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Shp2 Promotes Liver Cancer Stem Cell Expansion by Augmenting β-Catenin Signaling and Predicts Chemotherapeutic Response of Patients

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Src-homology 2 domain–containing phosphatase 2 (Shp2) has been reported to play an important role in the maintenance and self-renewal of embryonic and adult stem cells, but its role in cancer stem cells (CSCs) remains obscure. Herein, we observed high expression of Shp2 in both chemoresistant hepatocellular carcinomas (HCCs) and recurrent HCCs from patients. A remarkable increase of Shp2 was detected in sorted epithelial cell adhesion molecule–positive or cluster of differentiation 133–positive liver CSCs and in CSC-enriched hepatoma spheroids from patients. Up-regulated Shp2 facilitated liver CSC expansion by promoting the dedifferentiation of hepatoma cells and enhancing the self-renewal of liver CSCs. Mechanistically, Shp2 dephosphorylated cell division cycle 73 in the cytosol of hepatoma cells, and the dephosphorylated cell division cycle 73 bound β-catenin and facilitated the nuclear translocation of β-catenin, which promoted the dedifferentiation of hepatoma cells. Shp2 increased β-catenin accumulation by inhibiting glycogen synthase kinase 3β–mediated β-catenin degradation in liver CSCs, thereby enhancing the self-renewal of liver CSCs. Blockage of β-catenin abolished the discrepancy in liver CSC proportion and the self-renewal capacity between Shp2-depleted hepatoma cells and control cells, which further confirmed that β-catenin is required in Shp2-promoted liver CSC expansion. More importantly, HCC patients with low Shp2 levels benefited from transcatheter arterial chemoembolization or sorafenib treatment, but patients with high Shp2 expression did not, indicating the significance of Shp2 in personalized HCC therapy. *Conclusion:* Shp2 could promote HCC cell dedifferentiation and liver CSC expansion by amplifying β-catenin signaling and may be useful in predicting patient response to chemotherapeutics. (HEPATOLOGY 2017;65:1566-1580).

Hepatocellular carcinoma (HCC) is the sixth most common cancer in the world and the second leading cause of cancer death in men.[1] Despite recent advances in hepatic resection and transplantation, the long-term survival of HCC patients remains unsatisfactory due to frequent recurrence after surgical resection and the poor response of patients to conventional chemotherapy including transcatheter arterial chemoembolization (TACE) or the targeted agent sorafenib.[2,3] Accumulating evidence has demonstrated that chemoresistance and recurrence of HCC are closely associated with the

Abbreviations: CD, cluster of differentiation; CDC73, cell division cycle 73; CSC, cancer stem cell; DEN, diethylnitrosamine; EpCAM, epithelial cell adhesion molecule; GSK3β, glycogen synthase kinase 3β; HCC, hepatocellular carcinoma; HGDN, high-grade dysplastic nodule; NOD-SCID, non-obese diabetic–severe combined immunodeficient; PCR, polymerase chain reaction; sh, short hairpin; Shp2, Src-homology 2 domain–containing phosphatase 2; si, small interfering; STAT, signal transducer and activator of transcription; TACE, transcatheter arterial chemoembolization.

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existence of liver cancer stem cells (CSCs).[4,5] Therefore, a more effective therapeutic strategy could be developed if the molecular mechanism underlying CSC regulation is dissected.[6] Nevertheless, the detailed regulatory mechanism of CSC generation and expansion remains far from fully understood.

Src-homology 2 domain–containing phosphatase 2 (Shp2), encoded by *PTPN11*, was cloned in the early 1990s as a nonreceptor protein tyrosine phosphatase that contains two Src-homology 2 domains.[7] Intriguingly, distinct from other tyrosine phosphatases that play a negative role in proliferative signaling activation, Shp2 functions as a positive regulator of cell survival and growth.[8] Shp2 acts as a transducer of extracellular prosurvival and proliferative signals and is required for the full activation of extracellular signal–regulated kinase signaling.[9] Bard-Chapeau et al. generated hepatocyte-specific Shp2 knockout mice and observed that conditional Shp2 ablation dramatically attenuated extracellular signal–regulated kinase activation and liver regeneration following partial hepatectomy.[10] The effect of Shp2 on the modulation of the phosphoinositide 3-kinase/Akt pathway and the Janus kinase/signal transducer and activator of transcription (STAT) cascade could be cell type–specific or stimulus-specific.[11] In addition, genetic and sequencing data have indicated a broad role for Shp2 in development and disease.[12,13] *Ptpn11* mutation was detected in up to 50% of Noonan syndrome patients who possessed a high risk of leukemia.[14] Moreover, somatic mutation-elicited constitutive Shp2 activation was observed in different types of leukemia. Therefore, Shp2 has been considered to be a proto-oncogene in leukemia.[15,16] Given that *Ptpn11* mutation was scarcely detected in solid tumors, there have been studies elucidating the overexpression and pro-oncogenic function of Shp2 in glioblastoma, gastric carcinoma, and breast cancer.[17-19]

Our recent study demonstrated that Shp2 was highly expressed in the majority of human HCCs, while its expression level in normal liver was relatively low.[20] Shp2 overexpression correlated with advanced stage, poor differentiation, and metastasis of liver cancer.[20] We further elucidated that Shp2 could promote HCC growth and metastasis by coordinately activating the Ras/Raf/extracellular signal–regulated kinase pathway and the phosphoinositide 3-kinase/Akt/mammalian target of rapamycin cascade, which indicates the positive role of Shp2 in HCC progression.[20] Interestingly, we previously observed that hepatocyte-specific ablation of *Shp2* facilitated HCC initiation in aged or diethylnitrosamine (DEN)–treated mice, which is consistent with the hepatocyte conditional ablation of other oncogenic molecules including *IKKβ*, *Akt*, *STAT3*, *c-Met*, and *β-catenin*.[21,22] We proposed that hepatocyte-specific deletion of Shp2 promoted hepatic injury/necrosis and enhanced inflammatory signaling through the STAT3 pathway, resulting in regenerative hyperplasia and development of tumors in liver.[21] Actually, all of these pro-oncogenic molecules including *Shp2*, *IKKβ*, *Akt*, *STAT3*, *c-Met*, and *β-catenin* exert a prosurvival role in hepatocytes. Therefore, hepatocyte-specific deletion of any of these molecules in mice increases chronic liver injury and cell death, which triggers secondary inflammation and excessive compensatory hepatocyte proliferation, eventually enhancing HCC development in aged or DEN-treated mice.[22,23] In addition, a recent study demonstrated that hepatocyte-specific Shp2 ablation

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increased the synthesis of bile acid, which was reported to promote Yes-associated protein activation and HCC development.[24,25] Nevertheless, the regulatory role of Shp2 in liver CSCs remains unknown. Herein, we showed that Shp2 promoted liver CSC expansion by augmenting β-catenin activation in distinct patterns between liver cancer cells and liver CSCs. Consistently, Shp2 levels in human HCCs predicted the response of patients to conventional chemotherapy or sorafenib, suggesting that Shp2 might serve as a biomarker for HCC personalized therapy.

Materials and Methods

RECOMBINANT VIRUS AND CELL LINES

HCC cell lines SMMC-7721 and LM3 were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. Adenoviruses expressing Shp2 and green fluorescent protein were purchased from Vigene Bioscience Company (Jinan, China). SMMC-7721 short hairpin (sh) shp2 and LM3 shshp2 stable transfectants were established using the lentivirus expressing shShp2 and scramble control as described.[21,26] Sorafenib-resistant Huh7 and Bel7402 cells have been established.[27]

REAL-TIME POLYMERASE CHAIN REACTION AND WESTERN BLOT

The original amount of the specific transcripts was determined by real-time polymerase chain reaction (PCR) using the ABI PRISM 7300 sequence detector (Applied Biosystems). The primer sequences are listed in Supporting Table S1. Protein extracts of HCC cells or human HCC tissues were analyzed by western blot using an IRDye 800CW–conjugated antibody and the LI-COR imaging system (LI-COR Biosciences).[28] The antibodies used are provided in Supporting Table S2.

SPHEROID FORMATION ASSAY

Hepatoma cells were cultured in a 6-well or 96-well ultra-low attachment culture plate for 7 days, and the number of spheroids formed was counted under the microscope. For fresh clinical tissue specimens, single-cell suspensions of primary HCC were seeded in a six-well ultra-low attachment culture plate and infected with Lenti-shShp2/Lenti-Control. The number of spheroids formed was counted 7 days postinfection.

LIMITING DILUTION ASSAY

SMMC-7721 or LM3 shShp2 and control cells were seeded into 96-well ultra-low attachment culture plates at various cell numbers and incubated for 7 days. CSC proportions were analyzed using Poisson distribution statistics and the L-Calc Version 1.1 software program (Stem Cell Technologies, Inc., Vancouver, Canada) as described.[29]

EXPERIMENTAL ANIMAL MODELS

SMMC-7721 shShp2 and control cells were subcutaneously injected into nonobese diabetic–severe combined immunodeficient (NOD-SCID) mice at distinct cell numbers as indicated. The mice were sacrificed 7 weeks postinoculation, and the CSC proportions were analyzed as described.[29] DEN-induced mouse and rat hepatocarcinogenesis has been described.[28]

PATIENTS AND HCC SAMPLES

HCC tissues were obtained from 301 patients (cohort 1) who underwent surgical resection in the Eastern Hepatobiliary Surgery Hospital in Shanghai, China. The detailed clinical pathological characteristics of the patients are presented in Supporting Table S3. Immunohistochemistry of HCC paraffin sections or tissue microarrays was conducted as outlined.[20] Staining was assessed using Image-scope software (Aperio Technologies, Inc.) according to the percentage of positively stained cells and staining intensity. Disease-free survival was defined as the time interval between the dates of surgery and recurrence; if recurrence was not diagnosed, patients were censored on the date of death or the last follow-up. The procedure of human sample collection and analysis was approved by the Ethics Committee of Eastern Hepatobiliary Surgery Hospital.

TACE THERAPY AFTER SURGERY

A total of 114 HCCs (cohort 2) were collected from the patients who underwent TACE 1-2 months or not after hepatectomy in Eastern Hepatobiliary Surgery Hospital. Inclusion criteria of patients were as follows: preoperative World Health Organization performance status of 0-1; Child-Pugh class A; no distant metastasis, ascites, or encephalopathy; no chemotherapy or

radiotherapy before surgery; curative resection; and resected lesions identified as HCC by a pathologist. The characteristics of HCC patients included in this study are presented in Supporting Table S4. The regimen for the preventive adjuvant TACE consisted of 5-fluorouracil 0.75 g, cisplatin 60 mg, and the emulsion mixed with mitomycin C 16 mg and lipiodol 5 mL. Informed consent was obtained before surgery.

SORAFENIB TREATMENT FOLLOWING SURGICAL RESECTION

A total of 77 HCC samples were randomly retrieved from HCC patients (cohort 3) who underwent curative resection followed by sorafenib treatment in the Eastern Hepatobiliary Surgery Hospital from December 2008 to May 2010. Overall, 71 HCC samples were collected from control patients who underwent curative resection without sorafenib administration. The characteristics of HCC patients included in this study are described in Supporting Table S5. Sorafenib was given to the patients at a dose of 400 mg twice a day. Treatment interruptions and up to two dose reductions (200 mg twice a day and 200 mg once a day) were permitted for drug-related adverse effects. All of the above studies were approved by the Ethical Committee of the Second Military Medical University.

STATISTICAL ANALYSIS

Differences between variables were assessed by a two-tailed Student *t* test or analysis of variance. The patient survival of distinct subgroups was compared by Kaplan-Meier and log-rank analyses. $P < 0.05$ was considered statistically significant.

A description of additional materials and methods can be found in the Supporting Information.

Results

Shp2 EXPRESSION CORRELATES WITH CHEMORESISTANCE AND HCC RECURRENCE

Cisplatin-resistant HCC xenografts were established as described (Fig. 1A; Supporting Fig. S1A).[30] In comparison with control tumors, Shp2 expression was notably increased in the cisplatin-resistant HCC residual, indicating that Shp2 expression was associated with chemoresistance (Fig. 1B). Similarly, sorafenib-resistant HCC cells exhibited higher Shp2 levels than control cells (Supporting Fig. S1B), and enhanced Shp2 expression was detected in sorafenib-resistant HCC residual of a patient-derived xenograft model (Fig. 1C). Shp2 expression in HCC cells was not influenced by short-term treatment with cisplatin or sorafenib, excluding the possibility of direct induction of Shp2 by the two drugs (Supporting Fig. S1C). Coincidentally, we observed that Shp2 expression was notably enhanced in recurrent HCC compared with the primary lesion (Fig. 1D). Clinical investigations showed that patients with HCC recurrence exhibited enhanced Shp2 expression and that the patients with high Shp2 levels possessed a higher risk of HCC recurrence (Fig. 1E). Kaplan-Meier analysis showed that HCC patients with high Shp2 levels displayed a higher recurrence rate than patients with low Shp2 expression (Fig. 1F). Collectively, these data revealed the correlation between Shp2 expression and chemoresistance or HCC recurrence.

Shp2 IS HIGHLY EXPRESSED IN LIVER CSCs

Considering the close association of liver CSCs with chemoresistance and HCC recurrence, we investigated the expression of Shp2 in liver CSCs. Shp2 expression was significantly higher in CSC-enriched hepatoma spheroids compared with monolayer-cultured cells (Fig. 2A). Shp2 expression was further elevated in passaged hepatoma spheroids and could be restored to routine levels during reattachment and differentiation (Fig. 2B; Supporting Fig. S2A). Cluster of differentiation 133–positive ($CD133^+$) or epithelial cell adhesion molecule–positive ($EpCAM^+$) hepatoma cells, which have been considered liver CSCs, exhibited enhanced Shp2 expression compared with the $CD133^-$ or $EpCAM^-$ cells (Fig. 2C,D). Consistently, $CD133^+$ liver CSCs sorted from trypsinized spheroids of hepatoma cells displayed even higher Shp2 levels (Supporting Fig. S2B). As expected, expression of Shp2 in $CD133^+EpCAM^+$ HCC cells was significantly higher than that in control cells (Supporting Fig. S2C). Expression of Shp2 was significantly higher in spheroids than in monolayer-cultured primary HCC cells from patients (Fig. 2E). Interestingly, the correlation between Shp2 expression and the levels of CD133, EpCAM, Oct4, or Nanog was observed in spheroids derived from freshly isolated human HCC cells (Fig. 2F; Supporting Fig. S2D-F).

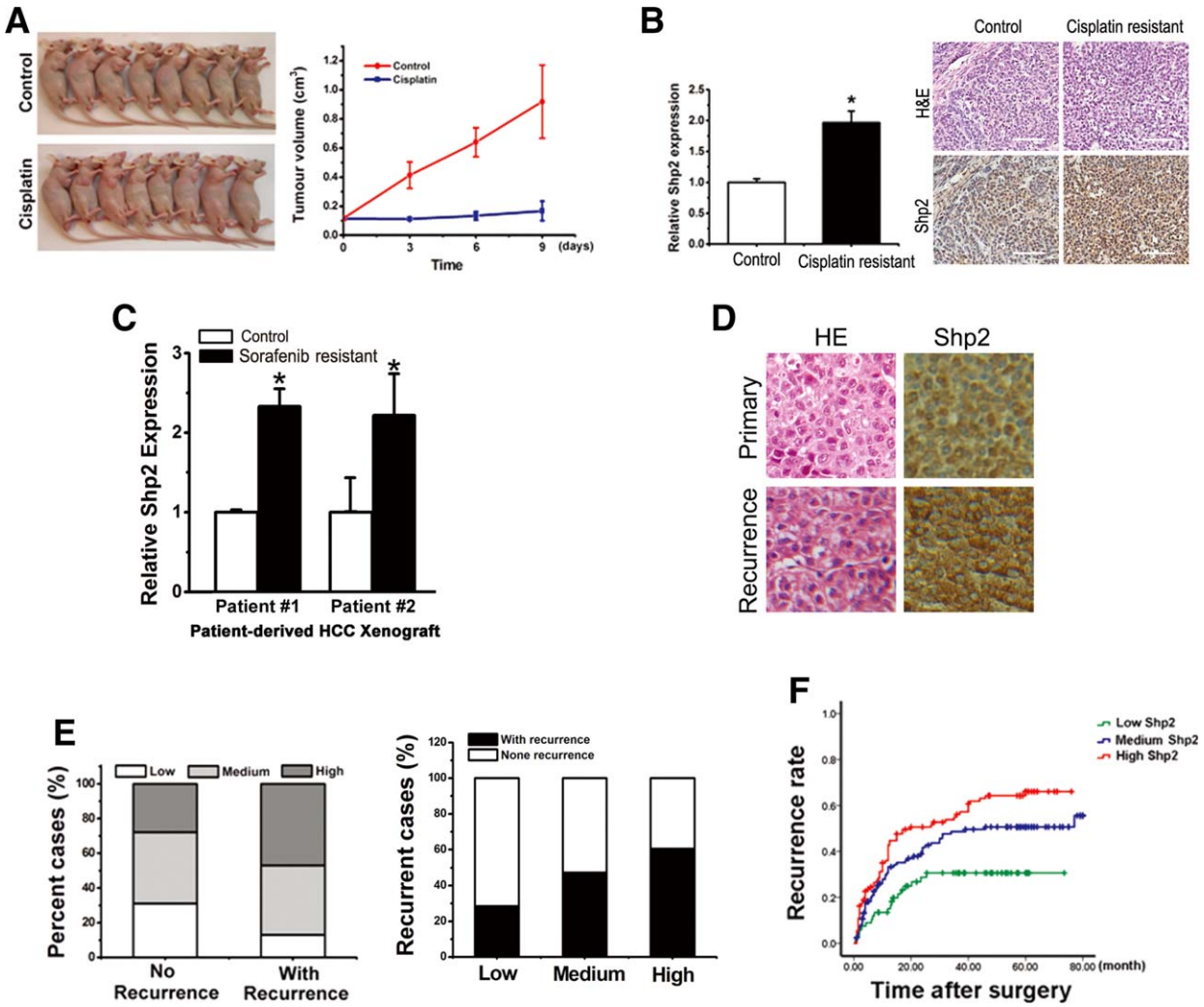


FIG. 1. Shp2 expression is associated with chemoresistance and recurrence of HCC. (A) SMMC-7721 cells (2×10^5) were subcutaneously inoculated into nude mice as a standard protocol. The SMMC-7721 cell–derived xenograft tumor was cut into 0.1 cm^3 volume and subcutaneously inoculated into 16 nude mice. The mice were then separated into a cisplatin group and a phosphate-buffered saline control group. Mice in the cisplatin group were administrated 2 mg/kg of cisplatin by intratumoral injection every other day five times. The residue tumor tissues enriched with cisplatin-resistant cells were resected for the following examination.[30] (B) Real-time PCR and immunohistochemical assays were performed to examine the expression of Shp2 in the cisplatin-resistant HCC xenografts. Magnification ×100. (C) Patient-derived xenografted HCCs were inoculated subcutaneously in nude mice, followed by sorafenib (30 mg/kg) treatment. Shp2 levels in xenografted tumors before (control) and after (resistant) sorafenib treatment were compared by real-time PCR. (D) Shp2 expression was compared in recurrent HCC and primary lesions of patient. Magnification ×400. (E) Immunohistochemical staining of Shp2 in 301 HCC patients was performed as described in Materials and Methods, and the expression levels were classified as low (0-0.2), medium (0.2-0.4), and high (0.4-0.6), according to the staining intensity. The correlation of Shp2 expression and HCC recurrence was analyzed. (F) Kaplan-Meier analysis revealed that HCC patients with Shp2 overexpression exhibited a higher recurrence rate ($P < 0.05$). All experiments were repeated at least three times, and representative data are shown. *$P < 0.05$. Abbreviation: H&E, hematoxylin and eosin.

Shp2 PROMOTES THE EXPANSION OF LIVER CSCs IN HEPATOMA CELLS

To explore the role of Shp2 in liver CSC regulation, shShp2-stable transfectants of hepatoma cells were used. Flow-cytometric analysis revealed a diminished proportion of liver CSCs in shShp2 stably transfected hepatoma cells (Fig. 3A). Hepatoma cells depleted of Shp2 expression formed fewer spheroids than control cells (Supporting Fig. S3A). Moreover, a dominant-negative mutant of Shp2[31] was transfected into

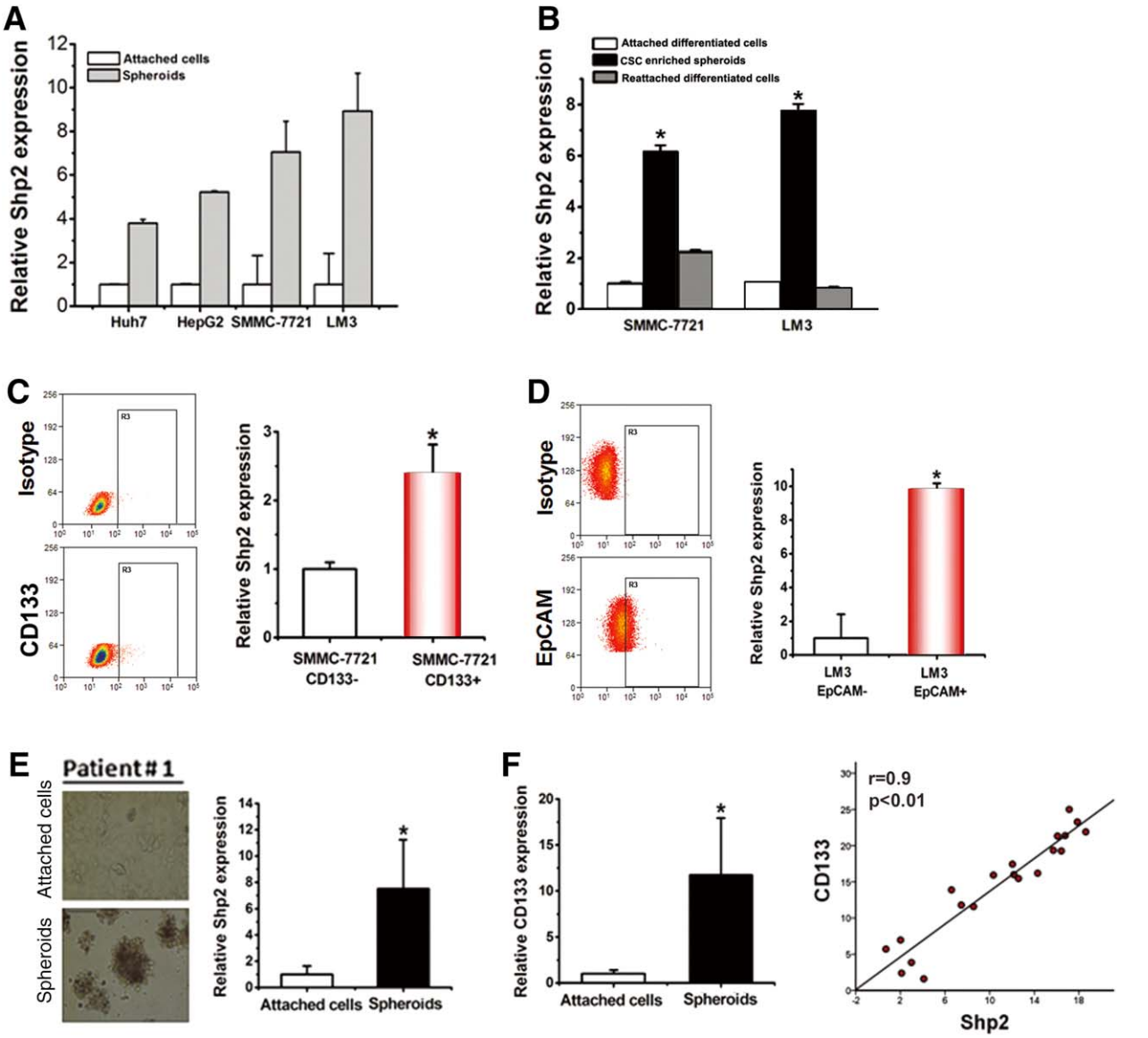
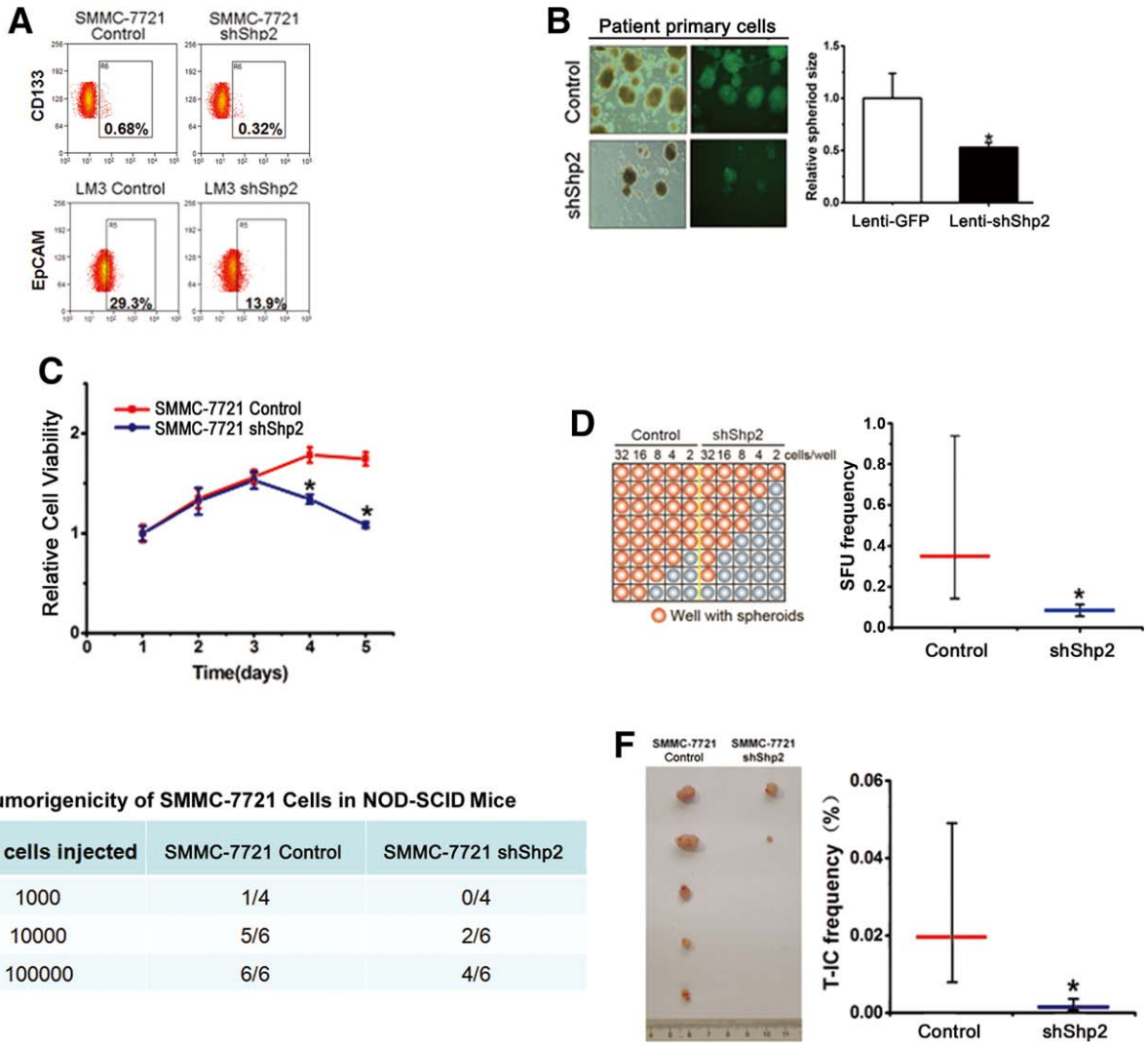


FIG. 2. Shp2 levels are elevated in liver CSCs. (A) Distinct HCC cell lines were cultured in monolayer or ultra-low attachment conditions. Shp2 expression in spheroids and attached cells was compared by real-time PCR. (B) Hepatoma cell–derived spheroids were trypsinized and cultured in attachment conditions. Shp2 expression in spheroids versus reattached cells was compared by real-time PCR. (C) $CD133^+$ HCC cells were sorted and subjected to real-time PCR assay. (D) $EpCAM^+$ HCC cells were sorted and subjected to real-time PCR assay. (E) HCC cells isolated from fresh HCC tissues were cultured in monolayers or ultra-low attachment conditions. The expression of Shp2 was analyzed by real-time PCR. (F) Significant correlation was observed between Shp2 and CD133 expression in the spheroids derived from freshly isolated patient HCC cells. All experiments were repeated at least three times, and representative data are shown. $^*P < 0.05$.

hepatoma cells, and consistent results were achieved (Supporting Fig. S3B,C). In addition, primary cultured patient hepatoma cells infected by lentivirus expressing shShp2 exhibited reduced spheroid formation compared to control cells (Fig. 3B), which further indicates that Shp2 depletion decreased the proportion of liver CSCs. As expected, the interference of Shp2 expression potently increased the sensitivity of



No. of cells injected	SMMC-7721 Control	SMMC-7721 shShp2
1000	1/4	0/4
10000	5/6	2/6
100000	6/6	4/6

FIG. 3. Shp2 depletion suppresses the self-renewal of liver CSCs. (A) The proportion of $CD133^+$ or $EpCAM^+$ HCC cells in Shp2 knockdown transfectants was evaluated by flow-cytometric assay. (B) Spheroid formation assay of patient primary hepatoma cells infected by lentivirus expressing green fluorescent protein and shShp2 or green fluorescent protein alone. A representative picture is shown. (C) SMMC-7721 shShp2 and control cells cultured in 96-well plates were treated with 1 μg/mL cisplatin, and cell viability was measured at the indicated time points using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). (D) The frequency of liver CSCs in SMMC-7721 shShp2 and control cells was compared by *in vitro* limiting dilution assay. (E,F) As for the *in vivo* limiting dilution assay, SMMC-7721 shShp2 and control cells were injected subcutaneously in NOD-SCID mice, and tumor incidence was evaluated at 7 weeks postinoculation. A representative picture is shown. All experiments were repeated at least three times, and representative data are shown. Abbreviations: GFP, green fluorescent protein; SFU, spheroid formation unit; T-IC, tumor initiating cell.

hepatoma cells to conventional chemotherapeutics (Fig. 3C; Supporting Fig. S3D). An *in vitro* limiting dilution assay illustrated that Shp2 depletion dramatically decreased the CSC population in hepatoma cells (Fig. 3D; Supporting Fig. S3E). Moreover, hepatoma cells expressing shShp2 displayed an attenuated tumor initiation capacity in NOD-SCID mice compared with control cells (Fig. 3E,F).

Shp2 PROMOTES DEDIFFERENTIATION OF HCC CELLS AND ENHANCES SELF-RENEWAL OF LIVER CSCs

It has been accepted that expansion of CSCs in a cancer cell population could be the result of dedifferentiation of cancer cells or enhanced self-renewal of

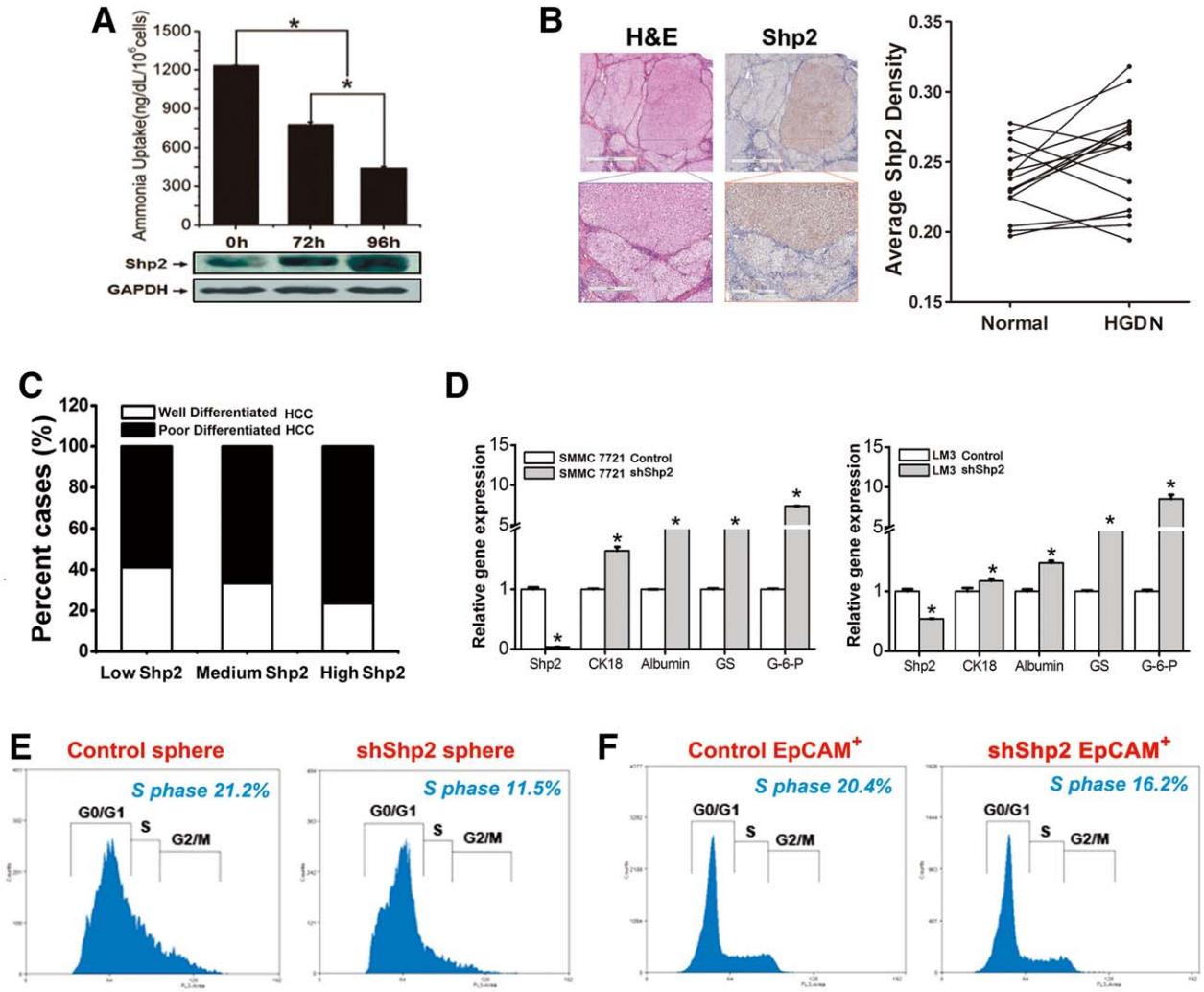


FIG. 4. Shp2 facilitates dedifferentiation of hepatoma cells and self-renewal of liver CSCs. (A) Primary rat hepatocytes were isolated as described previously and cultured on plates precoated with a solution of 250 μg/mL of rat tail collagen I for monolayer cultures, followed by ammonia uptake assay and western blot assay. (B) Shp2 levels were compared in 15 HGDNs and paired adjacent normal liver tissues by immunohistochemical staining. (C) Among 90 HCC patients, correlation between HCC differentiation status and Shp2 levels was analyzed. (D) SMMC-7721 shShp2 or LM3 shShp2 and their control cells were collected and subjected to real-time PCR. The primer sequences are listed in Supporting Table S1. (E) Spheroids from LM3-shShp2 cells were trypsinized, followed by flow-cytometric assay. (F) The cell cycle transition of EpCAM$^+$ LM3 cells was examined by Moflo XDP Coulter. All experiments were repeated at least three times, and representative data are shown. Abbreviations: CK18, cytokeratin 18; G-6-P, glucose 6-phosphate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GS, glycogen synthase; H&E, hematoxylin and eosin.

CSCs.[32] Primary cultured hepatocytes usually undergo a time-dependent dedifferentiation process after isolation.[33] Interestingly, Shp2 levels were progressively increased in parallel with hepatocyte dedifferentiation, indicating a potential role of Shp2 in hepatocyte dedifferentiation (Fig. 4A; Supporting Fig. S4A,B). High-grade dysplastic nodules (HGDNs) are the most dedifferentiated precancerous lesions, and the majority of human HCCs arise from HGDNs. Our data showed that Shp2 expression was significantly upregulated in 11 of 15 HGDNs, suggesting that Shp2 could be involved in the dedifferentiation process (Fig. 4B). Moreover, the proportion of poorly differentiated HCCs was significantly elevated concomitant with the increase of Shp2 levels (Fig. 4C; Supporting Fig. S4C), supporting a negative role of Shp2 in HCC differentiation. More importantly, Shp2 depletion upregulated the expression of hepatocyte-specific genes

and down-regulated the stemness-associated transcription factors in hepatoma cells (Fig. 4D; Supporting Fig. S4D). The uptake of ammonia for the production of urea is a unique function of hepatocytes.[34] Well-differentiated HCC cells could, to some extent, exert this hepatic function, while poorly differentiated HCC cells largely lose this ability. Shp2 overexpression reduced the urea production capability of hepatoma cells, further indicating the promoting effect of Shp2 in the dedifferentiation of hepatoma cells (Supporting Fig. S4E). Consistently, overexpression of the catalytically inactive Shp2 mutant increased the expression of hepatocyte-specific genes and decreased the stemness-associated transcription factors in hepatoma cells (Supporting Fig. S4F,G). Shp2 depletion significantly down-regulated the stemness-associated transcription factors in $EpCAM^+$ liver CSCs (Supporting Fig. S4H). In a further study, our data revealed that the interference of Shp2 reduced the S-phase proportion of liver CSCs in CSC-enriched spheroids and $EpCAM^+$ CSC population, which indicates that Shp2 could enhance the self-renewal of liver CSCs (Fig. 4E,F).

Shp2 ENHANCES β-CATENIN NUCLEAR TRANSLOCATION BY DEPHOSPHORYLATING CELL DIVISION CONTROL PROTEIN 73

Overexpression of Shp2 activated the luciferase reporter of β-catenin and the interference of Shp2 suppressed expression of the putative β-catenin target genes in hepatoma cells (Fig. 5A; Supporting Fig. S5A). Moreover, the nuclear translocation of β-catenin was significantly reduced in Shp2-depleted hepatoma cells (Fig. 5B). Consistently, forced Shp2 expression increased the nuclear accumulation of β-catenin in hepatoma cells (Supporting Fig. S5B). No obvious change in glycogen synthase kinase 3β (GSK3β) phosphorylation was detected in shShp2-stable transfectants compared with control cells (Supporting Fig. S5C). Importantly, a substrate analysis of Shp2 illustrated that cell division control protein 73 (CDC73) was dephosphorylated by Shp2 in hepatoma cells (Fig. 5C). A coimmunoprecipitation assay demonstrated the interaction between CDC73 and β-catenin (Fig. 5D). Dominant active mutation of Shp2 decreased the phosphorylation of CDC73 and enhanced activation of the β-catenin reporter, suggesting that the positive regulation of β-catenin by Shp2 in hepatoma cells depends on the phosphatase activity of Shp2 (Supporting Fig. S5D). A correlation between Shp2 up-regulation and β-catenin activation was observed in murine premalignant lesions and HCCs triggered by DEN exposure (Supporting Fig. S5E,F) as well as in HCC tissues from patients (Supporting Fig. S5G,H). To investigate the role of β-catenin in Shp2-mediated dedifferentiation of hepatoma cells, small interference RNA of β-catenin (si-β-catenin) was used (Supporting Fig. S5I,J). Shp2 depletion–mediated up-regulation of hepatocyte-specific genes and down-regulation of stemness-associated transcription factors were abrogated in hepatoma cells transfected with si-β-catenin (Fig. 5E; Supporting Fig. S5K). Consistently, Shp2-mediated suppression of urea production was abolished in hepatoma cells upon β-catenin inhibitor pretreatment or si-β-catenin transfection (Supporting Fig. S5L,M). As expected, Shp2 depletion not only suppressed β-catenin nuclear translocation but also enhanced the differentiation status of xenografted HCCs *in vivo* (Fig. 5F). Collectively, these data suggest that Shp2 augmented β-catenin activation through dephosphorylating CDC73 and thus promoting the dedifferentiation of hepatoma cells.

Shp2 PROMOTES β-CATENIN ACCUMULATION BY GSK3β IN LIVER CSCs

Given that Shp2 dephosphorylated phosphorylated CDC73 in hepatoma cells contributing to the β-catenin nuclear translocation, phosphorylation of CDC73 was extremely low in liver CSCs, indicating a distinct regulatory mechanism (Fig. 6A). Shp2 interference slightly reduced β-catenin levels in attached hepatoma cells but significantly reduced β-catenin expression in liver CSCs (Fig. 6B). Furthermore, Shp2 depletion notably suppressed the phosphorylation of $GSK3\beta^{Ser9}$ instead of $GSK3\beta^{Y216}$, indicating a canonical regulation of β-catenin stability by Shp2 in liver CSCs (Fig. 6C). Blockage of β-catenin by specific β-catenin inhibitor or siRNA abolished the difference in liver CSC proportion between shShp2 transfected hepatoma cells and control cells (Fig. 6D; Supporting Fig. S6A). Consistently, β-catenin inhibitor or siRNA entirely depleted the discrepancy of self-renewal capacity between Shp2 knockdown cells and control cells (Supporting Fig. S6B,C). Furthermore, β-catenin inhibitor or siRNA abrogated the Shp2-enhanced self-renewal of liver CSCs (Fig. 6E; Supporting Fig. S6D), indicating that β-catenin was required in Shp2-

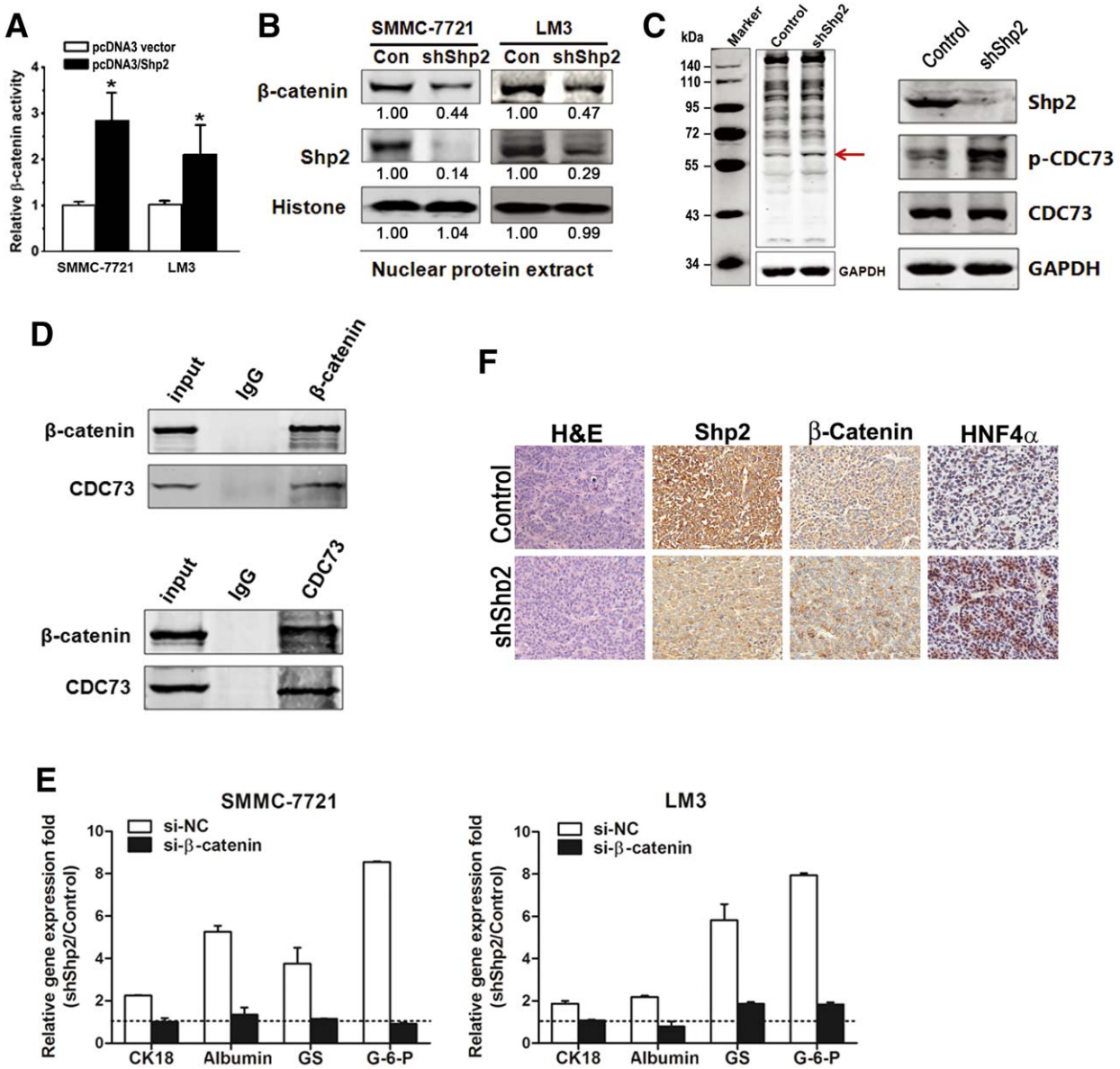


FIG. 5. Dephosphorylation of CDC73 by Shp2 facilitates *β*-catenin nuclear translocation. (A) SMMC-7721 cells and LM3 cells expressing *β*-catenin luciferase reporter were transfected with pcDNA3/Shp2 plasmid or vector control, followed by luciferase assay. Black bar indicates partial complementary DNA3/Shp2, and empty bar indicates vector control. (B) Nuclear protein of shShp2 stable transfectants was extracted and subjected to western blot assay. (C) Whole-cell lysates of SMMC-7721 shShp2 and control cells were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted by pY20 antibody (left) and antibodies as indicated (right). (D) Coimmunoprecipitation was performed as indicated, followed by immunoblotting. (E) SMMC-7721 shShp2 or LM3 shShp2 and their control cells were transfected with si-*β*-catenin or negative control, respectively, followed by real-time PCR assay. The results showed the relative gene expression fold of shShp2 cells versus control cells at the presence of negative control or si-*β*-catenin. (F) Immunohistochemical staining of Shp2 and *β*-catenin in the xenografted HCC of NOD-SCID mice inoculated with SMMC-7721 shShp2 or control cells. Magnification ×100. *Significant difference between the two groups ($P < 0.05$). All experiments were repeated at least three times, and representative data are shown. Abbreviations: CK18, cytokeratin 18; G-6-P, glucose 6-phosphate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GS, glycogen synthase; H&E, hematoxylin and eosin; HNF4*α*, hepatocyte nuclear factor 4*α*; IgG, immunoglobulin G; NC, negative control.

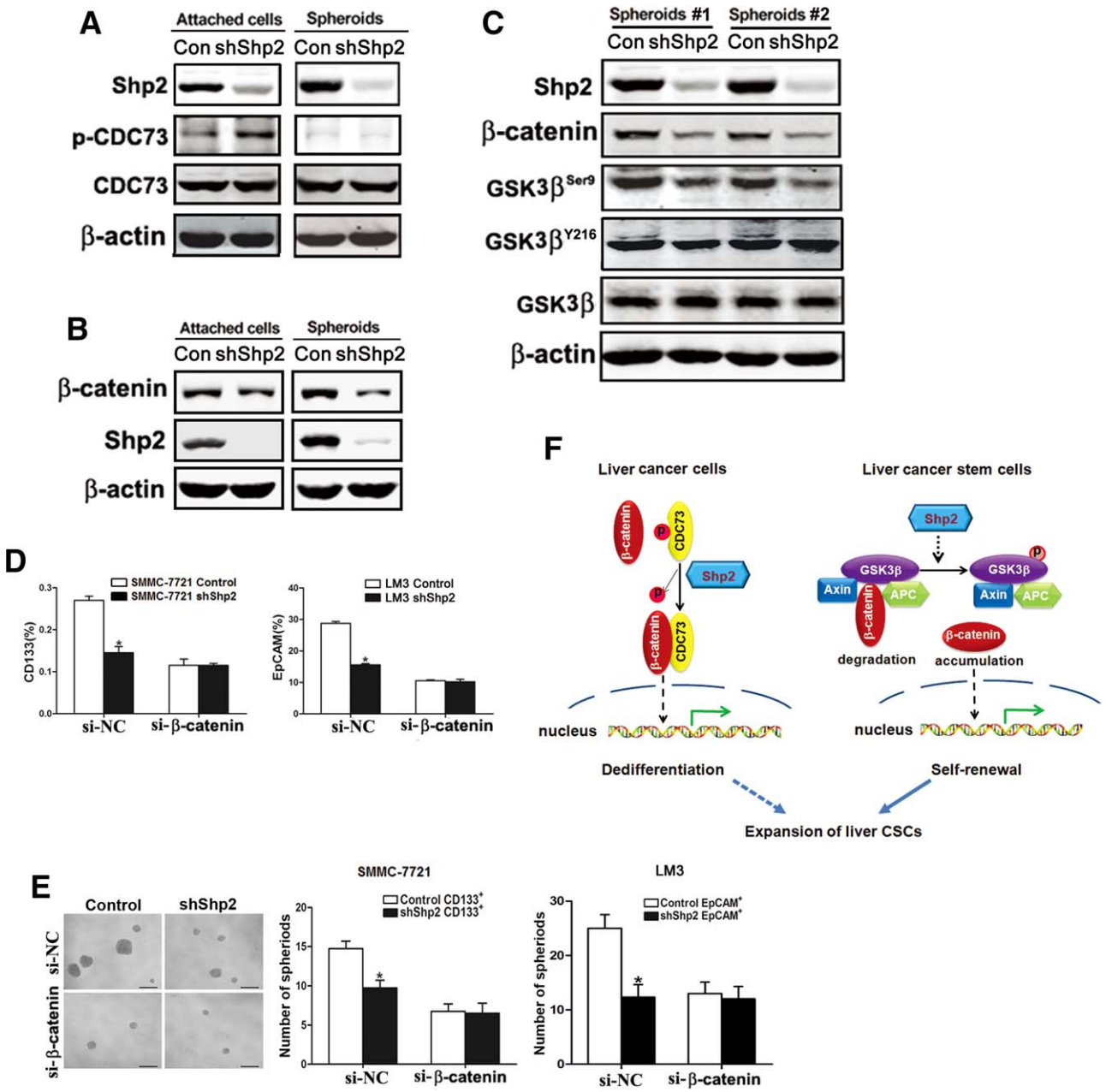


FIG. 6. Shp2 activates *β*-catenin in a GSK3*β*-dependent manner in liver CSCs. (A) Attached and spheroid SMMC-7721 shShp2 and control cells were subjected to western blot assay. (B) Expression of Shp2 and *β*-catenin was determined in attached and spheroid SMMC-7721 shShp2 and control cells. (C) Spheroids from SMMC-7721 shShp2 and control cells were examined by western blot assay. (D) SMMC-7721 shShp2 or LM3 shShp2 and their control cells were transfected with si-*β*-catenin or negative control, respectively, followed by flow-cytometric assay. (E) SMMC-7721 shShp2 or LM3 shShp2 and their control cells were transfected with si-*β*-catenin or negative control, respectively. $CD133^+$ or $EpCAM^+$ liver CSCs were sorted by flow cytometry and subjected to spheroid formation assay. (F) Schematic representation of distinct regulation of *β*-catenin by Shp2 in liver cancer cells and CSCs. All experiments were repeated at least three times, and representative data are shown. Abbreviations: APC, antigen-presenting cell; NC, negative control.

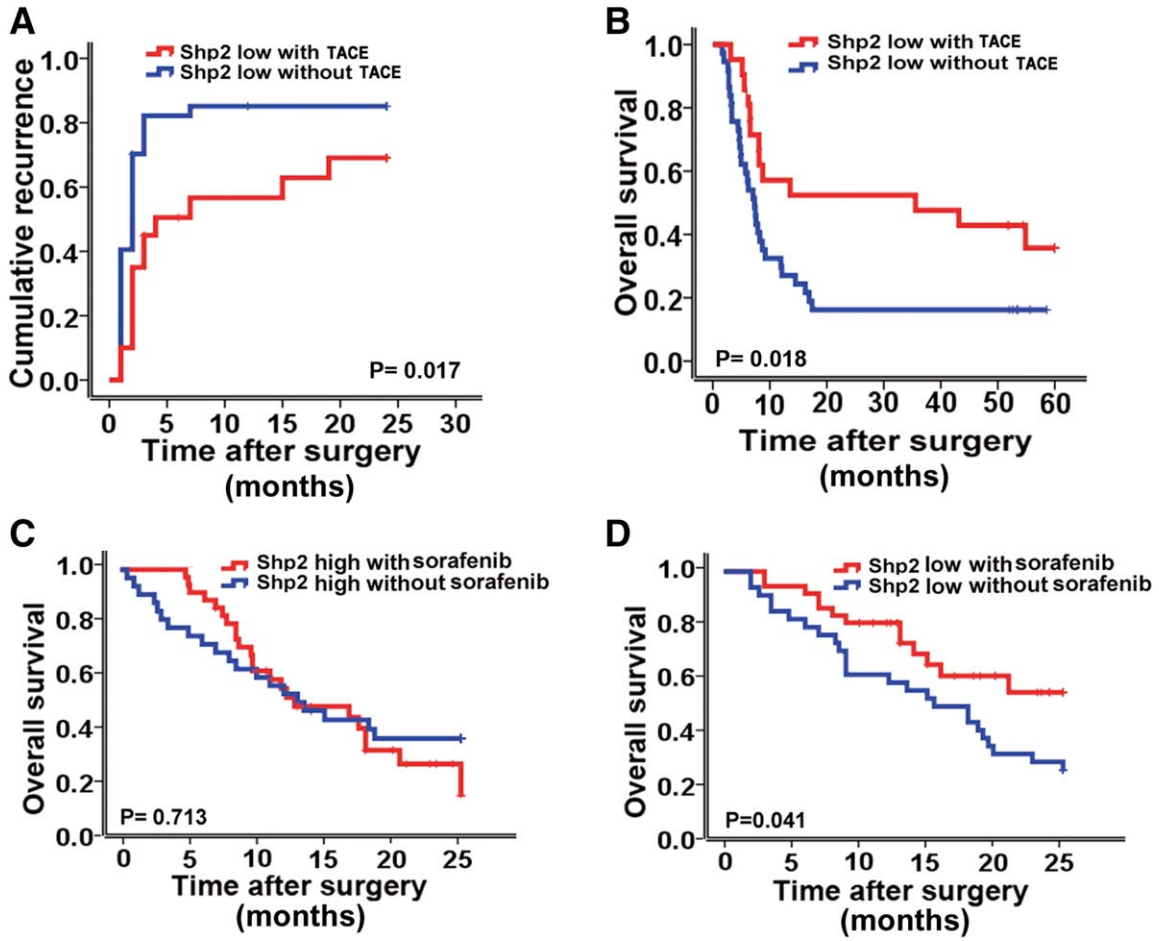


FIG. 7. Low Shp2 levels predict patient response to chemotherapy. (A) Recurrence rates of HCC patients with low Shp2 levels treated with TACE (21) or not (36) after surgery were compared using Kaplan-Meier analysis. (B) Overall survival rates of HCC patients with low Shp2 levels treated with TACE (21) or not (36) after surgery were compared using Kaplan-Meier analysis. (C) Overall survival rates of HCC patients with high Shp2 levels treated with sorafenib (38) or not (35) after surgical resection were compared using Kaplan-Meier analysis. (D) Overall survival rates of HCC patients with low Shp2 levels treated with sorafenib (39) or not (36) after surgery were compared using Kaplan-Meier analysis.

promoted liver CSC expansion. Taken together, these data suggest distinct regulation of β-catenin by Shp2 in liver CSCs and nonstem liver cancer cells (Fig. 6F).

LOW Shp2 EXPRESSION IS ASSOCIATED WITH PATIENT RESPONSE TO CHEMOTHERAPEUTICS

Considering the importance of liver CSCs in HCC chemoresistance and the role of Shp2 in liver CSC expansion, we further investigated the significance of Shp2 in patient response to conventional chemotherapeutics and the targeted agent sorafenib. HCC patients with low Shp2 expression displayed less recurrence and longer survival time following TACE treatment, while patients with high Shp2 levels exhibited no response to TACE therapy (Fig. 7A,B; Supporting Fig. S7). In addition, HCC patients with low Shp2 levels benefited from sorafenib administration, while patients with high Shp2 expression did not (Fig. 7C,D). Collectively, these data suggest that Shp2 could serve as a biomarker for patient response to chemotherapeutics, which is worthy of extended clinical investigation.

Discussion

Numerous HCC patients are diagnosed at an advanced stage and thus miss the opportunity for surgical treatment. Conventional chemotherapy including TACE is largely inefficient in these patients due to the chemoresistance of HCC.[2] For those patients who undergo surgical resection, frequent recurrence also leads to poor survival. The existence of liver CSCs is considered to be the origin of chemoresistance and HCC recurrence. It is therefore important to decipher the molecular mechanism underlying liver CSC regulation so as to develop novel therapeutic strategies targeting CSCs. In this study, we report that Shp2 plays a pivotal role in liver CSC expansion and may serve as a therapeutic target in personalized treatment of HCC.

It is proposed that CSCs could be enriched by chemotherapy due to their unique survival mechanisms.[30] In the current study, liver CSCs were enriched by establishing chemoresistant HCC xenograft tumors, and expression of Shp2 in these chemoresistant xenografts was notably up-regulated. More intriguingly, elevated Shp2 expression in recurrent HCC and a correlation between Shp2 levels and HCC recurrence were observed. Considering the importance of CSCs in tumor recurrence and chemoresistance, we investigated the influence of Shp2 on liver CSCs. Spheroid culture of cancer cells is a routine approach to enrich CSCs. We noted that Shp2 expression was highly expressed in hepatoma spheroids and was even higher in serially passaged spheroids. To date, EpCAM and CD133 have been accepted as the predominant biomarkers of liver CSCs, which also include CD90, CD24, CD44, CD13, aldehyde dehydrogenase, and OV6.[30] We found that $CD133^+$ cancer cells were present in most patient HCC tissues, but the proportion of these cells was around 0.1%-1%.[35] Positive expression of EpCAM was detected in ~40% of patient HCC tissues. The proportion of $EpCAM^+$ cancer cells varied from case to case, and most $EpCAM^+$ cases displayed >25% positive cells.[36] Our data showed that Shp2 levels increased in $EpCAM^+$ or $CD133^+$ liver CSCs and correlated with the expression of liver CSC markers.

Shp2 has been reported to play a prosurvival role in trophoblast stem cells, and homozygous inactivation of Shp2 led to early embryonic lethality in mice.[37] Deletion of Shp2 also caused the aberrant differentiation and death of neural stem cells[38]; in contrast, Shp2 deficiency enhanced the self-renewal of embryonic stem cells.[39] We also reported that Shp2 was required for the maintenance of hematopoietic stem cells in a gene dosage–dependent and cell-autonomous manner.[30,40] Nevertheless, the role of Shp2 in CSCs remains largely unclear. In the present study, we demonstrated that Shp2 promotes the dedifferentiation of hepatoma cells and enhances the self-renewal of liver CSCs. These findings indicate that Shp2 is critical for liver CSC expansion and that targeting Shp2 could be a promising strategy for HCC therapy.

It has been reported that overexpression of certain stemness-associated genes such as ZEB1,[41] transforming growth factor-β1,[42] Nanog,[43] and Oct4[44] could induce the dedifferentiation of cancer cells into CSCs. A recent study demonstrated that ATOH8 reduction was associated with HCC dedifferentiation and that HCC cells with ATOH8 depletion acquired a particular CSC property.[32] Aberrant β-catenin activation is frequently detected in human HCCs and correlates with HCC dedifferentiation.[45,46] Hyperactivation of β-catenin was also reported to increase the liver CSC population and influence the hierarchy of human HCCs.[47,48] Consistently, c-Myc, the major target gene of β-catenin, is closely associated with stemness regulation and might serve as a switch for HCC progression or differentiation.[49,50] Holczbauer et al. reported that c-Myc was required for the oncogenic dedifferentiation of hepatocyte and that knockdown of c-Myc in transformed hepatocytes reduced their CSC properties.[51] In the present study, we observed the positive regulation of β-catenin by Shp2 in liver cancer cells, which should be at least partially responsible for Shp2-mediated dedifferentiation of liver cancer cells into liver CSCs. Indeed, our data suggested that Shp2 could up-regulate stemness-associated transcription factors including c-Myc, Nanog, and Lin28. It was previously reported that the association of β-catenin and CDC73, a speculated substrate of Shp2 in the nucleus, epigenetically activated the transcription of Wnt/β-catenin target genes in AGS gastric cancer cells.[52] We speculated that Shp2 dephosphorylated CDC73 in the cytoplasm and facilitated the interaction and nuclear translocation of CDC73 and β-catenin, promoting the dedifferentiation of liver cancer cells. We also found that Shp2 enhanced β-catenin accumulation through the canonical Wnt/β-catenin pathway in liver CSCs, which led to the enhanced self-renewal of liver CSCs. To our knowledge, this is the first report concerning the distinct regulation and functions of one signaling cascade in cancer stem and nonstem cells, which synergistically promotes CSC expansion. This notion, we believe, could be extended to other signaling pathways and cancer types.

Our clinical investigation revealed that HCC patients with low Shp2 expression exhibited superior response to TACE treatment following surgical resection, but patients with high Shp2 levels showed no response. This observation was consistent with the promoting role of Shp2 in liver CSC expansion. We previously reported that among HCC patients administrated sorafenib, Shp2-low patients presented longer postoperative survival than Shp2-high patients. However, it was not clear whether Shp2-low patients benefited from sorafenib treatment or not because the patients with low Shp2 expression possessed superior survival even without sorafenib therapy. Herein, we observed that HCC patients with low Shp2 expression benefited from sorafenib administration after surgery, while patients with high Shp2 levels did not, which further indicates that Shp2 could serve as a biomarker in personalized therapy for HCC. Taken together, we found that Shp2 promotes the expansion of liver CSCs by augmenting β-catenin signaling and may serve as an optimal target in liver CSC-targeted therapy. The significance of Shp2 in HCC personalized medicine is worthy of further investigation.

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