Original Article

Promotion of hepatocellular carcinoma stemness and progression by abnormal spindle-like microcephaly-associated protein via the Wnt/β-catenin pathway

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Background: Cancer stem cells (CSCs) play a crucial role in tumor recurrence and metastasis, which are the primary causes of death in patients with hepatocellular carcinoma (HCC). Currently, no drug effectively blocks the recurrence and metastasis of liver cancer, leading to a poor prognosis for patients. To enhance treatment outcomes, there is an urgent need to investigate the molecular mechanisms behind the recurrence and progression of liver cancer, with the aim of identifying effective therapeutic targets. Targeting HCC stemness can improve the prognosis of patients with HCC. Abnormal spindle-like microcephaly-associated protein (*ASPM*) plays a pivotal role in regulating neurogenesis and brain size, which is a centrosome protein. *ASPM* has been implicated in tumorigenesis and tumor progression, but its regulatory role in HCC stemness is not well understood. This study aims to investigate the role of *ASPM* in liver cancer stemness and elucidate its potential molecular mechanisms.

Methods: Bioinformatics analysis was used to study the expression of *ASPM* and its clinical significance in HCC. *In vitro* and *in vivo* assays were conducted to clarify the impact of *ASPM* knockdown on HCC cell stemness. The correlation between *ASPM* and the Wnt/β-catenin pathway was examined through analysis of online databases and *in vitro* experiments.

Results: The bioinformatics analysis revealed significant upregulation of *ASPM* was significantly upregulated in HCC samples, with expression correlating with poor prognosis. *In vitro* experimental data confirmed elevated *ASPM* expression in HCC cells compared to normal hepatocytes. Knockdown of *ASPM* suppressed HCC cell growth, clone formation, spheroid formation, migration, invasion, and the expression of CSC markers *CD133* and *CD44*. This also inhibited the activation of the Wnt/β-catenin pathway. Reactivation of this pathway partially reversed the biological changes induced by *ASPM* knockdown in HCC cells. Additionally, *in vivo* data demonstrated that *ASPM* downregulation reduced the size and weight of xenografts in BALB/c mice, along with decreased expression of CSC markers.

Conclusions: These findings suggest that *ASPM* promotes HCC stemness and progression through the Wnt/β-catenin pathway. Targeting *ASPM* or the Wnt/β-catenin pathway may be a promising strategy to prevent HCC chemoresistance and recurrence, ultimately improving patient prognosis.

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Keywords: Abnormal spindle-like microcephaly-associated protein (*ASPM*); Wnt/β-catenin; hepatocellular carcinoma (HCC); stemness

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Introduction

Liver cancer is the fourth leading cause of cancer-related death and ranks sixth among new cases worldwide (1). It is predicted that more than one million individuals will be affected by liver cancer per year by the year 2025 (2), making it a global health challenge. Hepatocellular carcinoma (HCC) originates from hepatocytes and accounts for 90% of primary liver cancer cases (3). Although surgical treatment has long been the preferred therapy for providing the best outcome, approximately 70% of patients develop recurrence within 5 years after surgical treatment (4,5). To improve therapeutic outcomes, the molecular mechanisms underlying HCC pathogenesis and progression need to be urgently developed and effective therapeutic targets identified.

Cancer stem cells (CSCs) are a driving force in tumor recurrence and metastasis, leading to tumor relapse after treatment (6). A number of CSCs have been isolated from malignancies such as breast cancer, lung cancer, colon cancer, glioma, pancreatic cancer, prostate cancer, and liver carcinoma (7-10). Similar to normal stem cells, CSCs are characterized by self-renewal ability, differentiation potential, and long-term dormancy (11). The dormant cells are always resistant to therapeutic agents, resulting in chemoresistance and cancer relapse. Therefore, targeting CSCs to exit dormancy could enhance their sensitivity to treatment and prevent relapse. Furthermore, eradicating resident CSCs could contribute substantially to preventing relapse. Several pathways, including the Wnt pathway, Notch pathway, and Hedgehog pathways, regulate cancer cell stemness (12). Among them, the Wnt signaling pathway is highly complex and evolutionarily conserved. This pathway is critical for embryonic development and cancer progression. The activation of the Wnt pathway via different stimuli can promote the stemness of liver cancer, gastric, and prostate cancer (13-15). One study showed that polyphyllin I elevated the degradation of β-catenin, the downstream molecule of the Wnt pathway, leading to the attenuation of liver cancer stemness (16). Other molecules such as Nrf2 can promote hepatic stem cell proliferation and initiate tumorigenesis via the Wnt/β-catenin pathway (17).

Abnormal spindle-like microcephaly-associated protein (*ASPM*) is a positive regulator of Wnt signaling pathway (18). *ASPM* was initially found to be a centrosome protein that plays an essential role in regulating neurogenesis and brain size (19,20). Recently accumulated evidence suggests that *ASPM* is aberrantly upregulated in multiple tumors including gastric cancer, prostate cancer, lung carcinoma, and liver cancer, among others (21-28). *ASPM* and *TROAP* have been shown to be highly expressed in most cancers including liver cancer, and predicted poor prognosis in tumor patients (29). Multiple studies have shown that

Highlight box

Key findings

- Abnormal spindle-like microcephaly-associated protein (*ASPM*) promotes hepatocellular carcinoma (HCC) stemness and progression via the Wnt/β-catenin pathway. Targeting either *ASPM* or the Wnt/β-catenin pathway may prevent HCC chemoresistance and recurrence, thus improving patient prognosis.

What is known and what is new?

- Cancer stem cells are the main cause of HCC-related death. And *ASPM* is reported to figure prominently in tumorigenesis and progression.
- Our study demonstrated that *ASPM* is a potential prognostic indicator of HCC. *ASPM* downregulation remarkably restrained HCC stemness, invasion, and migration abilities, which could be partly overcome through the reactivation of the Wnt/β-catenin pathway.

What is the implication, and what should change now?

- *ASPM* plays an essential role in controlling HCC stemness and development through the canonical Wnt/β-catenin pathway. Targeting *ASPM* markedly suppresses the growth and stemness of HCC xenografts. This study suggests that targeting either *ASPM* or canonical Wnt signaling molecules may be a promising therapeutic approach.
- In order to further verify our findings, it is necessary to further explore the molecular mechanism through which *ASPM* promotes HCC stemness and progression via the Wnt/β-catenin pathway.

immune cell infiltration is a prognostic marker of cancer progression (30,31). The association between high ASPM expression and tumor-infiltrating immune cell cells has been demonstrated in bladder cancer (29,32). Studies have also found that *ASPM* is involved in the regulation of cancer stemness in prostate cancer and pancreatic ductal adenocarcinoma (22,23). Pai *et al.* reported that *ASPM* could stabilize β-catenin through combination with disheveled-3, a major upstream regulator of canonical Wnt signaling in prostate cancer cells (23). However, the role of *ASPM* in regulating HCC stemness through the Wnt pathway remains unclear.

Our bioinformatics analysis of data from The Cancer Genome Atlas (TCGA) database showed that high expression of *ASPM* was associated with a shorter overall survival (OS) period in patients with HCC. In other words, higher expression of *ASPM* predicts a poor prognosis in patients with liver cancer. Therefore, we reasonably hypothesized that *ASPM* may participate in regulating HCC stemness. In this study, we performed bioinformatics analysis, as well as *in vitro* and *in vivo* assays to characterize the role of *ASPM* in HCC stemness and progression and to clarify the related molecular mechanisms. We present this article in accordance with the ARRIVE and MDAR reporting checklists (available at https://jgo.amegroups.com/article/view/10.21037/jgo-24-406/rc).

Methods

Cell culture

Human HCC cell lines HepG2 and HCCLM3 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and the normal liver cell line HL-7702 was purchased from Nanjing Huaao Biomedical Technology Co., Ltd. (Nanjing, China). The cells were cultured in RPMI-1640 (Solarbio Life Sciences, Beijing, China) medium or Dulbecco's modified Eagle medium (DMEM; Corning Inc., Corning, NY, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 100 U/mL of penicillin, and 100 mg/L of streptomycin in an incubator (Thermo Fisher Scientific, Waltham, MA, USA) at 37 ℃ in a 5% CO_2 atmosphere.

Data collection and process

The messenger RNA (mRNA) sequencing data and corresponding clinical information of 369 patients with liver HCC (LIHC) and 50 normal samples were downloaded from the TCGA database (https://portal.gdc.cancer.gov/). The raw data were standardized and transformed into transcripts per million (TPM) using the "edgeR" R package in R software version 4.1.2 (The R Foundation of Statistical Computing, Vienna, Austria). Additionally, HCC-associated GSE84005 microarray datasets from 38 HCC samples and 38 normal liver samples were downloaded from the Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/). These data were normalized using $\log_2(x+1)$ to ensure uniformed standards via the "affy" R package. In addition, the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database and the Tumor Immune Estimation Resource (TIMER) database were utilized for correlation analysis. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Survival analysis

Correlations between liver cancer patient survival and *ASPM* expression (probe: 259266) were derived from the TCGA database. Prognostic values associated with *ASPM* and OS, were assessed in patients with liver cancer. In order to evaluate the prognostic values of *ASPM*, a total of 369 liver cancer samples were divided into the high and low *ASPM*-expression groups based on the cutoff value. The confidence interval was set as 95%, with statistical significance defined as a log-rank P value of <0.05.

Knockdown of ASPM via small interfering RNA (siRNA)

ASPM was knocked down by transfection with siRNA. HCC cells were transfected with siRNA using Lipo8000 reagent (Beyotime, Beijing, China) following the manufacturer's protocol. The siRNA sequences were as follows: si*ASPM*-3225 sense 5'-CGGCAAUAAGUCGUCUUCAAATT-3', si*ASPM*-3225 anti-sense 5'-UUUGAAGACGACUUAUUGCCGTT-3', si*ASPM*-5680 sense 5'-CCAAAGUUGUUGACCGUAUUUTT-3', si*ASPM*-5680 anti-sense 5'-AAAUACGGUCAACAACUUUGGTT-3', negative control siRNA sense 5'-CACAACUGGUCAUACUGGUAATT-3', and negative control siRNA anti-sense 5'-UUACCAGUAUGACCAGUUGUGTT-3'. The *ASPM* knockdown effect was assessed using Western blotting.

Construction of the HCC cell line with stable ASPM knockdown using short hairpin RNA (shRNA)

shRNA targeting *ASPM* and negative control shRNA were synthesized by Sangon Biotech (Shanghai, China). The shRNA sequences were as follows: sh*ASPM* sense 5'-GATCGCCGTTTGTTTACTTCTGAAACTCGAGTTTCAGAAGTAAACAAACGGCTTTTTG-3', sh*ASPM* anti-sense 5'-CCGGCAAAAAGCCGTTTGTTTACTTCTGAAACTCGAGTTTCAGAAGTAAACAAACGGC-3', negative control shRNA sense 5'-CCGGGATTTCGATCATGACTTTCGTCTCGAGACGAAAGTCATGATCGAAATCTTTTTG-3', and negative control shRNA anti-sense 5'-AATTCAAAAAGATTTCGATCATGACTTTCGTCTCGAGACGAAAGTCATGATCGAAATC-3'. The shRNA fragment was ligated into the response vector pLKO.1-GFP-puro producing pLKO.1-GFP-puro-ASPM and pLKO.1-GFP-puro-negative control. The sequences of the constructed plasmid were verified by DNA sequencing (Sangon Biotech).

The Lipo8000 reagent (Beyotime) was used to transfect each of the plasmids. The regulator vector, along with the pCMV-VSV-G (plasmid #8454) and pCMV-dR8.91 was transfected into 293T cells. The supernatant containing the lentivirus was collected and then mixed with HepG2 cells. After 48 hours, HepG2 cells with shRNAs were selected using 5 μg/mL of puromycin and purified through single-cell cloning. The depletion efficiency was assessed through Western blotting.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNAs were extracted from cells using TRIzol (TianGen Biotech, Beijing, China). The RNAs were then converted to complementary DNAs (cDNAs) using the HiScript 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China). RT-PCR was performed with BeyoFusion PCR Master Mix (Beyotime). The primers used were as follows: *ASPM* sense, 5'-AGAGTTAATGCAGCACTCGTCA-3', *ASPM* anti-sense 5'-CCTCCACATAGCCTGAATAAGTGA-3', glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense 5'-ACCACAGTCCATGCCATCAC-3', and GAPDH anti-sense 5'-TCCACCACCCTGTTGCTGTA-3'. The PCR products were then analyzed using agarose gel electrophoresis.

Western blot analysis

Cells were harvested and lysed in cold radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime) supplemented with phenylmethanesulfonylfluoride (PMSF; Beyotime) and phosphatase inhibitors [MedChemExpress (MCE), Monmouth Junction, NJ, USA]. Subsequently, 30 μg of protein samples were separated using 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) at 120 V for 1 hour. Protein samples were transferred to polyvinylidene difluoride (PVDF) membranes for 2 hours. The membranes were blocked in 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween 20 detergent (TBST) for 1 hour. Then, the membranes were incubated in blocking buffer overnight at 4 ℃ with primary antibodies, including *ASPM* (1:500; Proteintech, Rosemont, IL, USA), *CD133* (1:500; Proteintech), *CD44* (1:500; 15675-1-AP; Proteintech), β-catenin (1:700; Zen-Bioscience, Chengdu, China), Wnt3a (1:600; Zen-Bioscience), β-actin (1:5,000; Zen-Bioscience), and GAPDH (1:5,000; Zen-Bioscience), and with secondary antibodies for 2 hours after 3 washes. Sensitive enhanced chemiluminescence (ECL) Luminescence Reagent (MeilunBio, Dalian, China) was used to detect the result.

Sphere formation assay

In the sphere formation assay, HepG2 or HCCLM3 cells transfected with control siRNA or si*ASPM* were plated in six-well ultralow adherent plates (Corning Inc.) at 1,000 cells per well. Spheres were cultivated in serum-free DMEM/F12 medium containing 20 ng/mL of basic fibroblast growth factor (bFGF; MCE), 20 ng/mL of epidermal growth factor (EGF; MCE), 1% N-2 (Thermo Fisher Scientific), and 2% B27 (MCE). After 15 days, the number of tumor spheres was visualized and counted under a microscope.

Transwell invasion assay

Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) was thawed at 4 ℃ overnight and then diluted with serum-free medium at a ratio of 1:8. Following this, 100 μL of Matrigel was added to the upper chambers of the Transwell plate (Corning Inc.) and incubated at 37 ℃ for 4 hours until coagulated. HepG2 or HCCLM3 cells transfected with

control siRNA or si*ASPM* were resuspended in serum-free DMEM medium at a concentration of 4×10^4 cells/well and seeded in the upper chamber. Meanwhile, 800 µL of DMEM medium containing 20% FBS was added to the lower chamber. After 24 hours of incubation, the upper chamber was removed, cleaned twice with phosphate-buffered saline (PBS), and the cells were fixed using methanol. They were then stained with 0.1% crystal violet solution, cleaned with PBS, and each chamber was photographed and counted under a microscope.

Wound healing assay

HepG2 or HCCLM3 cells transfected with control siRNA or si*ASPM* were seeded into 12-well plates at 3×10^5 and cultivated at 37 ℃ for 18 hours to form confluent monolayers. A linear wound was produced by dragging a 10-µL aseptic gun head through the monolayer in each well. The cells were then cultured in serum-free DMEM at 37 ℃ for 72 hours. The wound healing rate of cells was determined as follows: wound healing rate (%) = (Ai – At)/Ai × 100%, where Ai is the initial wound area, and At is the terminal wound area.

In vivo xenograft experiments

Animal experiments were performed under a project license (No. 2023-KY-1111-002) granted by the ethics review committee of the First Affiliated Hospital of Zhengzhou University, in compliance with the First Affiliated Hospital of Zhengzhou University guidelines for the care and use of animals. A protocol was prepared before the study without registration. For this study, 12 nude mice (5 weeks old, male) were randomly divided into two groups (n=6), which were purchased from SiPeiFu Biotechnology Co., Ltd. (Beijing, China). Approximately 6×10^6 HepG2 cells with sh*ASPM* (HepG2-sh*ASPM*) or cells with negative control shRNA (HepG2-NC) (150 µL) in logarithmic growth phase were injected subcutaneously into each nude mouse to establish tumor xenografts. The volume of the tumors was measured every 5 days using calipers and calculated according to the following formula: tumor volume (mm^3) = ($m \times n^2$)/2, where m and n represent the longest and shortest diameters, respectively. After 30 days post-transplantation, the animals were euthanized, and the tumors were harvested, photographed, weighed, and sectioned for immunohistochemistry (IHC) and Western blotting.

IHC analysis

The tumor xenograft tissues were fixed with 4% paraformaldehyde. After being embedded in paraffin, the tissues were sectioned to a thickness of 5 µm, dewaxed, dehydrated, and subjected to antigen retrieval. The tissue sections were then incubated at 4 ℃ overnight with primary antibodies, including anti-*ASPM* (1:100; Proteintech), anti-*CD133* (1:50; Proteintech), and anti-*CD44* (1:50; Proteintech). Subsequently, the tissue sections were incubated with biotin-labeled secondary antibody (ZSGB-Bio, Beijing, China), visualized, and assessed under a microscope.

Statistical analysis

Results are presented as the mean ± standard deviation (SD). Analysis was performed using SPSS 21.0 software (IBM Corp., Armonk, NY, USA). Differences between groups were assessed using the Student *t*-test. The Kaplan-Meier method was used to establish survival cures for OS and DFS. The differences in survival of patients between high and low *ASPM* expression were assessed using the log-rank test. A P value less than 0.05 was considered to indicate a statistically significant difference.

Results

Aberrant expression of ASPM in HCC and its clinical significance

To assess the clinical significance of *ASPM* in HCC, we evaluated the difference in its expression between HCC samples and normal liver samples using the TCGA-HCC database and the GSE84005 microarray dataset. The box plots revealed that *ASPM* expression in HCC samples was significantly higher than in normal liver samples (*Figure 1A*). To confirm these bioinformatics findings, two HCC cell lines (HCCLM3, and HepG2) and one normal liver cell line HL-7702 were cultured. The reverse transcription-quantitative polymerase chain reaction (RT-qPCR) results showed that *ASPM* expression in three HCC cell lines was significantly elevated (*Figure 1B*). Data from the TCGA database showed that *ASPM* expression was negatively associated with the OS of patients with HCC (*Figure 1C*),

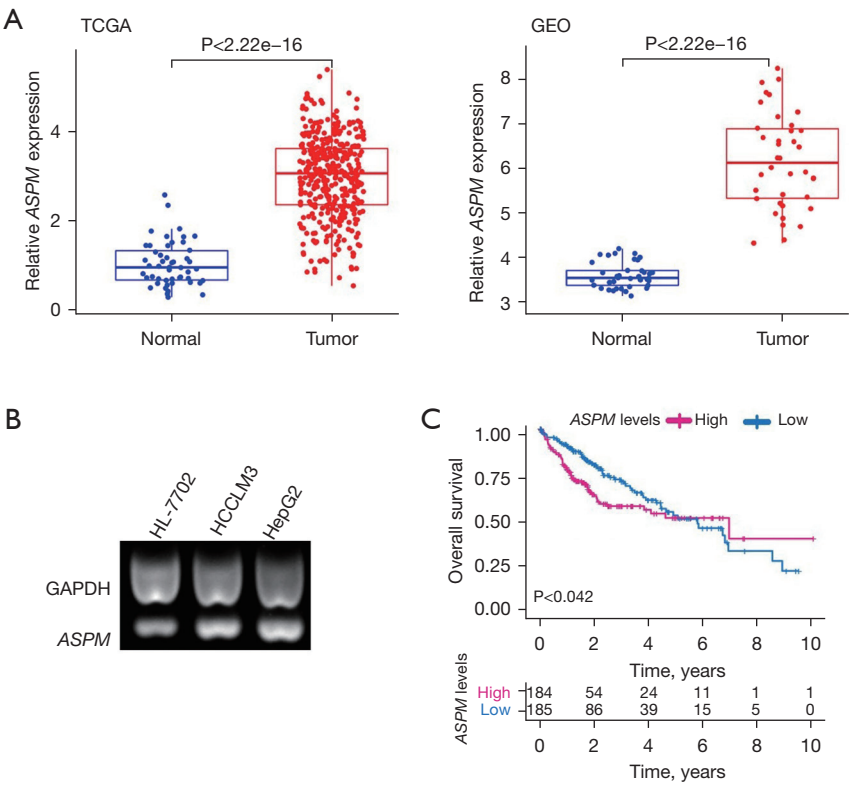

Figure 1 Elevated *ASPM* expression in HCC and its prognostic value. (A) Box plots showing the relative *ASPM* expression levels of *ASPM* in normal samples and HCC samples based on the TCGA database (left) and the GSE84005 GEO dataset (right). (B) Total RNA from two HCC cell lines (HCCLM3, and HepG2) and the normal liver cell line HL-7702 was subjected to RT-PCR. The products were analyzed using agarose gel electrophoresis. (C) The OS analyses in patients with HCC were performed using the TCGA database. *ASPM*, abnormal spindle-like microcephaly-associated protein; TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; RT-PCR, reverse transcription polymerase chain reaction; OS, overall survival.

indicating that *ASPM* could potentially serve as a prognostic indicator for HCC.

ASPM contributed to HCC cell stemness and malignant properties

To investigate the effect of *ASPM* on HCC stemness, si*ASPM* was utilized to reduce the expression of *ASPM*. Western blot analysis showed that both si*ASPM*-3225 and si*ASPM*-5680 significantly decreased *ASPM* expression in HCC cells (*Figure 2A*), leading to a notable decrease in the expression of stem cell markers (*CD133* and *CD44*) (*Figure 2B*). These results suggest that ASPM may play a role in promoting HCC stemness. Results from the spheroid formation assay revealed that *ASPM* knockdown resulted in a decrease in both the number and size of tumor cell spheroids (*Figure 2C*), further supporting the idea that ASPM is involved in regulating HCC cell stemness. Additionally, the Transwell invasion assay (*Figure 2D*) and wound healing assay (*Figure 2E*) demonstrated that *ASPM* knockdown obviously decreased the cell invasion and migration abilities, respectively.

ASPM was significantly correlated with the Wnt signaling pathway

Data from the STRING database showed that four key proteins related to Wnt pathway (CTNNB1, DVL3, FZD2, and FZD8) were significantly correlated with *ASPM* (*Figure 3A*). Additionally, data from the TIMER database showed a positive correlation between the expression of *ASPM* and that of CTNNB1, DVL3, FZD2, and FZD8 (*Figure 3B*). These

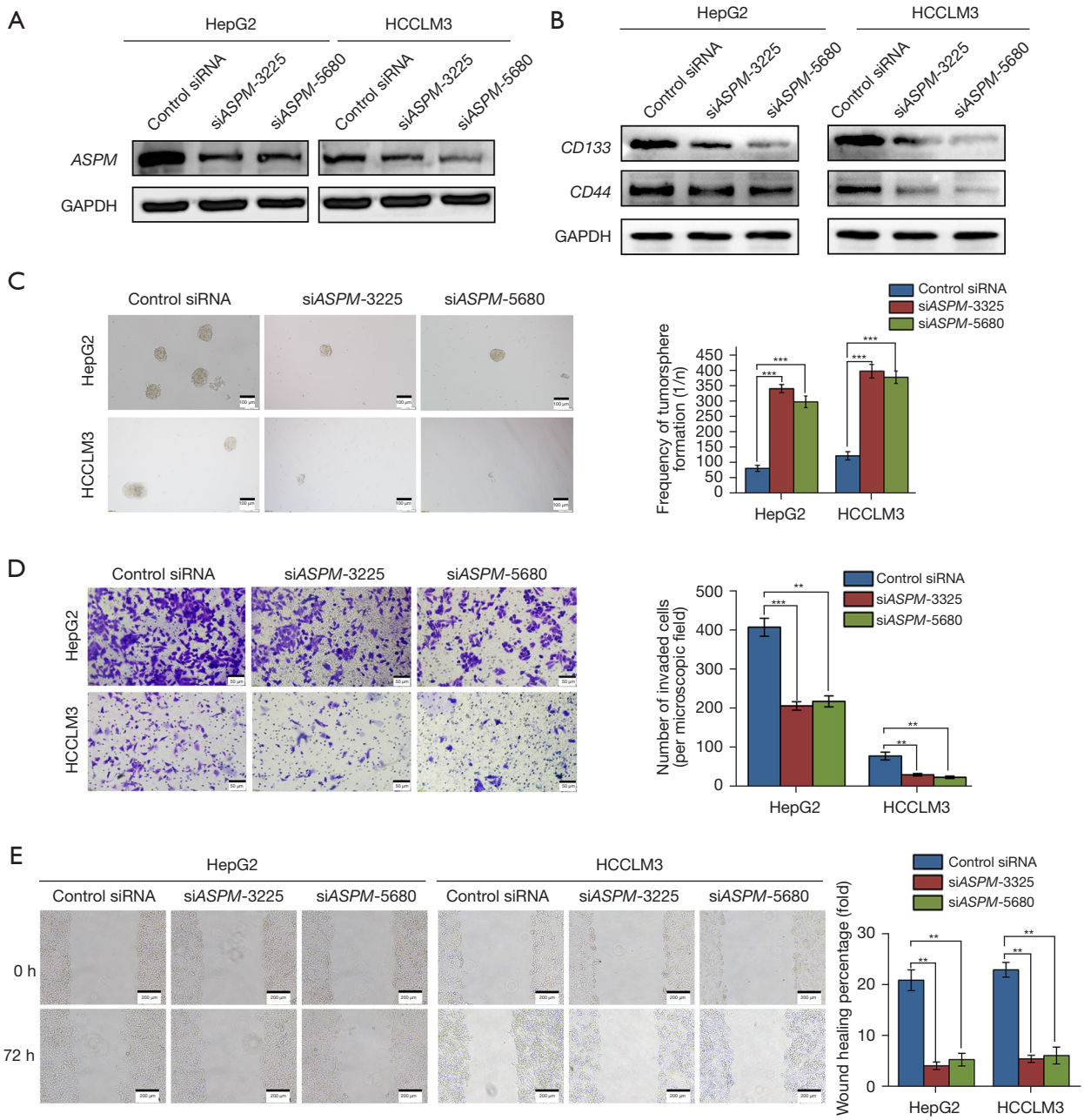

Figure 2 *ASPM* contributes to HCC cell stemness and malignant properties *in vitro*. (A,B) Total protein extracts from HepG2 or HCCLM3 cells were obtained after transfection with the indicated siRNA for 72 hours and subjected to Western blotting. GAPDH served as the loading control. (C) HepG2 or HCCLM3 cells transfected with control siRNA or si*ASPM* were cultured in serum-free and nonadherent culture plates for 15 days. HepG2 or HCCLM3 tumor cell spheroids were photographed with microscope. Scale bars, 100 μm. (D,E) *ASPM* knockdown in HepG2 or HCCLM3 cells impaired their abilities of invasion (D) and migration (E) according to Transwell invasion assay (scale bars, 50 μm) and wound healing assay (scale bars, 200 μm), respectively. The cells of the Transwell invasion assay were fixed with methanol, stained with 0.1% crystal violet solution, and cleaned with PBS. Each experiment was repeated in triplicate. **, P≤0.01; ***, P≤0.001. *ASPM*, abnormal spindle-like microcephaly-associated protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; si, small interfering; HCC, hepatocellular carcinoma.

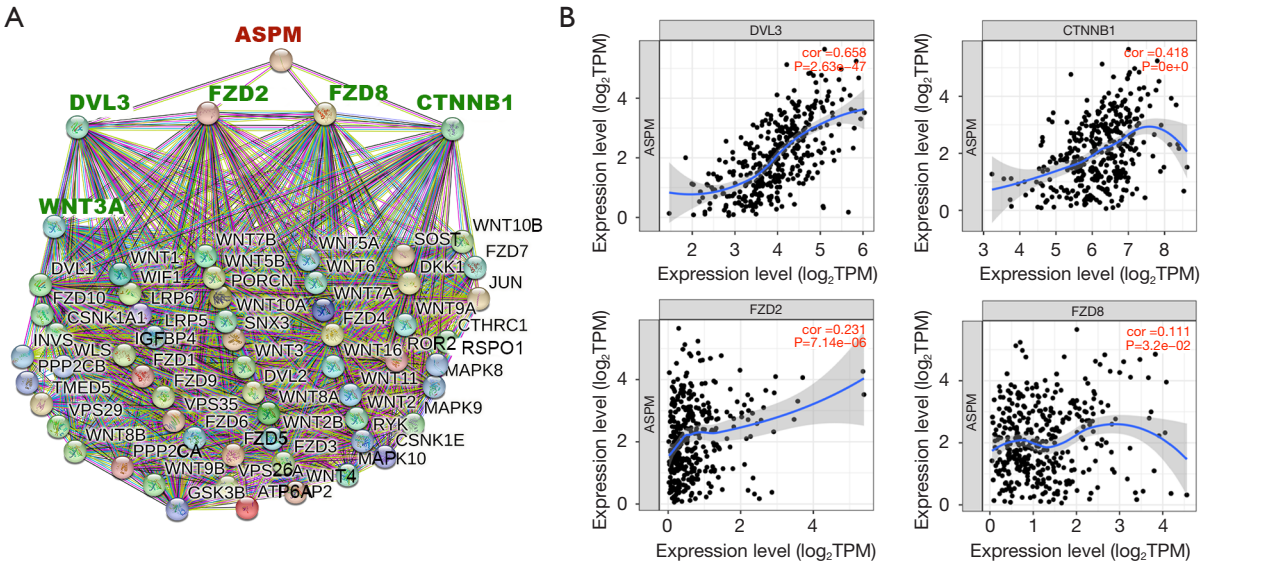

Figure 3 Analysis of the correlation between *ASPM* and the Wnt pathway. (A) The interactions between *ASPM* and Wnt/β-catenin pathway-related proteins were analyzed using the STRING database. (B) The correlation of *ASPM* with CTNNB1, DVL3, FZD2, and FZD8 was analyzed using the TIMER database. *ASPM*, abnormal spindle-like microcephaly-associated protein; TPM, transcripts per million; STRING, Search Tool for the Retrieval of Interacting Genes/Proteins; TIMER, Tumor Immune Estimation Resource.

results suggest that *ASPM* may play a role in regulating the Wnt pathway.

ASPM promoted HCC cell stemness and malignant properties via the Wnt/β-catenin pathway

To determine the involvement of the Wnt/β-catenin pathway in *ASPM*-promoted HCC cell stemness, the expressions of both Wnt3a and β-catenin were detected. As shown in *Figure 4A*, *ASPM* knockdown significantly reduced the expression of both Wnt3a and β-catenin in HepG2 and HCCLM3 cells, suggesting that *ASPM* may regulate the Wnt/β-catenin pathway. Meanwhile, the Wnt/β-catenin signaling-specific activator lithium chloride (LiCl) could partly overcome the inhibition of the Wnt/β-catenin pathway induced by *ASPM* knockdown (*Figure 4A*). *Figure 4B* shows that *ASPM* knockdown decreased the expression of HCC stem cell markers, *CD133* and *CD44*. Compared to *ASPM*-knockdown HepG2 and HCCLM3 cells, the expression of both *CD133* and *CD44* was markedly increased in HCC cells treated with LiCl in addition to *ASPM* knockdown. These results indicate that the activation of the Wnt/β-catenin pathway could restore HCC cell stemness upon *ASPM* knockdown. Similarly, *ASPM* knockdown reduced the number and size of the tumor cell spheroids (*Figure 4C*), as well as their invasion (*Figure 4D*) and migration ability (*Figure 4E*). Compared to *ASPM*-knockdown HepG2 and HCCLM3 cells, the number and size of the tumor cell spheroids (*Figure 4C*), as well as their invasion (*Figure 4D*) and migration ability (*Figure 4E*) were markedly elevated. These results suggest that *ASPM* can promote HCC cell stemness and malignancy via the Wnt/β-catenin pathway.

ASPM knockdown suppressed HCC tumor growth in vivo

To evaluate the role of *ASPM* knockdown in HCC tumor growth *in vivo*, we transfected the constructed plasmid pLKO.1-GFP-puro-*ASPM* (sh*ASPM*) into HepG2 cells to establish a cell line carrying a stable *ASPM* knockdown. The plasmid pLKO.1-GFP-puro-negative control (control shRNA) served as the control. *Figure 5A* shows that the *ASPM* expression in HepG2 cells transfected with sh*ASPM* was significantly lower than that in control cells. There was no significant difference in body weight between the mice with *ASPM* knockdown xenografts and control mice (*Figure 5B*). Tumor sizes in mice injected with *ASPM*-knockdown HCC cells were significantly decreased starting from 10 days after injection compared to the control group (*Figure 5C*). *Figure 5D,5E* show that tumor sizes and weights in mice injected with *ASPM* knockdown HCC cells were greatly reduced at 30 days after injection. These results indicate that *ASPM* knockdown can suppress HCC tumor growth *in vivo*.

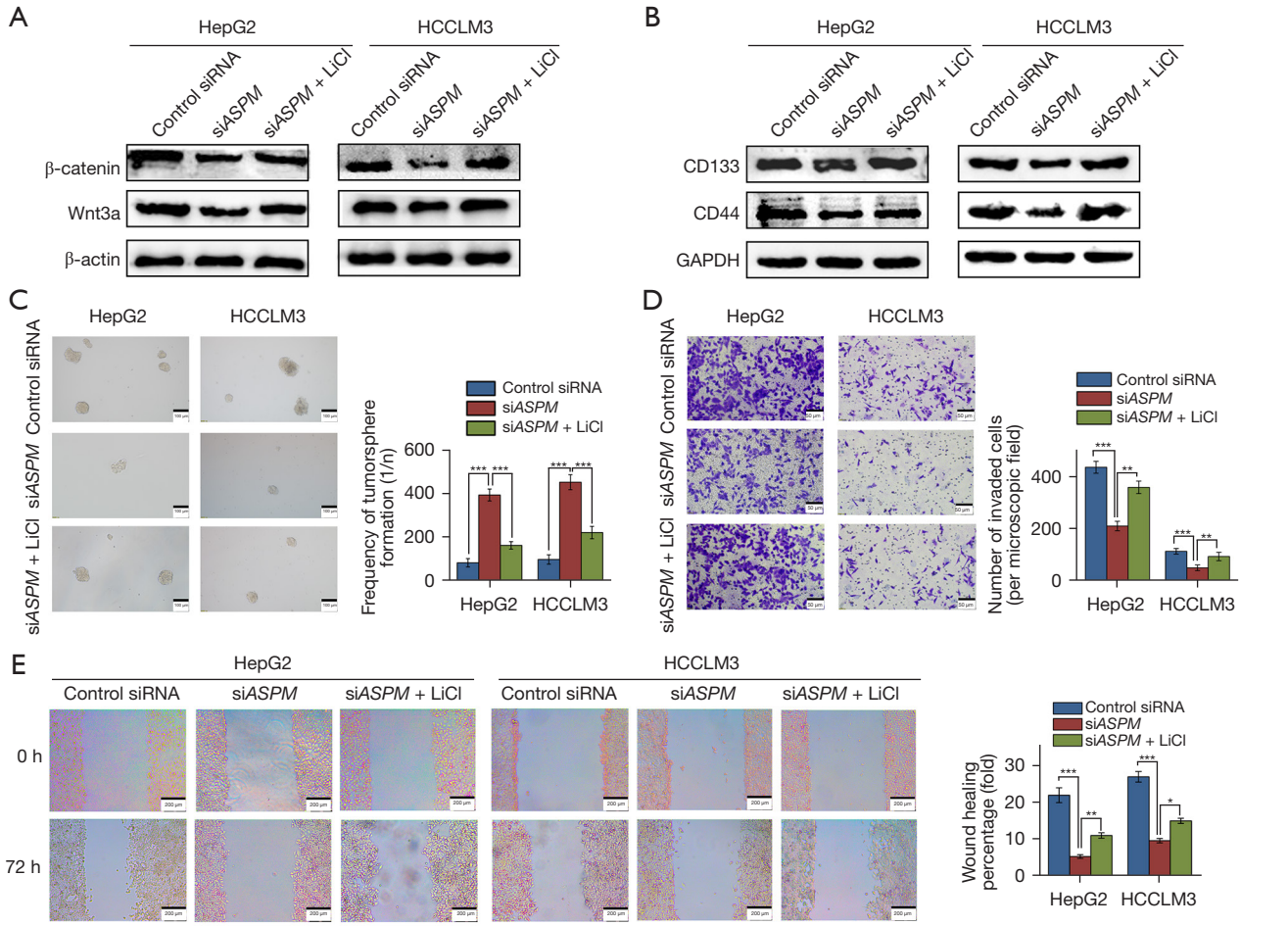

Figure 4 *ASPM* promotes HCC cell stemness and malignant properties through the Wnt/β-catenin pathway. (A,B) HepG2 and HCCLM3 cells were transfected with control siRNA, si*ASPM*, or si*ASPM* plus pretreatment with 20 mmol/L of LiCl for 24 hours. The expression of β-catenin, Wnt3a, *CD133*, and *CD44* was determined via Western blotting. β-actin or GAPDH served as the loading control. (C) HepG2 or HCCLM3 cells were transfected with control siRNA, si*ASPM*, or si*ASPM* plus 20 mmol/L of LiCl treatment and cultured in serum-free and nonadherent culture plates for 15 days. HepG2 or HCCLM3 tumor cell spheroids were photographed with microscope (scale bars, 100 μm). (D,E) HepG2 and HCCLM3 cells were treated in the same manner as described in (D) and (E). Transwell invasion assay (scale bars, 50 μm) and wound healing assay (scale bars, 200 μm) were employed to detect the invasion and migration abilities, respectively. The cells of the Transwell invasion assay were fixed using methanol, stained with 0.1% crystal violet solution, and cleaned with PBS. *, P≤0.05; **, P≤0.01; ***, P≤0.001. si, small interfering; *ASPM*, abnormal spindle-like microcephaly-associated protein; LiCl, lithium chloride; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCC, hepatocellular carcinoma.

ASPM knockdown suppressed HCC cell stemness in mouse xenografts

To determine whether *ASPM* knockdown affects HCC stemness *in vivo*, we examined the expression of HCC stemness markers. As shown in *Figure 6*, the expression of *ASPM* (*Figure 6A*), *CD133* (*Figure 6B*), and *CD44* (*Figure 6C*) in the mouse xenografts derived from sh*ASPM*-HepG2 cells was significantly reduced compared to control xenografts. These results indicate that *ASPM* knockdown can suppress HCC cell stemness in mouse xenografts.

Discussion

As the primary type of liver cancer, HCC remains a considerable global threat to human health. Drug resistance, tumor recurrence, and metastasis are the leading causes of

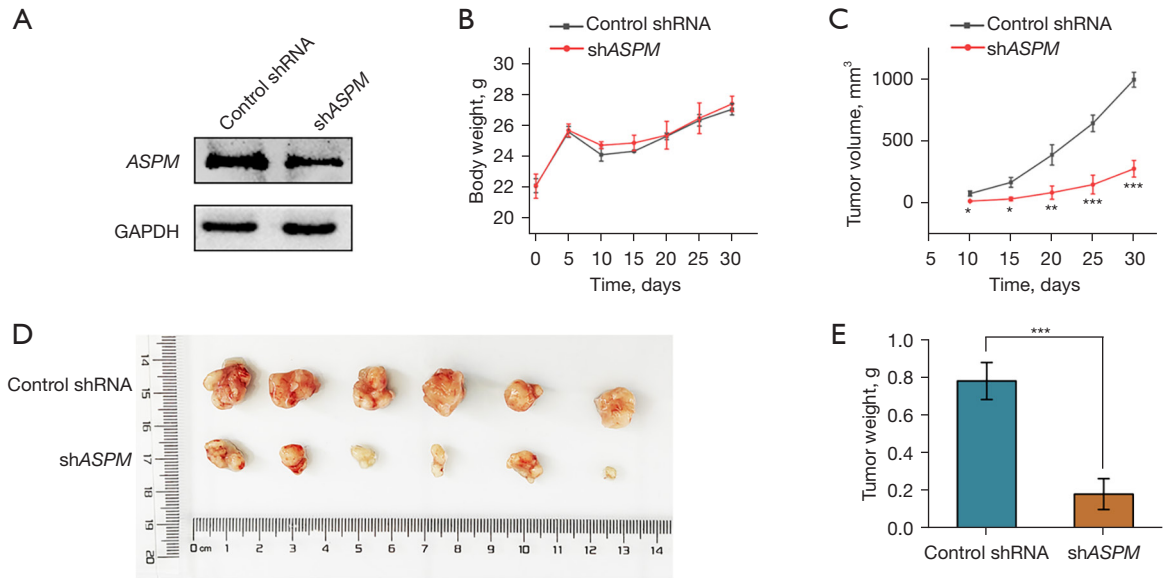

Figure 5 *ASPM* knockdown suppressed HCC tumor growth *in vivo*. (A) Total protein extracts from HepG2 cells transfected with either control shRNA or sh*ASPM* were subjected to Western blotting. GAPDH served as the loading control. (B,C) Mouse xenografts derived from control shRNA- or sh*ASPM*-transfected HepG2 cells were continuously observed for 30 days. Mouse body weight (B) and tumor volume (C) were measured and statistically analyzed every 5 days. (D,E) The mice were sacrificed on the 30th day after HCC cell injection. The tumors in the control and *ASPM* knockdown mice were photographed (D). The peeled tumor xenografts were measured and weighed (E). The bar plots represent the quantified data of the weight and volume as indicated. *, P<0.05; **, P<0.01; ***, P<0.001. *ASPM*, abnormal spindle-like microcephaly-associated protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; sh, short hairpin; HCC, hepatocellular carcinoma.

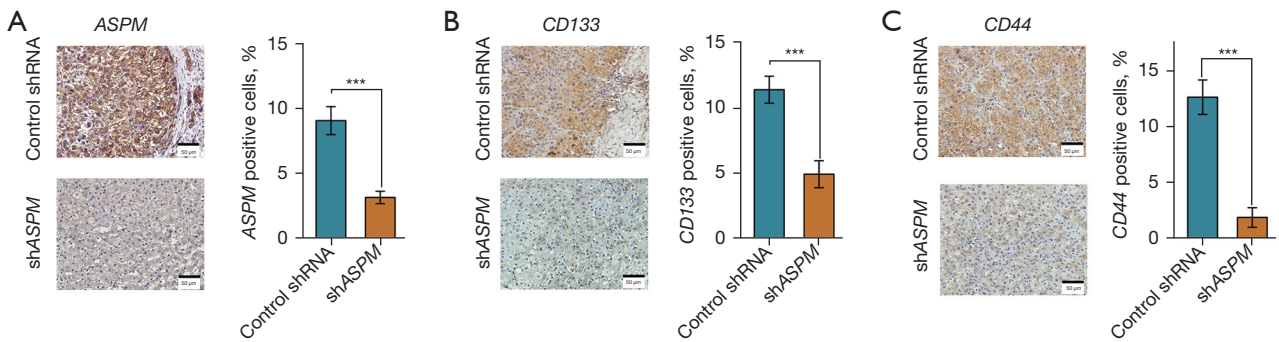

Figure 6 *ASPM* knockdown suppressed the expression of *CD133* and *CD44* in mouse xenografts. (A-C) The peeled tumor xenografts were subjected to IHC staining. The expression of (A) *ASPM*, (B) *CD133*, and (C) *CD44* in the tumor xenografts was determined. The bar plots represent the quantified protein expression data as indicated (scale bars, 50 μm). ***, P<0.001. *ASPM*, abnormal spindle-like microcephaly-associated protein; sh, short hairpin; IHC, immunohistochemistry.

HCC-related death (4,5). It is widely acknowledged that dormant CSCs are resistant to therapeutic agents, leading to drug resistance and cancer relapse after treatment (11). Therefore, eradicating the resident CSCs or suppressing HCC stemness may improve therapeutic outcomes. In this study, we demonstrated that *ASPM* is a potential prognostic indicator for HCC. *ASPM* downregulation significantly inhibits HCC stemness, invasion, and migration abilities, which can be partially overcome by reactivating the Wnt/β-catenin pathway.

As a centrosome protein, *ASPM* has received increased research attention for its role in tumor occurrence and progression. Initially, researchers focused on exploring *ASPM*'s role in neurogenesis and embryonic development, but more recently, its involvement in cancer has been closely scrutinized. Studies have shown that *ASPM* can accelerate the malignant development of various tumors (19-27). For example, *ASPM* promotes the progression of ovarian cancer and influences its clinical characteristics, such as tumor grade (33). Additionally, high expression of *ASPM* has been linked to poor prognosis in patients with pancreatic cancer (22). However, few studies have investigated its role in cancer stemness. It has been found that *ASPM* can interact with disheveled-2 in pancreatic ductal adenocarcinoma cells or disheveled-3 in prostate cancer cells, leasing to the stabilization of β-catenin and activation of the canonical Wnt signaling pathway (23,34).

Cancer stemness can be enhanced through the Wnt/β-catenin pathway (22,24,35,36). However, the role of *ASPM* in regulating Wnt/β-catenin pathway-mediated liver cancer stemness is still unclear. *ASPM* has been reported to be a recurrence, invasion, metastasis, and prognostic marker for HCC (37,38). High *ASPM* expression levels were significantly associated with shorter OS in HCC. Furthermore, *ASPM* was also significantly positively correlated with immune infiltration cells, including regulatory T cells (Treg), T follicular helper (TFH) cells, and macrophages M0, but negatively correlated with immune infiltrating cells, including monocytes. This further suggests that *ASPM* expression is associated with poor prognosis in HCC (39). Our bioinformatics analysis data showed that *ASPM* expression in HCC samples was significantly higher than in normal liver samples, indicating that *ASPM* may play a role in liver cancer development and progression. In addition, a higher *ASPM* expression was linked to shorter OS in liver cancer patients, suggesting that *ASPM* may be crucial involved in HCC progression. As tumor seeding cells, CSCs are responsible for tumor relapse. Thus, the stronger the stemness of HCC, the shorter the OS of the patient. Overall, *ASPM* may contribute to the regulation of HCC stemness and facilitate HCC tumor recurrence in HCC.

The recognized biomarkers of liver CSCs include *CD133*, *CD44*, *CD34*, and *ABCG2*, among others. *CD133* is a glycoprotein with five transmembrane structural domains and two large glycosylated extracellular loops. It has been used to identify and isolate liver CSCs (40). *CD44* is a cell surface glycoprotein. As a hyaluronan receptor, *CD44* mediates intercellular and extracellular matrix interactions (41). *CD133* and *CD44* are coexpressed in liver CSCs. *CD133*$^+$*CD44*$^+$ liver cancer cells have stem cell-like characteristics, including strong proliferative potential, self-renewal, and differentiation ability (42). In studies of non-obese diabetic (NOD)/severe combined immune deficiency (SCID) mice, a small quantity of *CD133*$^+$*CD44*$^+$ HCC cells were found to contribute to tumor growth, while *CD133*$^+$*CD44*$^-$ HCC cells did not produce this effect (42,43), suggesting that double positivity for *CD133* and *CD44* could more accurately define the live CSC subpopulation. It was found that both *CD133* and *CD44* expressions were greatly increased in patients with liver cancer recurrence, pointing to the importance of CSCs in liver cancer recurrence. Our *in vitro* data indicated that *ASPM* downregulation remarkably suppresses CSC-related spheroid formation, the expression of both *CD133* and CD44, and HCC cell invasion and migration. *In vivo* data confirmed that *ASPM* knockdown could inhibit HCC tumor growth and decrease the expression of both *CD133* and *CD44*, suggesting that *ASPM* knockdown may suppress HCC tumor growth via stemness inhibition. Our *in vivo* data showed that *ASPM* expression level varies in different cells of the HCC xenografts. *ASPM* inhibition could reduce the stemness of HCC cells, thus changes HCC cell differentiation status. Therefore, heterogeneous expression of ASPM may result in HCC intratumoral heterogeneity. It is a good point to mention the *ASPM* mutation status in HCC. However, although lots of studies showed that *ASPM* is the most commonly mutated gene of the primary microcephaly, *ASPM* mutation status in cancers is rarely reported. In this study, we didn't show *ASPM* mutation status in HCC and its relationship with either *ASPM* or intratumoral heterogeneity, which will be studied in future studies. Overall, our data demonstrate that *ASPM* may serve as a pivotal regulator of HCC stemness. Targeting *ASPM* may be a promising approach to preventing HCC tumor growth and recurrence.

Wnt signaling pathway is highly complex and evolutionarily conserved, including its canonical and noncanonical branches (44). The noncanonical pathway is essential for cell polarity in organogenesis, tumor invasion, and stemness (45). In the canonical Wnt pathway, β-catenin acts as a key downstream effector and is generally phosphorylated by the Axin complex, which is composed of adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK-3β), casein kinase 1 (CK-1), and the scaffolding protein Axin. Destruction of this complex typically leads

to the degradation of β-catenin through the ubiquitination and proteasome system (46). The Wnt/β-catenin signaling pathway is involved in governing the self-renewal and cell destiny of hematopoietic stem cells (47). It has been reported that β-catenin signaling is continuously activated in approximately 50% of HCC cases (48), indicating that this signaling pathway may play an important role during HCC progression. HCC is a heterogeneous tumor at both histopathological and molecular levels. The extensive heterogeneity of HCC is associated with a progressive loss of differentiation phenotype and acquisition of stemness properties (49,50). A subpopulation of HCC cells with stem cell properties named LCSC are mainly controlled by Wnt/β-catenin pathway (49-51). Wnt activity exhibited a high level of intratumoral heterogeneity in HCC. Wnt-activity^{high}ALDH1^{+}EPCAM^{+} cells were identified as highly competent CSCs in HCC (52). However, whether *ASPM* can regulate HCC stemness via this pathway remains unclear. Our findings confirm that ASPM downregulation can suppress the activation of the Wnt/β-catenin signaling pathway and that reactivation of this pathway can partly overcome the ASPM knockdown-induced suppression of HCC stemness and malignant progression. These results suggest that *ASPM* may regulate HCC stemness and malignant progression via the Wnt/β-catenin pathway.

Conclusions

ASPM may serve as a prognostic indicator for patients with HCC. It is critical for controlling HCC stemness and development through the canonical Wnt/β-catenin pathway. Targeting *ASPM* markedly suppressed the growth and stemness of HCC xenografts. The superior performance of *ASPM* in the prognostic prediction supports it as a novel Wnt-related marker of cancer stemness in HCC. *ASPM* is also a clinically useful and immediately applicable prognostic indicator, and can not only predict the prognosis and survival of HCC patients, but also guide future Wnt or CSC-targeted rational therapies.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE and MDAR reporting checklists. Available at https://jgo.amegroups.com/article/view/10.21037/jgo-24-406/rc

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://jgo.amegroups.com/article/view/10.21037/jgo-24-406/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Animal experiments were performed under a project license (No. 2023-KY-1111-002) granted by the ethics review committee of the First Affiliated Hospital of Zhengzhou University, in compliance with the First Affiliated Hospital of Zhengzhou University guidelines for the care and use of animals. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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