

# Identification of the ASPM-miR-26b-5p network associated with the aggressive traits of HCC cells

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## Primary research

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# Abstract

**Background:** Even though earlier reports have revealed that abnormal spindle-like microcephaly associated (ASPM) exert essential roles in diverse malignancies, its relationship between specific microRNAs (miRNAs) in regulation of hepatocellular carcinoma (HCC) progression has never been elaborated.

**Methods:** Bioinformatics analysis detected differentially expressed genes in HCC and normal. qRT-PCR was performed to detect expression of miR-26b-5p in HCC tissues and cells. HCC cells were transfected with plasmids and their proliferative ability and colony formation were detected with loss-of-function assay. The invasion of HCC cells was determined using Transwell assay. The expression of ASPM was detected by western blotting. Luciferase reporter gene assay was performed to detect the interaction between miR-26b-5p and ASPM. ASPM silencing cells were injected into mice to establish xenograft tumor model.

**Results:** Herein, we proved that ASPM was upregulated in HCC and higher level of ASPM was significantly associated with worse survival in HCC patients. ASPM silencing restrained HCC cell proliferation, migration and invasion capacities in vitro. In vivo, downregulation of ASPM also suppressed HCC cells growth. Mechanistic analyses illustrated that ASPM was a directly target of miR-26b-5p. The expression of ASPM was negatively modulated by miR-26b-5p. Rescues assays displayed that miR-26b-5p inhibited HCC cells growth and invasion via modulating the expression of ASPM.

**Conclusions:** Our work validated that miR-26b-5p restrained the aggressiveness of HCC cells through targeting ASPM.

## Background

Hepatocellular carcinoma (HCC), the common type of liver malignancies, is the leading cause of cancer-associated mortality [1]. A high postsurgical recurrence rate and metastasis lead to the poor prognosis of HCC patients. Unfortunately, limited effective treatments are available and patients often miss the optimal opportunities for clinical interventions, because HCC is frequently diagnosed at the advanced stages [2, 3]. As the molecular mechanisms underlying HCC pathogenesis have not yet been completely understood, elucidating the pivotal cancer-promoting events would contribute to the comprehension of hepatocarcinogenesis and the development of novel effective targeted treatments [4].

Assembly factor for spindle microtubules (ASPM), also referred to as abnormal spindle microtubule assembly, is located on chromosome 1q31 and encodes the ASPM protein [5, 6]. It has been reported that the ASPM gene is overexpressed in glioblastoma and malignant glioma compared with normal brain tissue [7]. Abnormalities in ASPM expression are associated with numerous cancer types [8]. ASPM mRNA is overexpressed in HCC and non-small cell lung cancer (NSCLC) tissues. It has also been proposed that upregulation of ASPM expression increases the invasive capacity of melanoma cells.

However, to date, the potential role of ASPM in the growth and metastatic-related phenotypes of HCC cells remains unknown.

MiRNAs are endogenous non-coding RNAs, with ~22 nucleotides, which can bind to the 3'-untranslated region (3'-UTR) of target mRNA to regulate gene expression, leading to the degradation of the mRNA or translational inhibition of functional proteins [9, 10]. An increasing number of evidences had revealed that miRNAs were extensively involved in numerous pathological processes, including diabetes mellitus, atherosclerosis, and tumors [11]. Meanwhile, there are many reports of miRNAs related to HCC, such as miR-17, miR-21, and miR-492. Currently, miR-26b-5p has been reported to act as tumor suppressor in many cancers. However, little is known about the regulatory mechanism of miR-26b-5p/ASPM axis in the progression of HCC.

In this study, we revealed an interaction between miR-26b-5p and ASPM which modulated HCC cells growth and invasion. Based on the results, a novel regulatory pathway composed of miR-26b-5p/ASPM was provided for HCC therapy.

## Materials And Methods

### HCC tissues and cell lines

HCC samples and adjacent tissues were obtained from Shandong Provincial Hospital Affiliated to Shandong University. The clinicopathological features of patients with HCC were summarized in Table 1. Written consent was obtained from all participants who were involved in the study. All tissues were frozen in liquid nitrogen after surgery and stored at -80 °C. This study was approved by the Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong University. The HCC cell lines (HepG2, SNU449, SMMC-7721, Huh7 and PLC/PRF/5), HEK-293T and normal liver cell lines (LO2) were purchased from the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in DMEM containing with 10% FBS (Thermo Fisher Scientific, Waltham, MA, USA) and 100 U/ml penicillin/streptomycin in a humid incubator at 37 °C.

### Cell Transfection

pcDNA3.1 vector, pcDNA3.1 vector carrying ASPM (ASPM), miR-26b-5p mimic, negative control oligonucleotides (miR-NC), miR-26b-5p inhibitor (anti-miR-26b-5p), negative control oligonucleotide (anti-miR-NC), small interfering RNA of ASPM (si-ASPM) and scramble siRNA (si-Con) were bought from RiboBio (Guangzhou, China). Cells were transfected utilizing lipofectamine 3000 (Thermo Fisher Scientific) following to manufacturer's protocol. sh-NC and shRNA targeting ASPM (sh-ASPM) were purchased from RiboBio (Guangzhou, China) and transfected into SMMC-7721 cell. To stably overexpress miR-26b-5p in SMMC-7721 cell, the lentiviral packaging kit was used. Lentivirus containing miR-26b-5p was packaged following the manufacturer's manual. Lentivirus was packaged in HEK-293T cell and secreted into culture medium. SMMC-7721 cell was infected by lentivirus carrying miR-26b-5p with the presence of polybrene (Sigma) and selected by puromycin (Sigma) for two weeks. To

overexpress ASPM in SMMC-7721 cells, lentivirus carrying ASPM cDNA (GeneCopoeia) were packaged and used to infect cell following to manufacturer's protocol.

## qRT-PCR

Total RNAs were extracted using Trizol reagent (Thermo Fisher Scientific). miRNAs were extracted using RNAsimple Total RNA kit (Tiangen Biotech Co., Ltd., Beijing, China). cDNA was synthesized using a TransScript First-Strand cDNA Synthesis SuperMix (TransGen, Beijing, China). qPCR reactions were performed using PowerUp SYBR Green kit (ABI, Foster City, CA, United States) and QuantStudio 6 System (ABI). For miR-26b-5p, total RNA (2  $\mu$ g) was transcribed into cDNA using a microRNA First-Strand cDNA Synthesis Kit (Sangong Biotech, Shanghai, China) according to the manufacturer's protocol. Data was analyzed using the comparative Ct method ( $2^{-\Delta\Delta Ct}$ ). U6 or GAPDH was served as the internal control. Primers used were listed below: ASPM, forward primer (5'-GGCCCTAGACAACCCTAACGA-3') and reverse primer (5'-AGCTTGTTGTTTCAGAACATCA-3'); GAPDH, forward primer (5'-TGGATTTGGACGCATTGGTC-3') and reverse primer (5'-TTTGCCTGGTACGTGTTGAT-3'); miR-26b-5p, forward primer (5'-TTCAAGTAATTCAGGATAGGT-3') and reverse primer (5'-CTTCGCAGCATACT-3'); U6, forward primer (5'-CTCGCTTCGGCAGCACA-3') and reverse primer (5'-AAAATATGCAACGCTTCACG-3').

## Luciferase Assay

ASPM 3'-untranslated region (3'-UTR) carrying the binding sites for miR-26b-5p was cloned into psiCHECK-2 vector (Promega, Madison, WI, USA) to construct wild-type (wt) reporter vector (wt-ASPM) or mutant (mut) reporter vector (mut-ASPM). The ASPM 3'-UTR-mut was constructed by mutating the binding sites. Luciferase vector plasmid and miR-26b-5p mimics were cotransfected into SMMC-7721 and PLC/PRF/5 cells. After 48 h, the luciferase activity was detected using Luciferase Assay Kit (Promega).

## Cell Proliferation And Colony Formation Assays

Cell growth was detected using CCK-8 Kit (Beyotime, Nanjing, China) according to the protocol. In colony formation assay, cells were cultured into six-well plates and incubated for two weeks. After that, cells were stained and photographed.

## Migration And Invasion Assays

Cell migration assay was performed using transwell chamber (BD, Franklin Lakes, NJ, United States).  $5 \times 10^4$  SMMC-7721 and PLC/PRF/5 cells were seeded into the upper chambers and the lower chambers were filled with medium with 20% FBS. Cell migration was assessed by counting migrative cells under an inverted microscope. Cell invasion assay was conducted using transwell chamber coated with Matrigel (BD).  $5 \times 10^4$  SMMC-7721 and PLC/PRF/5 cells were seeded into the upper chambers and the lower chamber was filled with DMEM with 20% FBS. The number of invasion cell was counted under microscope.

## Tumor Xenografts

miR-NC or miR-26b-5p transfected SMMC-7721 cells were subcutaneously implanted into nude mice (Beijing Vital River Laboratory, Beijing, China). Tumor volume =  $0.5 \times \text{length} \times \text{width}^2$ . Tumor volume was examined every week. Mice were euthanized at 5 weeks post implantation. The tumor tissues were extracted for further immunohistochemical staining (IHC). All procedures involving experimental animals were performed in accordance with protocols that were approved by the Committee for Animal Research of Shandong Provincial Hospital Affiliated to Shandong University and complied with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 86 – 23, revised 1985).

## Immunoblotting Assay

Proteins were extracted from cells using RIPA buffer. 20  $\mu\text{g}$  protein lysis were loaded onto 10% SDS-PAGE. After separated, the proteins were transferred to PVDF membrane (Millipore, Braunschweig, Germany). The membrane was incubated with anti-GAPDH (ab8245, Abcam, Cambridge, UK) or ASPM (26223-1-AP, Proteintech Group, Rosemont, IL, USA) at 4 °C overnight. After washed with TBST, the membrane was incubated with secondary antibody for 2 h and the bands were detected using Enhanced chemiluminescence (ECL) reagent (Pierce, Rockford, IL, USA).

## Statistical analysis

GraphPad Prism software was used for statistical analyses. Data were presented as Mean  $\pm$  SD. Differences were calculated using Student's t-test or Mann-Whitney U test. The correlation analysis was performed by Spearman test.  $P < 0.05$  was statistically significant.

# Results

## ASPM is upregulated in HCC

Firstly, we assessed the GEO database GSE54236 and GSE14520. The volcano plot and heatmaps showed the dysregulated genes, including upregulated genes and downregulated genes (Fig. 1A-1B). ASPM was selected for investigation owing to its level was significantly upregulated in both GEO database and its biological roles in HCC needs to be explored (Fig. 1C). To confirm this observation, we measured the expression levels of ASPM in 46 pairs of HCC and adjacent samples using qRT-PCR. Consistent with the results of bioinformatics analysis, ASPM was significantly upregulated in HCC tissues (Fig. 1D). The IHC data illustrated that ASPM expression was distinctly increased in HCC tissues as compared to that in adjacent tissues (Fig. 1E) Furthermore, overexpression of ASPM was associated with the aggressive clinic phenotypes (Fig. 1F-G). ASPM was also more highly expressed in HCC with advanced tumor stages (II-III) than that in HCC with early tumor stages (I). Using the online bioinformatics tool GEPIA (<http://gepia.cancer-pku.cn/index.html>), we found that patients with increased ASPM expression exhibited poor overall survival (OS) (Fig. 1H). All these results revealed that ASPM might exert a critical role in HCC.

## **Knockdown of ASPM reduces HCC cells growth and invasion ability**

To reveal the function of ASPM in HCC, we explored its expressions in HCC cells (HepG2, SNU449, SMMC-7721, Huh7 and PLC/PRF/5) and normal liver cell line (LO2). The result of qRT-PCR implied that the levels of ASPM were higher in HCC cell lines than that in LO2 cell (Fig. 2A). Next, we knocked-down the ASPM in SMMC-7721 and PLC/PRF/5 cells using siRNA (Fig. 2B). CCK-8 assay (Fig. 2C) and colony-formation assay (Fig. 2D) displayed that HCC cells growth was significantly inhibited after ASPM knockdown. Meanwhile, the migration and invasive capacity were decreased in si-ASPM transfected SMMC-7721 and PLC/PRF/5 cells as demonstrated by transwell migration (Fig. 2E) and invasion assay (Fig. 2F). Then, SMMC-7721 cells stable transfected with sh-ASPM were implanted into BALB/c nude mice. sh-ASPM markedly restrained tumor growth after cells implantation (Fig. 2G-2H). Nevertheless, we proved that upregulation of ASPM enhanced HCC cell proliferation and invasion of HCC cells in vitro (Fig. 2I-2K). All findings suggest the oncogenic role of ASPM in HCC.

## **miR-26b-5p binds to ASPM and negatively regulates its expression**

We predicted the miRNAs that have complementary base pairing with ASPM using miRTarBase (Fig. 3A) and we found that miR-26b-5p formed complementary base pairing with ASPM. Interestingly, after searching The Cancer Genome Atlas Program (TCGA), miR-26b-5p was significantly lower in HCC when compared with in normal (Fig. 3B). To analyze the relationship between ASPM and miR-26b-5p, luciferase reporter gene assay was conducted. Transfection of miR-26b-5p restrained the luciferase activities in HCC cells transfected with wt-ASPM. Nevertheless, miR-26b-5p had no inhibitive effect on the luciferase activities in HCC cells transfected with mut-ASPM (Fig. 3C). In addition, miR-26b-5p was downregulated in 46 cases of HCC samples (Fig. 3D) and lower level of miR-26b-5p was positively associated with advanced stage (Fig. 3E) and metastasis (Fig. 3F). Meanwhile, miR-26b-5p level was negatively connected to the mRNA level of ASPM in HCC (Fig. 3G). To verify the expression of ASPM was negatively modulated by miR-26b-5p, SMMC-7721 and PLC/PRF/5 cells were transfected with miR-26b-5p mimics (Fig. 3H) and we found that the expressions of ASPM were reduced by miR-26b-5p (Fig. 3I). In addition, upregulation of miR-26b-5p inhibited the growth and aggressive traits of HCC cells (Fig. 3J and K). All these observations indicate that miR-26b-5p negatively regulates the growth and invasion abilities of HCC cells.'

## **miR-26b-5p suppresses HCC cells growth and invasion by targeting ASPM**

In xenograft tumor model, the tumor growth in nude mice inoculated with miR-26b-5p expressed SMMC-7721 cell was significantly inhibited (Fig. 4A). The result of IHC staining illustrated that ASPM expression was significantly declined in tumor tissue from mice injected with miR-26b-5p stable expressed SMMC-7721 cell (Fig. 4B). To study whether miR-26b-5p regulate HCC cells progression by modulating ASPM, SMMC-7721 cell was cotransfected with miR-26b-5p and pcDNA3.1-ASPM plasmid. The western blotting analysis disclosed that the level of ASPM was rescued in SW480 cells cotransfected with miR-26b-5p and pcDNA3.1-ASPM when compared to cells transfected with miR-26b-5p alone (Fig. 4C). Upregulation of miR-26b-5p suppressed the colony formation and invasion of SMMC-7721 cell, which could be rescued

by pcDNA3.1-ASPM (Fig. 4D-4E). PLC/PRF/5 cell was cotransfected with anti-miR-26b-5p and si-ASPM plasmid. The level of ASPM was decreased in PLC/PRF/5 cell cotransfected with anti-miR-26b-5p and si-ASPM when compared with cell transfected with anti-miR-26b-5p alone (Fig. 4F). Downregulation of miR-26b-5p enhanced the colony formation and invasion of PLC/PRF/5 cell, which could be neutralized by si-ASPM (Fig. 4G-4H). These data suggest that miR-26b-5p regulates HCC cells growth and invasion by modulating ASPM.

## Discussion

Increasing evidences have demonstrated that miR-26b-5p is associated with the progression of malignant tumors. For instance, miR-26b-5p suppresses the proliferation and induces apoptosis in multiple myeloma cells by targeting Jagged Canonical Notch Ligand 1 (JAG1) [12]. miR-26b-5p suppresses the growth and metastatic-associated traits of human papillary thyroid cancer (PTC) in a  $\beta$ -Catenin-dependent manner [13]. MiRNA-26b-5p enhances T cell responses through targeting Pim-2 Proto-Oncogene (PIM-2) in HCC [14]. All these findings manifest that miRNA-26b-5p acts as an oncogenic miRNA in a variety of tumor types. Recently, ASPM has been identified to be upregulated in cancers and is involved into cancer cells process [15–17]. ASPM promotes prostate cancer stemness and progression by augmenting Wnt-Dvl-3- $\beta$ -catenin signaling [18]. ASPM is a novel marker for vascular invasion, early recurrence, and poor prognosis of HCC [19]. However, the biological actions of miR-26b-5p-ASPM axis in HCC had not been reported so far.

Herein, we confirmed that ASPM was significantly upregulated in HCC tissues when compared to that in non-cancerous samples. Furthermore, we seek the association between ASPM level and the clinical-pathological parameters in HCC and verified that higher level of ASPM was related with advance malignant stage and more metastasis. Meanwhile, higher expression of ASPM resulted into worse OS of patients with HCC. All data displayed that ASPM served as a cancer-promoting gene in HCC. In vitro functional experimental assays, ASPM silencing markedly impaired HCC cells growth and the migration, invasion abilities of HCC cells. Consistently, knockdown of ASPM inhibited the tumor growth of HCC cells in mice. However, upregulation of endogenous ASPM caused completely opposite results.

By using bioinformatics prediction tools and luciferase reporter experiment, we certified that ASPM was a target of miR-26b-5p, which has been confirmed as a suppressive miRNA in a variety of cancers. The immunoblotting assay confirmed that ASPM was negatively modulated by miR-26b-5p in HCC cell. In human HCC tissues, we also revealed that miR-26b-5p was downregulated in cancer samples and its level was inversely associated with the expression of ASPM. Consistent with early reports, we demonstrated that miR-26b-5p impaired the aggressive phenotypes of HCC cells [12, 13]. The correlation analysis further highlights the negative correlation between miR-26b-5p level and ASPM level in HCC tissues. Finally, the rescue experiments indicated that miR-26b-5p restrained the growth and invasion in HCC cells

via intervening ASPM. However, the promoting impacts of miR-26b-5p inhibitor was neutralized by ASPM silencing.

Early studies disclosed that miRNAs have one or more downstream target genes and modulate mRNAs degradation or translational inhibition [20]. Our current study has several limitations. Although we revealed that miR-26b-5p suppressed the aggressive phenotypes of HCC cell through blocking ASPM, it might be better to examine other targets of miR-26b-5p. Additional, the biological function of miR-26b-5p-ASPM in other biological processes, including HCC cells apoptosis need to be further explored.

## **Conclusion**

Altogether, our observations contribute to the understanding of the role of miR-26b-5p-ASPM axis involved into the aggressive-related traits of HCC cells.

## **Declarations**

### **Acknowledgements**

None.

### **Authors' contributions**

Hong-Guang Li and Heng-Jun Gao wrote the paper and conceived and designed the experiments. Fang-Feng Liu and Jun-Liu analyzed the data. All authors read and approved the final manuscript.

### **Funding**

Not applicable.

### **Ethics approval and consent to participate**

This study was approved by the Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong University. All procedures involving experimental animals were performed in accordance with protocols that were approved by the Committee for Animal Research of Shandong Provincial Hospital Affiliated to Shandong University and complied with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 86-23, revised 1985).

### **Consent for publication**

Not applicable.

### **Competing interests**

The authors declare that they have no competing interests.

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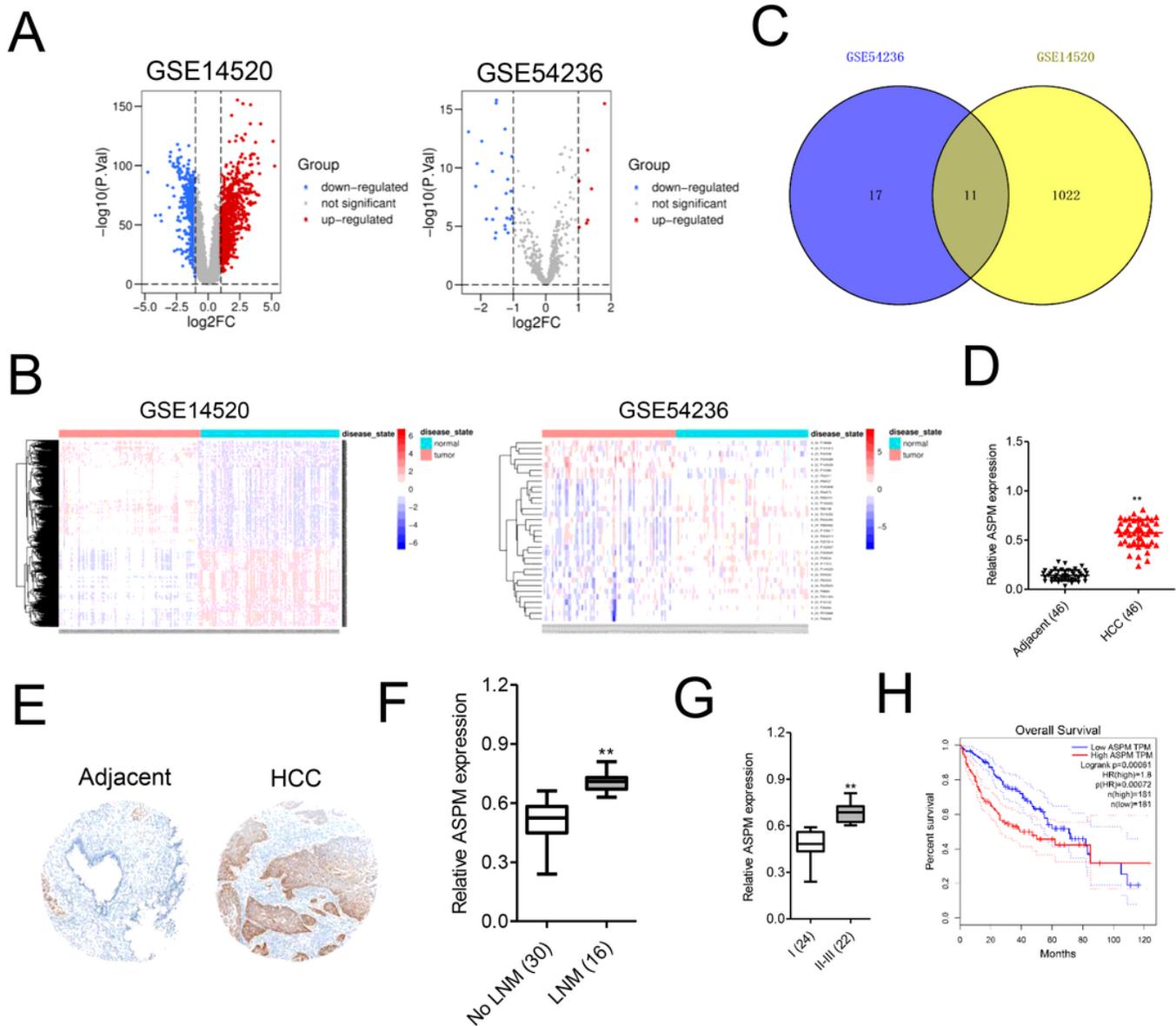
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## References

1. Wong CM, Tsang FH, Ng IO. Non-coding RNAs in hepatocellular carcinoma: molecular functions and pathological implications. *Nature reviews Gastroenterology & hepatology*. 2018 Mar;15(3):137-151.
2. Giannelli G, Koudelkova P, Dituri F, et al. Role of epithelial to mesenchymal transition in hepatocellular carcinoma. *Journal of hepatology*. 2016 Oct;65(4):798-808.
3. Zhu Z, Zhang X, Wang G, et al. Role of MicroRNAs in Hepatocellular Carcinoma. *Hepatitis monthly*. 2014 Aug;14(8):e18672.
4. Vasuri F, Visani M, Acquaviva G, et al. Role of microRNAs in the main molecular pathways of hepatocellular carcinoma. *World journal of gastroenterology*. 2018 Jul 7;24(25):2647-2660.
5. Saleh AA, Gohar SF, Hemida AS, et al. Evaluation of ASPM and TEF Gene Expressions as Potential Biomarkers for Bladder Cancer. *Biochemical genetics*. 2020 Jun;58(3):490-507.
6. Timaner M, Shaked Y. Elucidating the roles of ASPM isoforms reveals a novel prognostic marker for pancreatic cancer. *The Journal of pathology*. 2020 Feb;250(2):123-125.
7. Chen X, Huang L, Yang Y, et al. ASPM promotes glioblastoma growth by regulating G1 restriction point progression and Wnt-beta-catenin signaling. *Aging*. 2020 Jan 6;12(1):224-241.
8. Johnson MB, Sun X, Kodani A, et al. Aspm knockout ferret reveals an evolutionary mechanism governing cerebral cortical size. *Nature*. 2018 Apr;556(7701):370-375.
9. Li C, Zhang Y, Zhao W, et al. miR-153-3p regulates progression of ovarian carcinoma in vitro and in vivo by targeting MCL1 gene. *Journal of cellular biochemistry*. 2019 Nov;120(11):19147-19158.
10. Li Q, Wu X, Guo L, et al. MicroRNA-7-5p induces cell growth inhibition, cell cycle arrest and apoptosis by targeting PAK2 in non-small cell lung cancer. *FEBS open bio*. 2019 Nov;9(11):1983-1993.
11. Yang X, Zhao H, Yang J, et al. MiR-150-5p regulates melanoma proliferation, invasion and metastasis via SIX1-mediated Warburg Effect. *Biochemical and biophysical research communications*. 2019 Jul 12;515(1):85-91.
12. Jia CM, Tian YY, Quan LN, et al. miR-26b-5p suppresses proliferation and promotes apoptosis in multiple myeloma cells by targeting JAG1. *Pathology, research and practice*. 2018 Sep;214(9):1388-1394.
13. Zhou A, Pan H, Sun D, et al. miR-26b-5p Inhibits the Proliferation, Migration and Invasion of Human Papillary Thyroid Cancer in a beta-Catenin-Dependent Manner. *OncoTargets and therapy*. 2020;13:1593-1603.

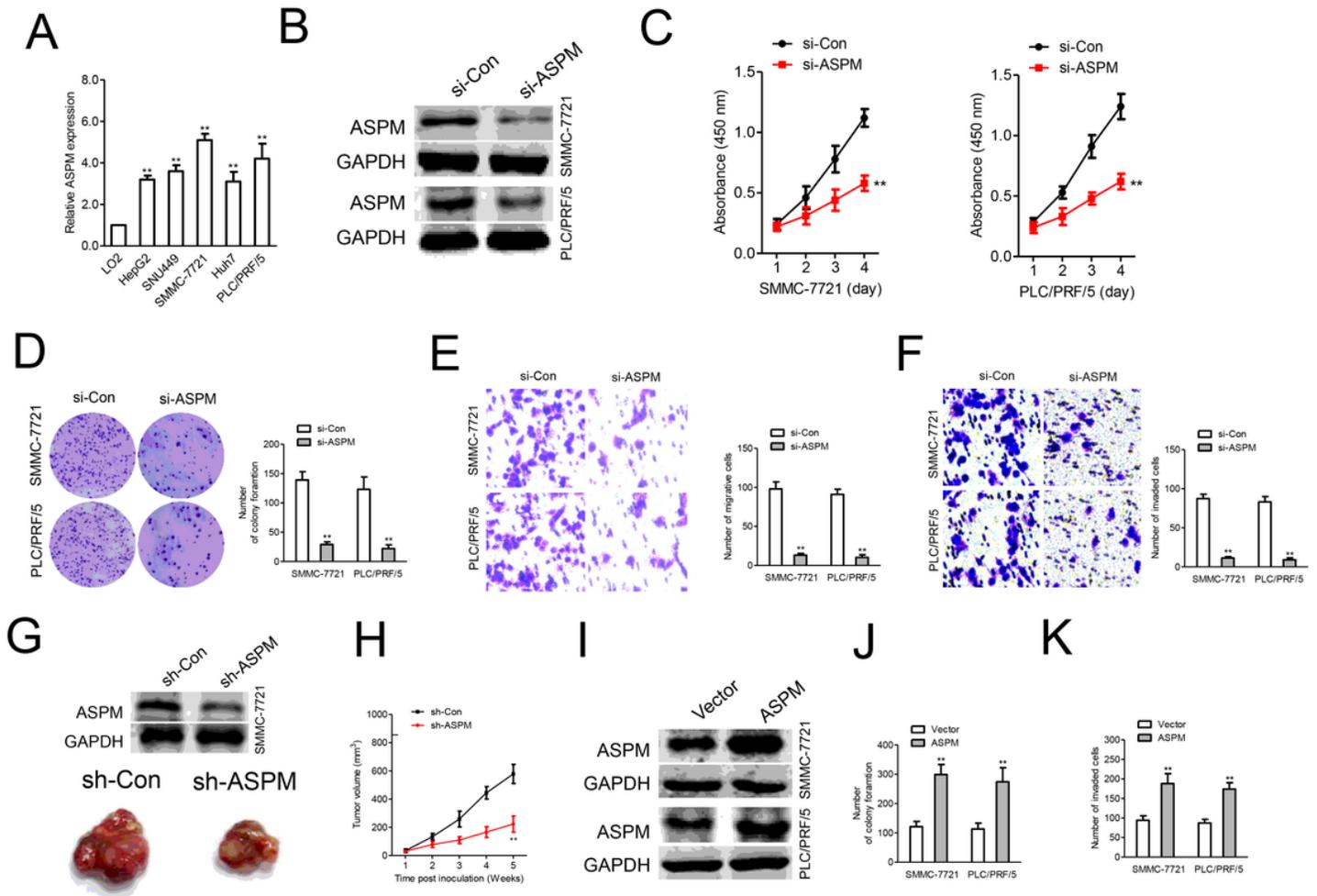
14. Han W, Li N, Liu J, et al. MicroRNA-26b-5p enhances T cell responses by targeting PIM-2 in hepatocellular carcinoma. *Cellular signalling*. 2019 Jul;59:182-190.
15. Zhou JW, Wang H, Sun W, et al. ASPM is a predictor of overall survival and has therapeutic potential in endometrial cancer. *American journal of translational research*. 2020;12(5):1942-1953.
16. Tang J, Lu M, Cui Q, et al. Overexpression of ASPM, CDC20, and TTK Confer a Poorer Prognosis in Breast Cancer Identified by Gene Co-expression Network Analysis. *Frontiers in oncology*. 2019;9:310.
17. Xu Z, Zhang Q, Luh F, et al. Overexpression of the ASPM gene is associated with aggressiveness and poor outcome in bladder cancer. *Oncology letters*. 2019 Feb;17(2):1865-1876.
18. Pai VC, Hsu CC, Chan TS, et al. ASPM promotes prostate cancer stemness and progression by augmenting Wnt-Dvl-3-beta-catenin signaling. *Oncogene*. 2019 Feb;38(8):1340-1353.
19. Lin SY, Pan HW, Liu SH, et al. ASPM is a novel marker for vascular invasion, early recurrence, and poor prognosis of hepatocellular carcinoma. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2008 Aug 1;14(15):4814-20.
20. Zhang Y, He Y, Lu LL, et al. miRNA-192-5p impacts the sensitivity of breast cancer cells to doxorubicin via targeting peptidylprolyl isomerase A. *The Kaohsiung journal of medical sciences*. 2019 Jan;35(1):17-23.

## Figures



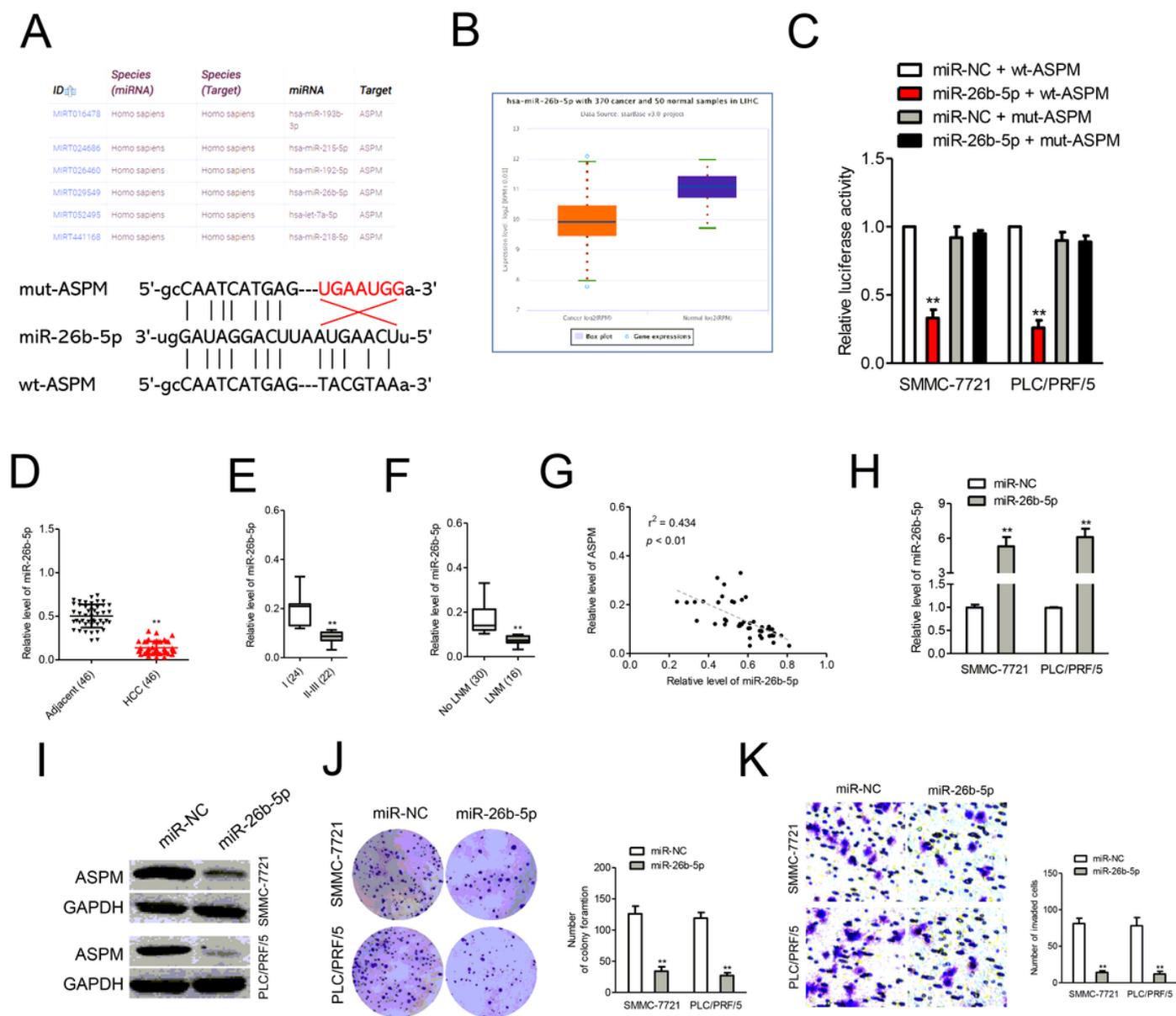
**Figure 1**

Overexpression of ASPM correlates with clinicopathological features and poor prognosis in HCC patients. (A) Volcano plot showing the differential genes (red dots and blue dots) in the expression microarray. (B) Heatmap of GSE54236 and GSE14520. (C) Venn diagram showing the common upregulated genes in two GEO dataset. (D) qRT-PCR analysis of ASPM expression in 46 human HCC tissues and the paired adjacent normal tissues. (E) The expressions of ASPM in HCC and adjacent tissues were measured by IHC staining. (F) The correlation between the expression levels of ASPM with tumor metastasis. (G) The correlation between the expression levels of ASPM with stage. (H) Kaplan-Meier survival analysis of overall survival rate between HCC patients with low or high of ASPM.



**Figure 2**

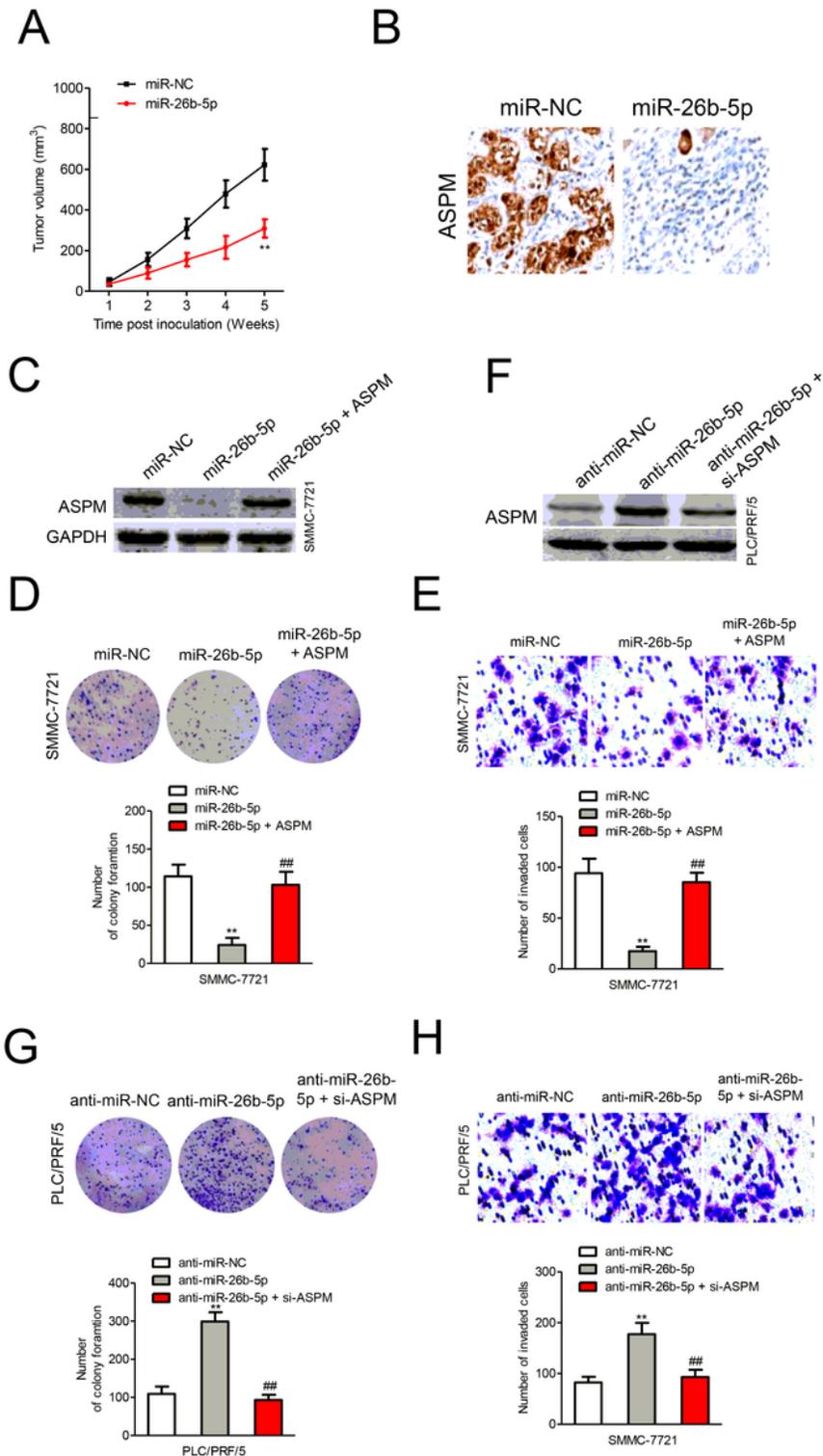
Knockdown of ASPM suppresses HCC cell proliferation, migration and invasion. (A) qRT-PCR analysis of expression in normal liver cell lines (LO2) and HCC cell lines (HepG2, SNU449, SMMC-7721, Huh7 and PLC/PRF/5). (B) Western blot analysis of expression in SMMC-7721 and PLC/PRF/5 cells transfected with si-Con or si-ASPM. (C-D) Cell proliferation capacity was analyzed by CCK-8 assay and colony formation assay. (E-F) The migration and invasion capability of SMMC-7721 and PLC/PRF/5 cells were analyzed by transwell assay. \*\*P<0.01 compared with si-Con. (G) Western blot analysis of expression in SMMC-7721 cells stable transfected with sh-Con or sh-ASPM (upper panel). Quantitative analysis of xenografted tumor volume (lower panel). \*\*P<0.01 compared with sh-Con. (I) Western blot analysis of expression in SMMC-7721 and PLC/PRF/5 cells transfected with vector or pcDNA3.1-ASPM. (J) Cell growth was analyzed using colony formation assay. (K) The invasion capability of SMMC-7721 and PLC/PRF/5 cells transfected with vector of ASPM plasmid were analyzed by transwell assay. \*\*P<0.01 compared with vector.



**Figure 3**

ASPM is a direct target of miR-26b-5p. (A) The target genes of miR-1908 were predicted by miRTarBase. The potential binding sites between miR-26b-5p and ASPM were shown. (B) The level of miR-26b-5p was decreased in HCC tissues when compared with that in normal from TCGA dataset. (C) Relative luciferase activity from SMMC-7721 and PLC/PRF/5 cells cotransfected with reporter plasmid and miR-26b-5p. (D) qRT-PCR analysis of miR-26b-5p expression in 46 human HCC tissues and the paired adjacent normal tissues. (E-F) The correlation between the expression levels of ASPM with tumor metastasis and stage. (G) The level of miR-26b-5p was negatively associated with ASPM expression in HCC tissues. (H) qRT-PCR analysis of miR-26b-5p level in SMMC-7721 and PLC/PRF/5 cells transfected with miR-NC or miR-26b-5p. (I) Western blot analysis of ASPM expression in SMMC-7721 and PLC/PRF/5 cells transfected with miR-NC or miR-26b-5p. (J) Cell growth was analyzed using colony formation assay. (K) The invasion

capability of SMMC-7721 and PLC/PRF/5 cells transfected with miR-NC or miR-26b-5p were analyzed by transwell assay. \*\*P<0.01 compared with miR-NC.



**Figure 4**

The effect of miR-26b-5p/ASPM axis on HCC cells biological behavior in vitro. (A) Tumor growth curves were measured after injection of SMMC-7721 cells transfected with miR-NC or miR-26b-5p. Tumor volume was calculated every week. Data are presented as Mean  $\pm$  SD. (n = 5). \*\*P<0.01 compared with

anti-miR-NC. (B) Tumors developed from miR-26b-5p transfected SMMC-7721 cells showed a lower level of ASPM protein than tumors developed from miR-NC transfected cells. (C) miR-26b-5p and pcDNA3.1-ASPM were cotransfected into SMMC-7721 cells, the expression of ASPM was detected using western blot. (D-E) The colony formation and invasion ability of SMMC-7721 cells were assessed by colony formation assay and transwell assay. \*\*P<0.01 compared with miR-NC, ##P<0.01 compared with miR-26b-5p. (F) anti-miR-26b-5p and si-ASPM were cotransfected into SMMC-7721 cells, the expression of ASPM was detected using western blot. (G-H) The colony formation and invasion ability of SMMC-7721 cells were assessed by colony formation assay and transwell assay. \*\*P<0.01 compared with anti-miR-NC, ##P<0.01 compared with anti-miR-26b-5p.