bar=50 μm. F. Co-localization ratio of β-catenin and SH3GL1

shown in panel E. G. SH3GL1 physically interacts with β-catenin determined by Co-IP assay in HepG2 cells. H. The representative images of immunofluorescence staining for β-catenin in SH3GL1 knocking down cells and their corresponding negative control cells. Scale bar=50 mm. I. The quantitative results of panel H. J. Transcriptional activity of β-catenin was dramatically inhibited following silencing SH3GL1. K. Silencing SH3GL1 significantly decreased nuclear β-catenin expression. LaminB1 was used as the of nuclear protein loading control. Control or shRNA suggest that indicated cells expression with control shRNA or SH3GL1 shRNA. *** indicate p < 0.0001.

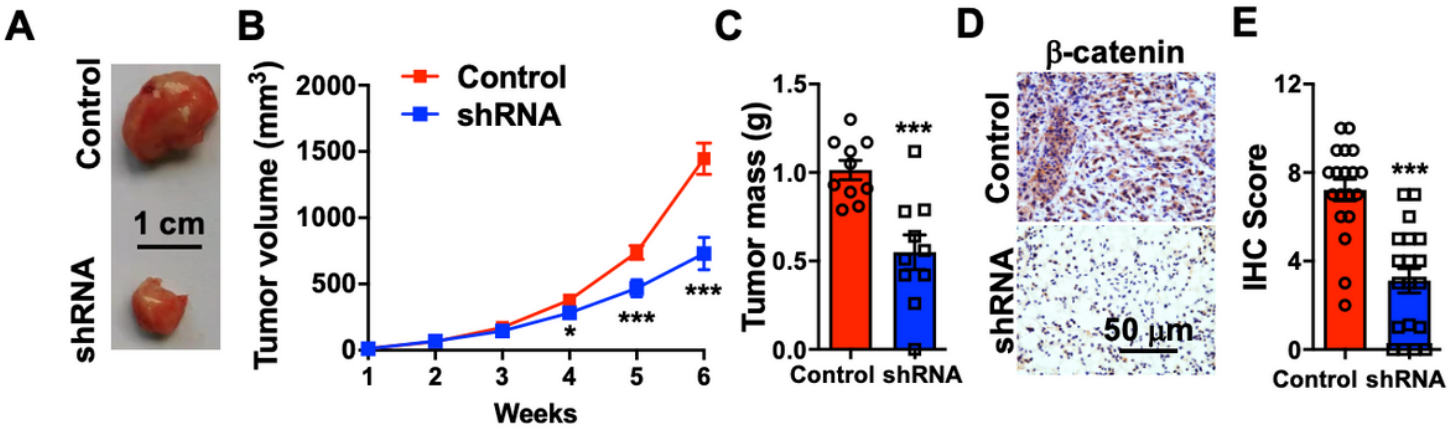

Figure 4

Silencing SH3GL1 induces regression on xenograft tumor growth in vivo. A. The representative images of xenograft tumors derived from Huh-7 cells. Scale bar=1 cm. B. Xenograft tumor volume derived from Huh-7 cells expressing with or without SH3GL1 shRNA. C. Xenograft tumor mass derived from Huh-7 cells expressing with or without SH3GL1 shRNA. D. The representative images of immunohistochemical staining for β-catenin in tumors derived from SH3GL1 control or shRNA cells. Scale bar=50 μm. E. The quantification data pf panel D. Control or shRNA suggest that indicated cells expression with control shRNA or SH3GL1 shRNA, respectively. * and *** indicate p < 0.05 and p < 0.0001, respectively.