

SKA2 promotes proliferation and invasion of hepatocellular carcinoma cells via activating the β -catenin signaling pathway

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Spindle and kinetochore associated complex subunit 2 (SKA2) is a part of the spindle and kinetochore associated (SKA) complex, which has been reported in various cancers, including the breast cancer, lung cancer, and glioma. However, its role remains unclear in hepatocellular carcinoma (HCC). Our study found that SKA2 mRNA levels and immunohistochemistry staining were significantly increased in HCC tissues compared with normal tissues. The role of SKA2 in cell proliferation and invasion was also determined. Overexpression of SKA2 significantly promoted cell proliferation and invasion, while knocking down of SKA2 expression inhibited the growth and invasion of HCC cells. In experiments investigating the underlying mechanism, overexpression of SKA2 may increase the expression levels of total β -catenin, and knockdown of SKA2 may decrease the expression levels of total β -catenin. Our studies thus suggest that SKA2 may promote proliferation and invasion of hepatocellular carcinoma cells by activating the β -catenin signaling pathway, which can serve as a potential target in the diagnosis and/or treatment of HCC.

Key words: SKA2, proliferation, invasion, hepatocellular carcinoma, β -catenin

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors with high morbidity and mortality. It is the fifth most commonly occurring cancer and the third leading cause of cancer deaths worldwide [1]. Multiple treatments such as liver resection, transarterial chemoembolization (TACE), radiotherapy and sorafenib can be employed for HCC patients. However, the prognosis of HCC patients remains poor due to the prognosis difficulty, high recurrence and early vascular invasion [2, 3]. More and more evidence revealed that HCC was associated with multi-gene mutation [4], therefore, the molecular target is now as a novel therapy for advanced HCC patients, as it showed a favorable curative effect and significantly prolonged the patient's survival time [5]. Hence, understanding HCC development and finding new molecular targets for HCC treatment become a significant focus.

Spindle and kinetochore associated complex subunit 2 (SKA2) located on human chromosome 17q 23.2 is a part of the spindle and kinetochore associated (SKA) complex, a heterotrimeric complex formed from SKA1, SKA2, and SKA3 [6, 7]. SKA2 is involved in the formation of Ska complex and plays an important role in the maintenance of the mitotic

mid-plateau and shutdown of the spindle checkpoint [6, 8–10]. Recent evidence showed that overexpression of SKA2 promoted the proliferation of human breast cancer, whereas SKA2 knockdown in human lung epithelial cells reduced transactivation and suppressed dexamethasone inhibition of proliferation [11, 12]. Furthermore, the function of SKA2 has been observed in various cancers, including breast cancer, lung cancer, and glioma [13–16]. However, the role of SKA2 in HCC still remains unclear.

In our study, we focused on the importance of SKA2 in the progression and metastasis of HCC. We demonstrated that SKA2 was highly expressed in human HCC tissues and enhanced the proliferation and invasion of HCC cell lines *in vitro*. Additionally, we evaluated the molecular mechanism of SKA2 in HCC growth and metastasis.

Patients and methods

HCC tissue samples. Thirty-five pairs of primary HCC tissues and their paired adjacent normal tissues were obtained from patients who underwent hepatectomy between February 2017 and July 2018 at the Affiliated

Hospital of Traditional Chinese Medicine, Xinjiang Medical University. All of them signed informed consent. All specimens were immediately snap-frozen in liquid nitrogen and maintained at -80°C until use. These 30 paired samples were subjected to RNA extraction for quantitative real time PCR (qRT-PCR) analysis, and the other 5 paired samples were used for immunohistochemistry. Ethical approval for the study was obtained from the Research Ethics Committee of the Affiliated Hospital of Traditional Chinese Medicine, Xinjiang Medical University.

Immunohistochemistry. Clinical HCC tissues and paired non-cancerous tissues were fixed in formalin, embedded in paraffin, and cut into $5\ \mu\text{m}$ thick consecutive sections. After deparaffinization and antigen recovery (in a sodium citrate solution, pH 6.0, 20 min, 98°C), the sections were washed thrice with 0.01 mol/l PBS for 5 min each time, blocked for 1 h in 0.01 mol/l PBS containing 0.3% Triton X-100 and 5% BSA, and incubated with an anti-SKA2 (1:100, ab75345, Abcam) antibody at 4°C overnight. After brief washes with 0.01 mol/l PBS, the sections were incubated with 0.01 mol/l PBS containing a horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:500, SA00001-2, Proteintech) for 2 h, followed by development with 0.003% H_2O_2 and 0.03% 3, 30-diaminobenzidine in 0.05 mol/l Tris-HCl (pH 7.6). Two histopathologists blindly reviewed the slides and scored the staining.

RNA preparation and real-time PCR. Preparation of RNA and complementary DNA (cDNA) samples were performed as described earlier [17]. The specimens were snap-frozen in liquid nitrogen and stored at -80°C . The total RNA of tumor tissues and adjacent noncancerous tissues from the 30 patients was isolated using Trizol reagent (Invitrogen, Carlsbad, CA), and reverse-transcribed to cDNA using the PrimeScript RT reagent Kit (DRR037A, Takara, Japan) according to the manufacturer's instructions. The real-time PCR was performed using the SYBR Premix Ex Taq (Takara). The SKA2-specific primers were 5'-GGAAGTGGTCCAGAAAGCTG-3' (forward) and 5'-AGCTCCAGGTCTGTTTGCTT-3' (reverse), and the GAPDH primers were 5'-ATGACCCCTTCATTGACCTCA-3' (forward) and 5'-GAGATGATCACCCTTTTGCT-3' (reverse). GAPDH acted as an internal control. The relative mRNA level of target genes to that of GAPDH in clinical samples was calculated according to the methods described [18].

Cell culture. Human HCC cell lines YY-8103, YY-8103, HepG2, and Huh7 were purchased from the cell bank of the Shanghai Biology Institute, Chinese Academy of Science (Shanghai, China). Cell lines were maintained in a humidified atmosphere containing 5% CO_2 at 37°C in DMEM medium supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% fetal bovine serum (FBS; Gibco).

Cell transfection. Lentiviral constructs of SKA2 and the nucleotide sequences of shRNA against SKA2 were described previously [19]. The sequences of the shSKA2 were 5'-CCGGCAAACCTTTGTATGCCCGCTTTCTCGAGA-

AAGCGGGCATACAAAGTTTGTGTTTTG-3' (forward) and 5'-AATTCAAAAACAACTTTGTATGCCCGCTTTCTCGAGAAAGCGGGCATACAAAGTTTGTG-3' (reverse). YY-8103 and LM3 cells were infected with p23-ZsGreen-SKA2 and HepG2 and Huh7 cells were infected with pLKO.1-shRNA. Overexpressed and silenced cells were sorted using flow cytometry or selected by puromycin ($4\ \mu\text{g}/\text{ml}$) for at least 4 days. All cells were routinely cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Gibco) in a humidified incubator containing 5% CO_2 at 37°C .

Western blot. Western blot analysis was performed as previously described [20]. Total cell lines protein was harvested and lysed with RIPA buffer (Beyotime, Beijing, China) according to the manufacturers' instructions. Primary antibodies against the following proteins were used as follows: SKA2 from Abcam; β -catenin (cat. #8480), Flag (cat. #14793) and GAPDH (cat. #5174) were purchased from Cell Signaling Technology. The images were captured using the Gel Dox XR system (Bio-Rad, Philadelphia, PA).

Cell proliferation analysis. Cell proliferation was detected with crystal violet assay or MTT staining method described by manufacturers' instructions. Briefly, in crystal violet assay, 1×10^3 cells were seeded into 6-well plates, the cells were cultured in medium with 10% FBS, changed the medium every three days and 2 weeks later the cells were stained with crystal violet. After staining for 10 min, the fixed cells were washed with phosphate-buffered saline (PBS) and photographed. Then, 1 ml glacial acetic acid was added to the cells and the optical density (OD) was detected at 570 nm by a microplate reader.

In MTT assay, 1×10^3 cells were seeded into 96-well plates, and cell viability was detected by MTT, after 1, 2, 3, 4, 5, 6, or 7-day incubation, $20\ \mu\text{l}$ of a 5 mg/ml MTT solution was added to each well, and the plate was incubated at 37°C for further 4 hours. Then, the medium was removed, $200\ \mu\text{l}$ DMSO was added to dissolve the generated formazan. The measurement process was performed every 24 hours for 7 days to generate a cell growth curve. The optical density (OD) was measured at 490 nm by a microplate reader.

Transwell assay. The invasion of HCC cells was examined by transwell with polyethylene terephthalate membranes (24-well inserts, $8.0\ \mu\text{m}$, Corning). $150\ \mu\text{l}$ cell suspension contained 2×10^5 cells were loaded into the upper well coated with Matrigel (BD Biosciences). Next, $500\ \mu\text{l}$ DMEM medium with 10% FBS was placed into the bottom of the well to induce invasion. 72 h later, invasive cells were stained with 0.1% crystal violet. Randomly selected four fields and counted the cells, experiments were repeated three times and the data are presented as the means \pm SD.

Statistical analysis. Statistical evaluations were performed with GraphPad software (www.graphpad.com), and results were shown as mean \pm SD unless otherwise stated. Statistical significance was set at a p value of <0.05 and marked with an asterisk.

Results

SKA2 is upregulated in HCC tissues. Firstly, we examined the mRNA level of SKA2 in 30 pairs of HCC tissues and the corresponding normal tissue, and we found that the mRNA expression of SKA2 was significantly increased in 22 of 30 HCC tissues compared to the matched normal tissues (Figure 1A). The immunohistochemistry assays showed that SKA2 was mainly located in the cytoplasm, and the tumor tissues showed stronger staining when compared to the paired normal tissues in five patient cases (Figures 1B, S1), which was consistent with the results of real-time PCR. From the TCGA database, we found that the expression of SKA2 was significantly upregulated in 11% (40/360) HCC tissues (Figure 1C). In addition, the overall survival (OS) rate was significantly higher in cases without SKA2 alteration group compared to cases with SKA2 alteration group ($p < 0.05$, Figure 1D). Taken together, we can confirm that SKA2 is upregulated in HCC tissues based on our analysis and TCGA database.

Successful overexpression and knockdown of SKA2 in HCC cell lines. Based on the clinical data, we hypothesized that SKA2 might influence the growth of HCC cells. Firstly,

we checked the SKA2 levels in several common HCC cell lines at the protein level. We found that the expression level of SKA2 was significantly increased in HepG2 and Huh7 cell lines compared to YY-8103 and LM3 cell lines (Figure 2A). To identify the function of SKA2 in HCC cells, we transfected the plasmids containing either an empty p23 vector or SKA2 overexpression vectors (Flag-SKA2) into YY-8103 and LM3 HCC cell lines, which have relatively low expression of SKA2. And we used specific shRNA targeting SKA2 in HepG2 and Huh7 cell lines, which have relatively high expression of SKA2. Western blotting analyses showed that we successfully established the overexpression and knockdown of SKA2 in HCC cell lines (Figures 2B–E).

SKA2 overexpression promoted growth and invasion of HCC cell lines. The MTT assays showed that the absorbance values of the LM3 cells after 4, 5 and 6 days post-transfection with SKA2 overexpression vectors was significantly higher than those of the untreated cells ($p < 0.01$, Figure 3A). Similarly, the absorbance values of the YY-8103 cells after 5 and 6 days post-transfection with SKA2 overexpression vectors were also significantly higher than those of the untreated cells ($p < 0.01$, Figure 3A). Furthermore, the crystal violet assays

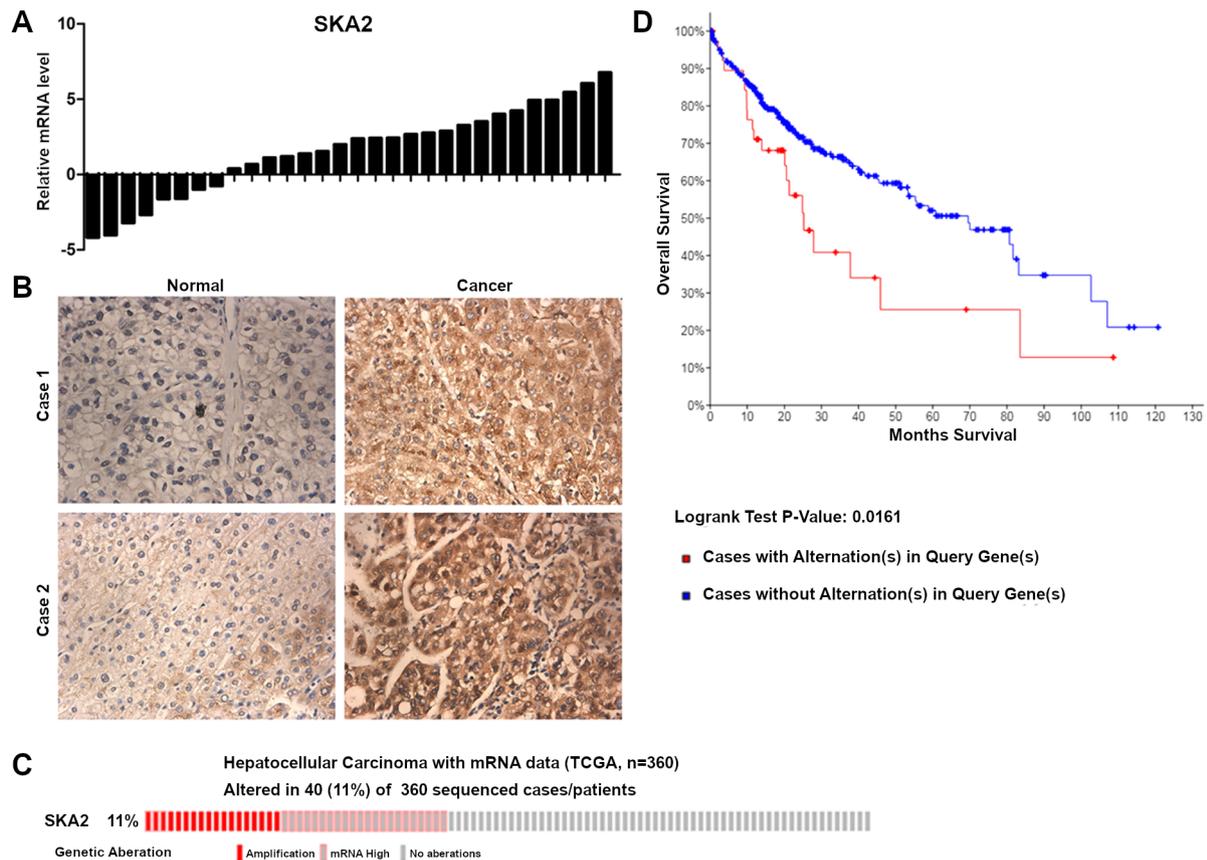


Figure 1. SKA2 expression was upregulated in HCC tissues and correlated with HCC patient survival. A) SKA2 mRNA levels in 30 pairs of tumor samples (T) and matched normal hepatic tissues (N) determined by q-PCR. B) Immunohistochemistry staining of SKA2 in paired N and T tissues from two patients. C) SKA2 was altered in 11% of HCC patients (from TCGA database). D) SKA2 with alterations was correlated with worse overall survival in HCC patients (from TCGA database).

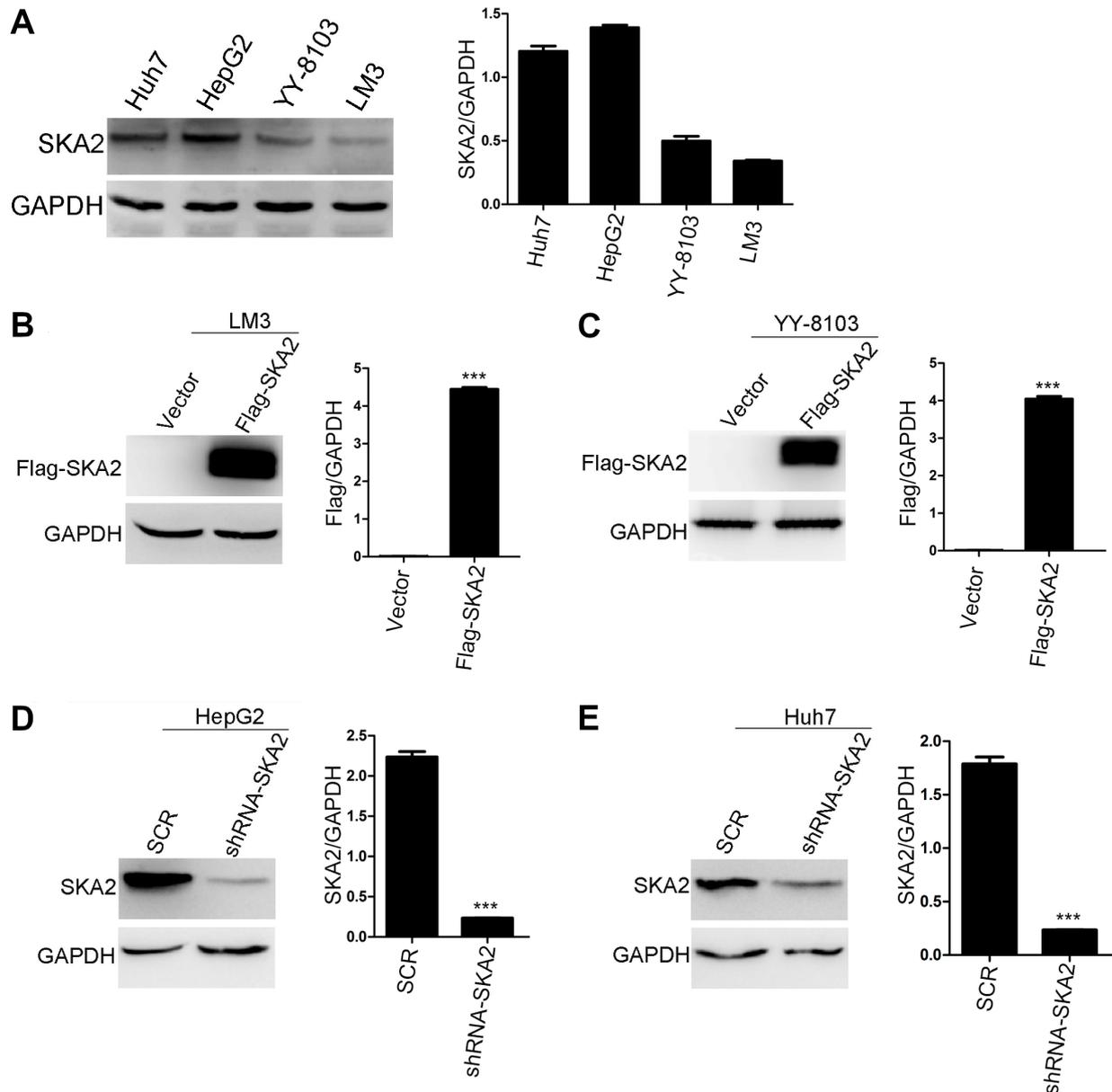


Figure 2. Overexpression and knockdown of SKA2 in HCC cell lines. **A)** Western blots showing SKA2 expression in four HCC cell lines: YY-8103, LM3, HepG2, and Huh7. Western blots showing the overexpression of SKA2 in **B)** LM3 cells and **C)** YY-8103 cells. Western blots showing the knocking down of SKA2 in **D)** HepG2 cells and **E)** Huh7 cells. Left panel: Result of western blot. Right panel: Grey analysis results of the western blot bands. GAPDH was used as a loading control.

showed that absorbance values of the LM3 and YY-8103 cells after transfection with SKA2 overexpression vectors were significantly higher than those of the untreated cells (all $p < 0.001$, Figure 3B). In addition, the transwell assays showed that the overexpression of SKA2 significantly promoted the invasion of LM3 and YY-8103 cells (Figure 3C).

Knocking down SKA2 expression inhibited the growth of HCC cell lines. Similar to the overexpression experiments, we tested the growth and invasion of the control and SKA2-shRNA cell lines. The MTT assays showed that the

absorbance values of the HepG2 cells at 3, 4, 5 and 6 days after SKA2 downregulation were significantly lower than those of the untreated cells ($p < 0.01$, Figure 4A). Similarly, the absorbance values of the Huh-7 cells at 4, 5 and 6 days after SKA2 downregulation were also significantly lower than those of the untreated cells ($p < 0.01$, Figure 4A). In addition, the crystal violet assays showed that absorbance values of the HepG2 and Huh-7 cells after SKA2 downregulation were significantly lower than those of the untreated cells (all $p < 0.001$, Figure 4B). Moreover, the transwell assays

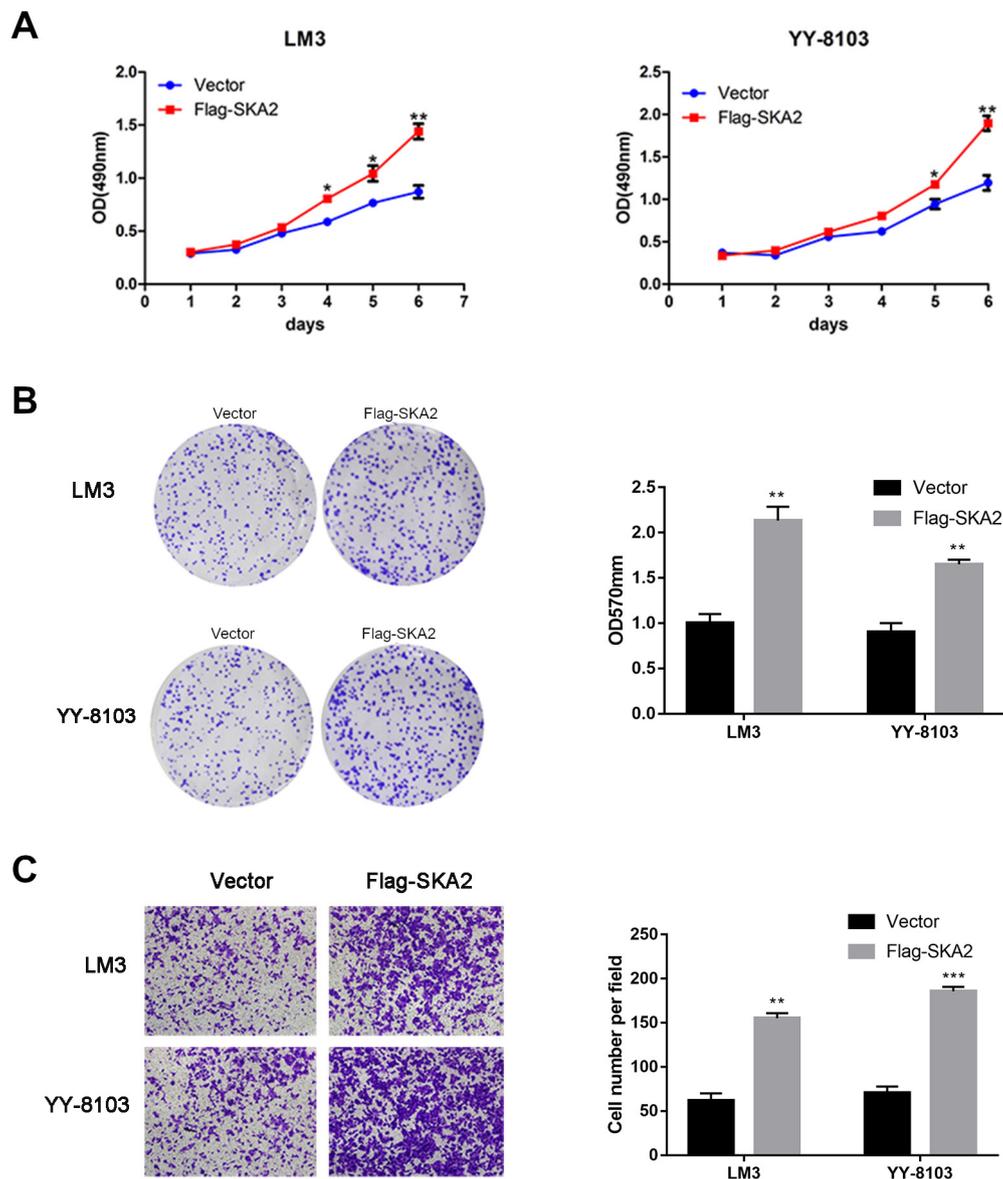


Figure 3. SKA2 overexpression promoted growth and invasion in HCC cells. **A)** The effects of SKA2 overexpression on the proliferation capacity of LM3 and YY-8103 cells were assessed by MTT assays. **B)** The effects of SKA2 overexpression on the proliferation capacity of LM3 and YY-8103 cells were assessed by crystal violet assays. **C)** The effects of SKA2 overexpression on the invasion of LM3 and YY-8103 cells were assessed by transwell assays. Quantitative blot analysis was shown on the right. Data shown are mean \pm SD from three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with the Vector group.

demonstrated that the knocking down of SKA2 significantly suppressed the invasion of HepG2 and Huh7 cells (Figure 3C).

SKA2 activated the β -catenin Signaling Pathway in HCC cells. It has been reported that Wnt/ β -catenin signaling pathway activation plays an important role in the development and progression of HCC. To explore the molecular mechanism of SKA2 on HCC, western blot analysis was performed to investigate the expression levels of total β -catenin protein levels in SKA2 overexpression and knock-

down cell lines. As shown in Figure 5A–B, overexpression of SKA2 significantly increased the expression of total β -catenin in LM3 and YY-8103 cell lines. In turn, SKA2 downregulation significantly decreased the level of total β -catenin (Figures 5C–D). In addition, the expression level of Snail and Vimentin that were relevant to the tumor progression and metastasis were significantly increased by overexpression of SKA2 in LM3 and YY-8103 cells. However, the opposite effects were observed in HepG2 and Huh7 cells. Moreover, we also examined the expression level of some markers of

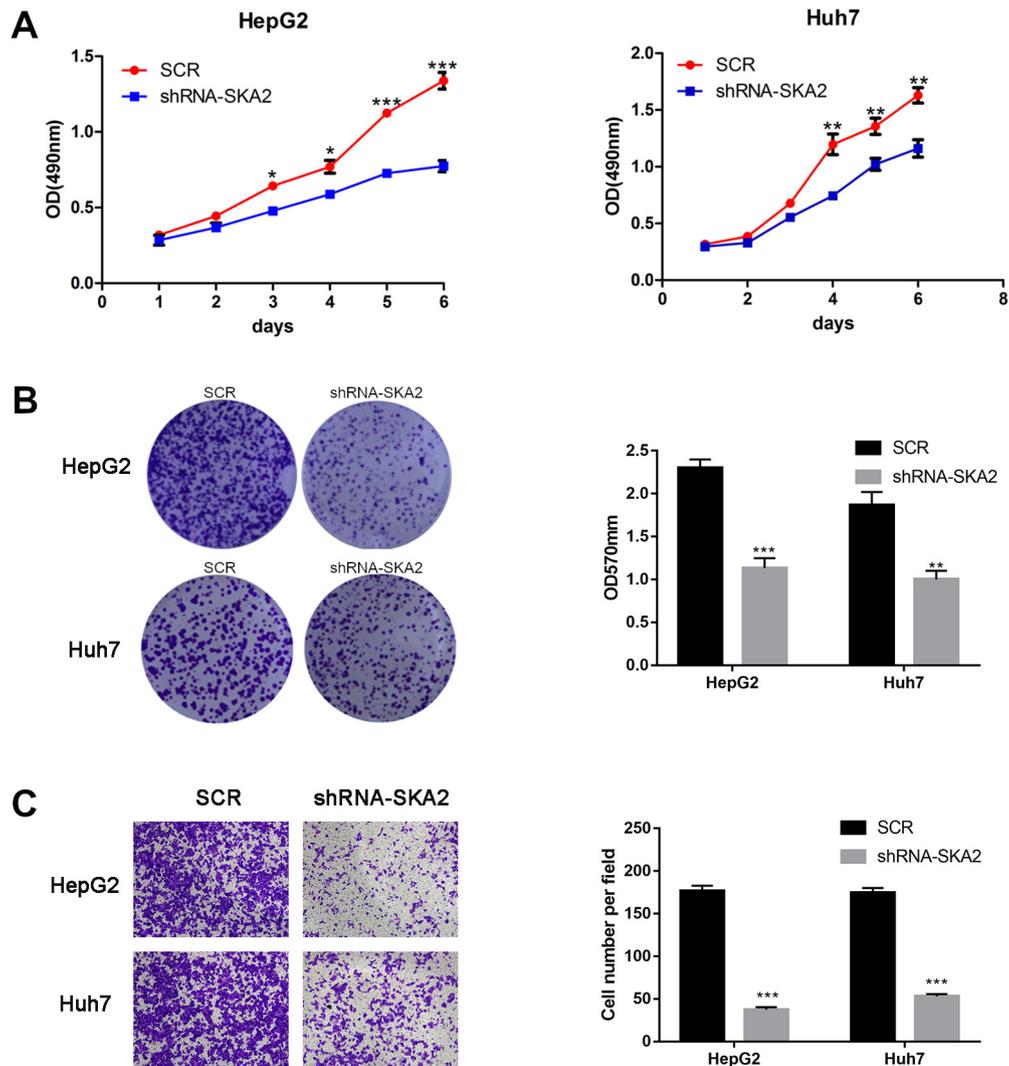


Figure 4. SKA2 knockdown inhibited growth in HCC cells. **A)** The effects of SKA2 knockdown on the proliferation capacity of HepG2 and Huh7 cells were assessed by MTT assays. **B)** The effects of SKA2 knockdown on the proliferation capacity of HepG2 and Huh7 cells were assessed by crystal violet assay. **C)** The effects of SKA2 knockdown on the invasion of HepG2 and Huh7 cells were assessed by transwell assays. Quantitative blot analysis is shown on the right. Data shown are mean \pm SD from three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with the shRNA group.

other important signaling pathways, such as p-ERK in the RAF/MEK/ERK pathway, p-AKT, and p-mTOR in the PI3K/AKT/mTOR pathway (Figure S2). We found that SKA2 did not influence these signaling pathways to promote the proliferation and invasion of HCC cells, which supported the finding that Wnt/ β -catenin pathway is exclusively involved in the upregulation of cell proliferation and invasion of hepatocellular carcinoma.

Discussion

The prognosis of patients with metastatic or recurrent HCC remains poor, with a 5-year survival rate of $<15\%$, despite some progress has been achieved in the diagnosis and

treatment of HCC [21]. Therefore, exploring new regulating cancer genes and finding the new regulatory mechanism has become the key points in the field of HCC. Emerging evidence has shown that SKA2 was involved in tumorigenesis of various cancers, including breast cancer, lung cancer, and glioma [13–16].

In the current study, it is documented that the SKA2 mRNA levels and immunohistochemistry staining are significantly increased in HCC tissues, compared with normal tissues. Furthermore, the western analysis confirmed the successful overexpression and knockdown of SKA2 in HCC cell lines. Cell proliferation and invasion were significantly promoted following the overexpression of SKA2. In addition, the knocking down of SKA2 expression inhibited the growth

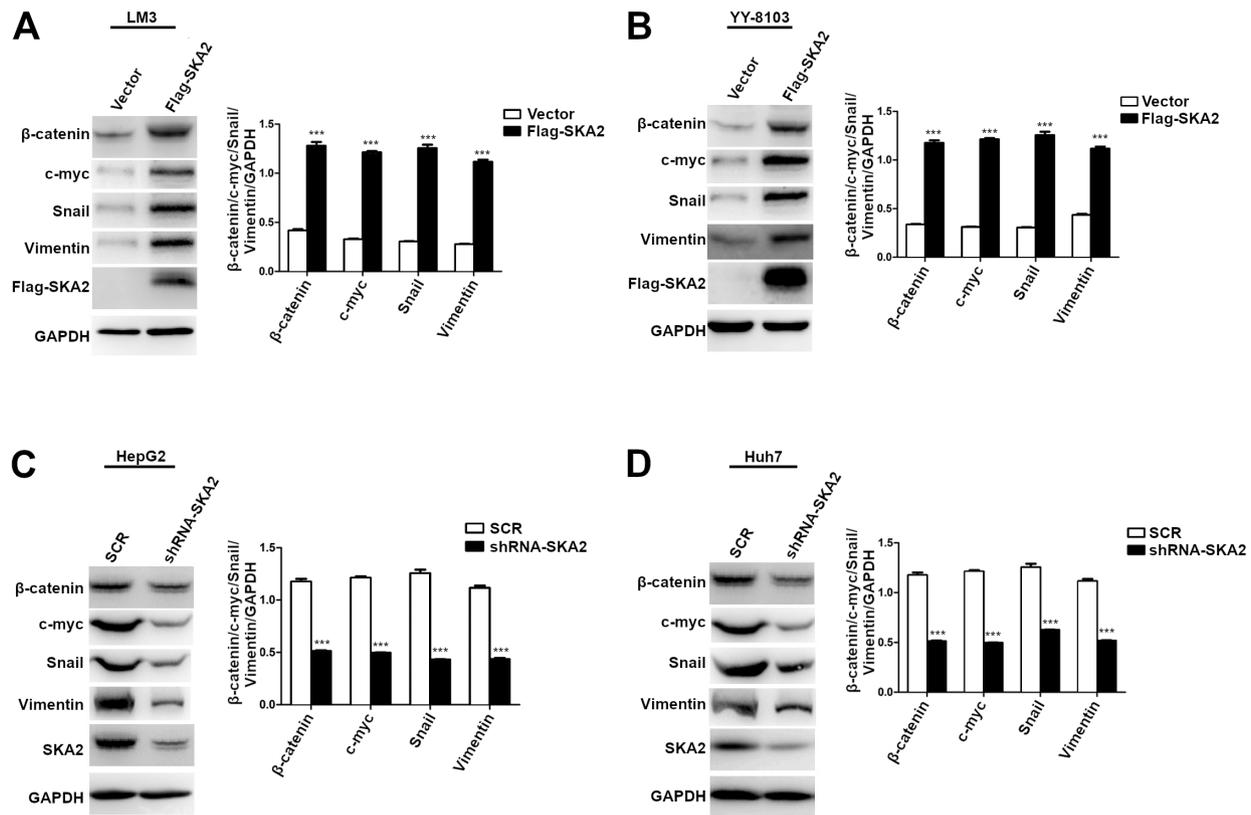


Figure 5. SKA2 activated the Wnt/ β -catenin signaling pathway. The overexpression SKA2 respectively increased the expression of total β -catenin, c-myc, Snail, and Vimentin in LM3 (A) and YY-8103 cells (B) according to the western blot. Left panel: Result of western blot. Right panel: Grey analysis results of the western blot bands. GAPDH was used as a loading control. The knockdown SKA2 respectively decreased the expression of total β -catenin, c-myc, Snail, and Vimentin in HepG2 (C) and Huh7 (D) cells according to the western blot. Left panel: Result of western blot. Right panel: Grey analysis results of the western blot bands. GAPDH was used as a loading control.

and invasion of HCC cell lines. In experiments investigating the underlying mechanism, expression levels of total β -catenin were significantly increased by overexpression of SKA2. Meanwhile, the knockdown of SKA2 decreased the expression of total β -catenin that was relevant to the tumor progression. The present study revealed that SKA2 might promote proliferation and invasion of HCC cells by activating the Wnt/ β -catenin signaling pathway. It has been well reported that Wnt/ β -catenin signaling pathway was involved in cancer development and progression [22]. β -catenin is a core component of the canonical Wnt signaling pathway that regulates the transcription of several Wnt downstream target genes, such as c-myc, cyclinD1, and vimentin, leading to cell metastasis and invasion [23]. Moreover, our study found that the overexpression of SKA2 could increase the expression of Snail and Vimentin that were relevant to the tumor progression and metastasis. To the best of our knowledge, this is the first study revealing how SKA2 is involved in the proliferation and invasion of HCC.

In summary, our study demonstrated that SKA2 plays a critical role in the proliferation and invasion of HCC cells.

These results provide a better understanding of the mechanisms for the role of SKA2 in tumor development and might serve as a potential target in the diagnosis and/or treatment of HCC.

Supplementary information is available in the online version of the paper.

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Supplementary Information

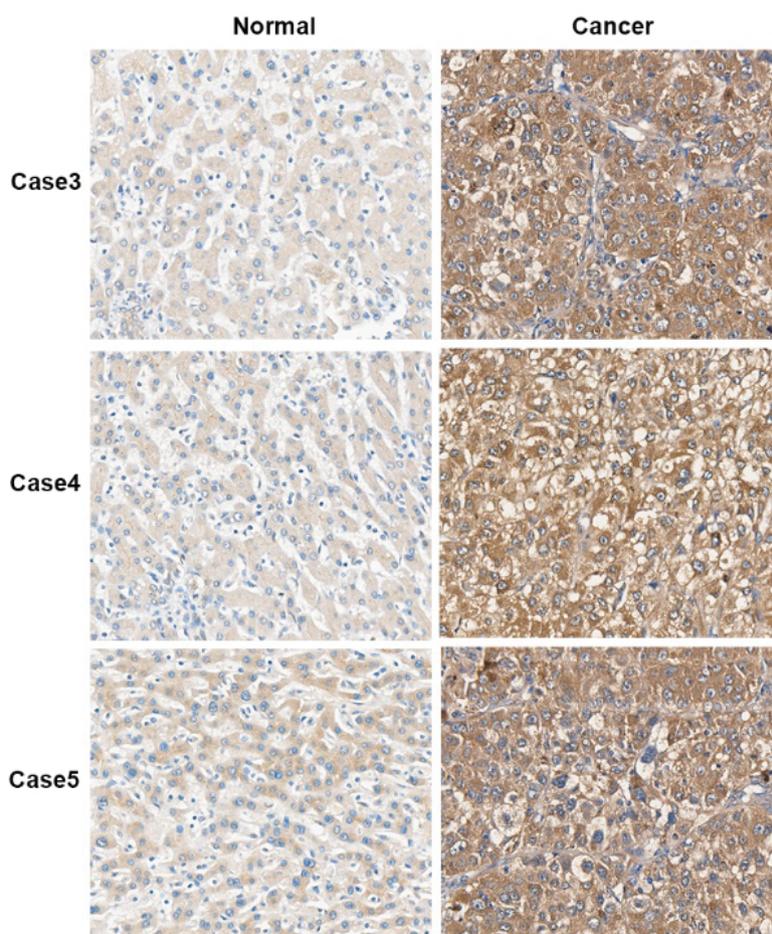


Figure S1. Immunohistochemistry staining of SKA2 in paired normal (N) and tumor (T) tissues from the other three patients.

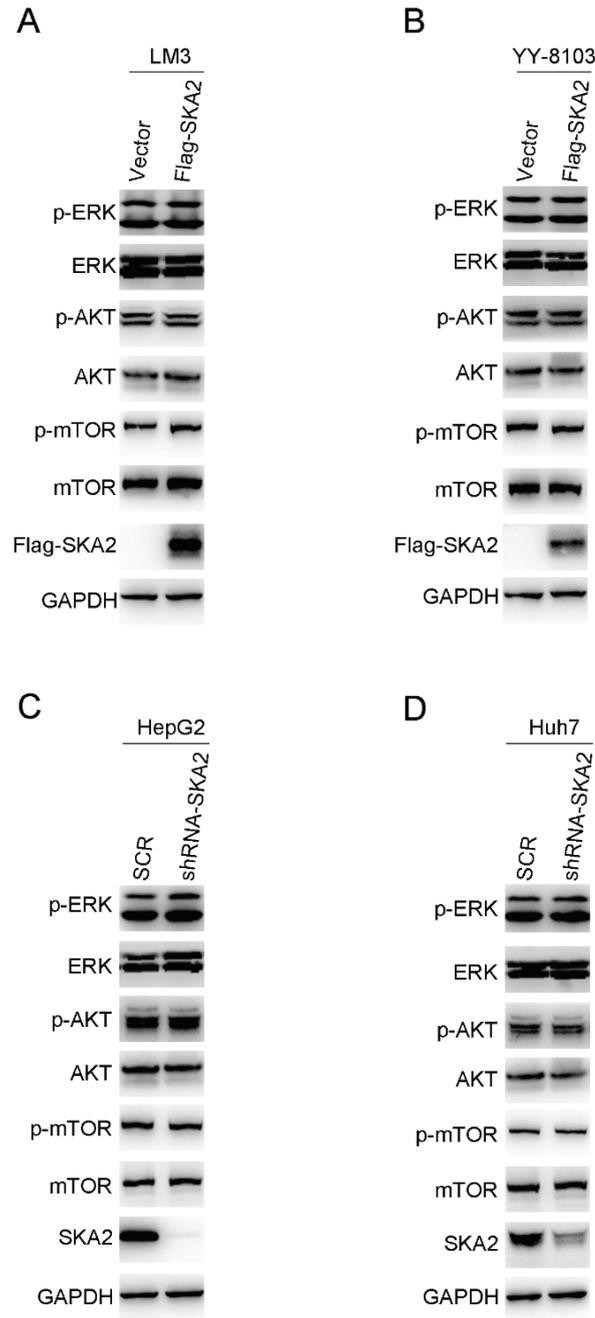


Figure S2. The expression level of p-ERK, ERK, p-AKT, AKT, p-mTOR, and mTOR among SKA2 overexpression and vector control groups were detected in LM3 (A) and YY-8103 cells (B) according to Western blot. GAPDH was used as a loading control. The expression level of p-ERK, ERK, p-AKT, AKT, p-mTOR, and mTOR among SKA2 knockdown and SCR control groups were detected in HepG2 (C) and Huh7 (D) cells according to Western blot. GAPDH was used as a loading control.