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SSH3 promotes malignant progression of HCC by activating FGF1-mediated FGF/FGFR pathway

Q.-S. SHI, Y.-H. ZHANG, J. LONG, Z.-L. QIAN, C.-X. HU

Department of Oncology Minimally Invasive Interventional Radiology, Beijing Youan Hospital, Capital Medical University, Beijing, China

Abstract. – OBJECTIVE: To investigate the impact of silencing SSH3 on the expression of FGF/FGFR pathway-related genes FGF1, FGFR1, and FGFR2 in hepatocellular carcinoma (HCC) cell line, so as to further understand the role of SSH3 in proliferation and apoptosis of HCC cells.

PATIENTS AND METHODS: TWe first detected SSH3 expression in 51 pairs of tumor tissue specimens and adjacent tissues collected from HCC patients through quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and analyzed the interplay between SSH3 expression and clinical characteristics of HCC patients. *In vitro*, after SSH3-silenced human HCC cell line was constructed by lentiviral transfection, Cell Counting Kit-8 (CCK-8), cell cloning assay, and flow apoptosis methods were conducted to explore the HCC cell functions. Finally, whether SSH3 exerts its biological characteristics through the FGF/FGFR pathway and the mutual regulation mechanism between SSH3 and FGF1 were further uncovered.

RESULTS: It was found that SSH3 expression was remarkably higher in tumor tissues of HCC patients than that in normal tissues. Meanwhile, in comparison to patients with low expression of SSH3, patients with high expression of SSH3 had higher pathological grade and larger tumor size. In addition, after silencing SSH3, HCC cell proliferation ability was attenuated while the apoptosis ability was enhanced in comparison to the control group. Moreover, the protein levels of FGF1/FGFR pathway-related genes FGF1, FGFR1, and FGFR2 were markedly inhibited by the downregulation of SSH3. Meanwhile, cell recovery experiment demonstrated that the overexpression of FGF1 reversed the impact of SSH3 silencing on the proliferation and apoptosis of HCC cells.

CONCLUSIONS: In summary, SSH3 is capable of accelerating the malignant progression of HCC by activating FGF1-mediated FGF/FGFR pathway, thus becoming a new molecular target for HCC therapy.

Key Words:
SSH3, FGF1, Hepatocellular carcinoma, Malignant progression.

Introduction

As the third largest cancer killer in the world, the mortality and morbidity of hepatocellular carcinoma (HCC) are increasing year by year around the world[1,2]. In China, it has also become one of the most common tumors with the highest degree of malignancy, and more than 300,000 patients die of this cancer every year, which is related to its biological characteristics of prone to recurrence and metastasis[3,4]. The development of HCC is a complex process involving multiple genes *in vivo* and coordinated by multiple steps[5,6]. At present, surgery is one of the most effective methods for HCC treatment, however, the high incidence of intrahepatic metastasis and vascular invasion and the lack of effective non-surgical treatment, result in poor prognosis of most HCC patients[7,8]. Therefore, it is urgent to carry out in-depth research on the mechanism of HCC progression so as to provide basis for identifying new molecular targets and developing new targeted drugs[9,10].

The Slingshot family includes SSH1, SSH2, and SSH3[11,12]. Lu et al[13] have found that both SSH1 and SSH2 have phosphatase activity *in vitro*, which can dephosphorylate phosphorylation, while SSH3 does not show this activity. In addition, literature has reported that that both SSH1 and SSH2 can be co-located with micro-filaggrin, while the localization relationship between SSH3 and micro-filaggrin is not clear[14]. Currently, there are still very few studies on mechanisms whereby SSH3 is engaged in tumor development, and only one article reported that SSH3 can accelerate metastasis of colorectal tumor cells *via* affecting LIMK1/Rac1 signal transduction pathway[15]. Therefore, this study intends to specify the impact of SSH3 on HCC cell functions by constructing a recombinant adenovirus of SSH3 and using an *in vitro* SSH3-knocked down cell model, so as to further clarify the molecular pathogenic mechanism of SSH3 participating in the occurrence of HCC.

Corresponding Author: Jiang Long, MD; e-mail: jack1971425@sohu.com

Patients and Methods

Patients and HCC Samples

51 cases of HCC tumor tissue specimens and paired adjacent ones were obtained from HCC patients aged from 30 to 78 in our hospital. All cases were diagnosed by two senior directors of pathologists. This study was approved by the Ethics Committee of Beijing Youan Hospital, Capital Medical University. Signed written informed consents were obtained from all participants before the study.

Cell Lines and Reagents

Six human HCC cell lines Bel-7402, HepG2, MHCC88H, SMMC-7221 and Huh7, Hep3B, and one human normal liver cell line LO2 [American Type Culture Collection (ATCC; Manassas, VA, USA)] used in this study were cultured in Dulbecco's Modified Eagle's Medium (DMEM) high glucose medium (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), penicillin (100 U/mL; Thermo Fisher Scientific, Waltham, MA, USA), and streptomycin (100 μg/mL; Thermo Fisher Scientific, Waltham, MA, USA) in a 37°C, 5% CO_2 incubator.

Transfection

sh-NC and sh-SSH3 containing the silent SSH3 lentiviral sequence (Shanghai GenePharma Company, Shanghai, China) were used in the transfection experiments. When cell density reached 30-40%, lentiviral transfection was carried out according to the manufacturer's instructions. After 48 h, the stale cell lines were harvested for quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) analysis and cell functional assays.

Cell Proliferation Assay

The transfected cells were seeded in 96-well plates (2000 cells/well) with 100 μL culture medium. Cell Counting Kit-8 (CCK-8) assay was carried out according to the manufacturer's protocol (Dojindo Molecular Technologies, Kumamoto, Japan).

Colony Formation Assay

After 48 h of transfection, the cells were cultured with complete medium for 2 weeks, and then, fixed in 2 mL of methanol for 20 minutes. After the methanol was aspirated, the cells were stained with 0.1% crystal violet staining solution (Beyotime, Shanghai, China) for 20 minutes, washed 3 times with phosphate-buffered saline (PBS), photographed, and counted under a light-selective environment.

Flow Cytometry Analysis

The method of binding with Annexin V-fluorescein isothiocyanate (FITC; Merck, Billerica, MA, USA) and propidium iodide (PI) was used to be detected by flow cytometry. The cell density was adjusted to about 1×10^6 cells/mL. After the medium was discarded, the cells were washed twice with PBS and gently resuspended with 0.5 mL of pre-cooled 1 × binding buffer, and then, 1.25 Ul Annexin V-FITC was added for incubation at room temperature and light-proof reaction for 15 min. Subsequently, the cells were centrifuged at 1000 × g for 5 min at room temperature, and the supernatant was removed. After gently resuspending the cells with 0.5 mL of pre-cooled l × binding buffer, 10 Ul PI was added, and the sample was placed on ice and stored in the dark, and then, immediately analyzed by flow cytometry (BD, Franklin Lakes, NJ, USA).

QRT-PCR Assay

Total RNA was extracted from HCC cell lines and tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). SSH3 and FGF1 quantitation were performed by SYBR® Premix Ex Taq™, with GaphPad as internal standard (TaKaRa, Otsu, Shiga, Japan). The primers are: SSH3 F: 5'-TCCAGGTATTGCACCAAGC-3'; R: 5'-GCCATAGCCGTCCACTCAT-3'. FGF1 F: 5'-CCCCGTCAGATAATCTGTG-3'; R: 5'-CTTGTCAGATACGGGAGG-3'. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) F: 5'-GGUGACUAUUCAACCGCAUTT-3'; R: 5'-AUGCGGUUGAAUAGUCACCTT-3'.

Western Blot

The transfected cells were lysed using cell lysis buffer, shaken on ice for 30 minutes, and centrifuged at 14,000 × g for 15 minutes at 4°C. The total protein concentration was calculated by bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL, USA). The extracted proteins were separated using a 10% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Immunoblotting was carried out based on instructions.

Dual-Luciferase Reporting Assay

Cells were seeded in 24-well plates and co-transfected with SSH3 overexpression and blank vector and pMIR Luciferase reporter plasmid (Yeasen, Shanghai, China). The plasmid was then introduced into the cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 h, the reporter Luciferase activity was normalized.

Statistical Analysis

GraphPad Prism 5 V5.01 software (La Jolla, CA, USA) was applied to perform statistical analysis. The differences between the two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using one-way ANOVA test followed by post-hoc test (Least Significant Difference). $p<0.05$ indicated the significant difference.

Results

Expression of SSH3 in HCC and its Association with Clinicopathological Factors of HCC Patients

The results regarding the association of SSH3 expression with clinicopathologic characteristics of hepatocellular carcinoma were shown in Table I. QPCR analysis revealed that in comparison to the paracancerous normal tissues, the HCC tumor tissues contained a higher SSH3 expression (Figure 1A), suggesting that SSH3 may serve as an oncogene in HCC. Similarly, *in vitro*, SSH3 also showed a higher expression in HCC cell lines Bel-7402, HepG2, MHCC88H, SMMC-7221, Huh7, and Hep3B, especially in Hub7 and HepG2, than in LO2 cells (Figure 1B).

Additionally, according to the expression of SSH3, we divided the above tumor tissue specimens into high and low groups to clarify the association between SSH3 levels and some clinical indicators, including pathological stage, tumor size, incidence of lymph node or distant metastasis, age, and gender of HCC patients. As a result, pathological stage and tumor size (Figure 1C) showed a marked difference between the two groups, indicating that SSH3 expression has close relevance to these two indicators.

Downregulation of SSH3 Inhibited Cell Proliferation, and Promoted Cell Apoptosis in HCC

We then constructed SSH3 silencing model in Hub7 and HepG cell lines and identified it at the transcriptional level (Figure 2A) and verified the effect of SSH3 knockdown on the proliferation and apoptosis of HCC cells by CCK-8, colony formation assay, and flow apoptosis assays. As

Table I. Association of SSH3 expression with clinicopathologic characteristics of hepatocellular carcinoma.

Parameters	Number of cases	SSH3 expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.438
<60	23	14	9	
≥60	28	14	14	
Gender				0.877
Male	25	14	11	
Female	26	14	12	
T stage				0.016
T1-T2	25	18	7	
T3-T4	26	10	16	
Tumor size (cm)				0.010
<3	34	23	11	
≥3	17	5	12	
Lymph node metastasis				0.382
No	30	18	12	
Yes	21	10	11	
Distance metastasis				0.405
No	32	19	13	
Yes	19	9	10	

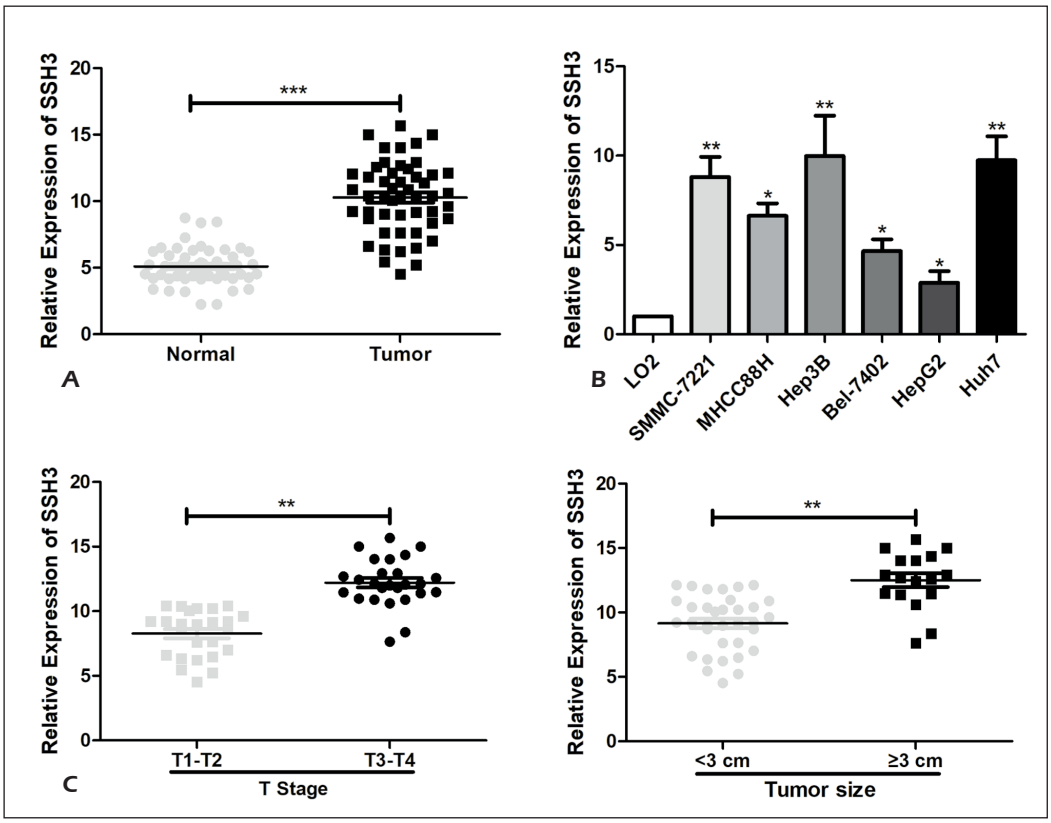

Figure 1. Volcano of differential genes, red represents upregulated genes, green represents downregulated genes. Figure 1. SSH3 is significantly highly expressed in hepatocellular carcinoma tissues and cell lines. **A**, qRT-PCR was used to detect the difference of SSH3 expression in tumor tissues and adjacent tissues of patients with hepatocellular carcinoma. **B**, qRT-PCR was used to detect the expression level of SSH3 in hepatocellular carcinoma cell lines. **C**, qRT-PCR was used to analyze the expression of SSH3 in HCC patients with different pathological stages and different tumor sizes. Data are mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

shown in Figure 2B, a decrease in the proliferation of HCC cells was found in sh-SSH3 group, and the same tendency was also observed in the clone formation assay (Figure 2C). Moreover, flow cytometry assay revealed an increase in cell apoptosis in HCC cell lines with stable SSH3 knockdown (Figure 2D).

Downregulation of SSH3 Decreased the Expressions of Related Genes in FGF/FGFR Pathway

In sh-SSH3 group, the expression of FGF/FGFR pathway related genes FGF1, FGFR1, and FGFR2 in HCC cells showed a significant reduction, measured by Western blotting (Figure 3A). In addition, in comparison to normal tissues, FGF1 expression showed an increase in HCC tumor tissues (Figure 3B), which was positively correlated with SSH3 levels (Figure 3C). Finally, Luciferase reporter assay was carried out to confirm that SSH3 can indeed bind to FGF1-specific sites (Figure 3D).

SSH3 Modulated FGF1 in Human HCC Cells

To further explore how SSH3 and FGF1 interact to regulate the malignant progression of HCC, we transfected FGF1 overexpression plasmid in Hub7 and HepG cell lines with SSH3 knockdown. It was found by Western blotting that SSH3 expression in the sh-SSH3+pcDNA3.1-FGF1 group was remarkably increased in comparison to the sh-SSH3+pcDNA3.1 group (Figure 4A). Subsequently, cell proliferation and apoptosis detection demonstrated that the overexpression of FGF1 significantly improved the decreased proliferation of HCC cells and the enhanced apoptosis rate induced by silencing SSH3 (Figure 4B, 4C, and 4D), suggesting that SSH3 may promote the malignant

progression of HCC cells *via* the modulation of FGF1.

Discussion

The development of HCC is a complicated process involving multiple genes and multiple signaling pathways. However, the precise molecular basis and mechanism of HCC proliferation and metastasis remain to be clearly determined, and studies[5-7] on its mechanism have been a hot topic at home and abroad. Therefore, it is of great significance of performing an in-depth study to find new targets for the improvement of HCC treatment[8,9].

Malignant degree of tumor is closely relevant to cell proliferation rate, and distant metastasis has occurred in most tumor patients during diagnosis or treatment, leading to poor prognosis[16,17]. Therefore, detection of genes engaged in the malignant progression of HCC is particularly important for diagnosis of HCC patients in early stage[8,9]. In this study, we found that SSH3 showed a significant higher expression in HCC tumor tissues in comparison to paired adjacent tissues, and the same tendency was observed in *in vitro* cell lines, indicating an extremely vital role of SSH3 in the occurrence of this cancer. Meanwhile, high expression of SSH3 was found to be positively correlated with pathological stage and tumor size

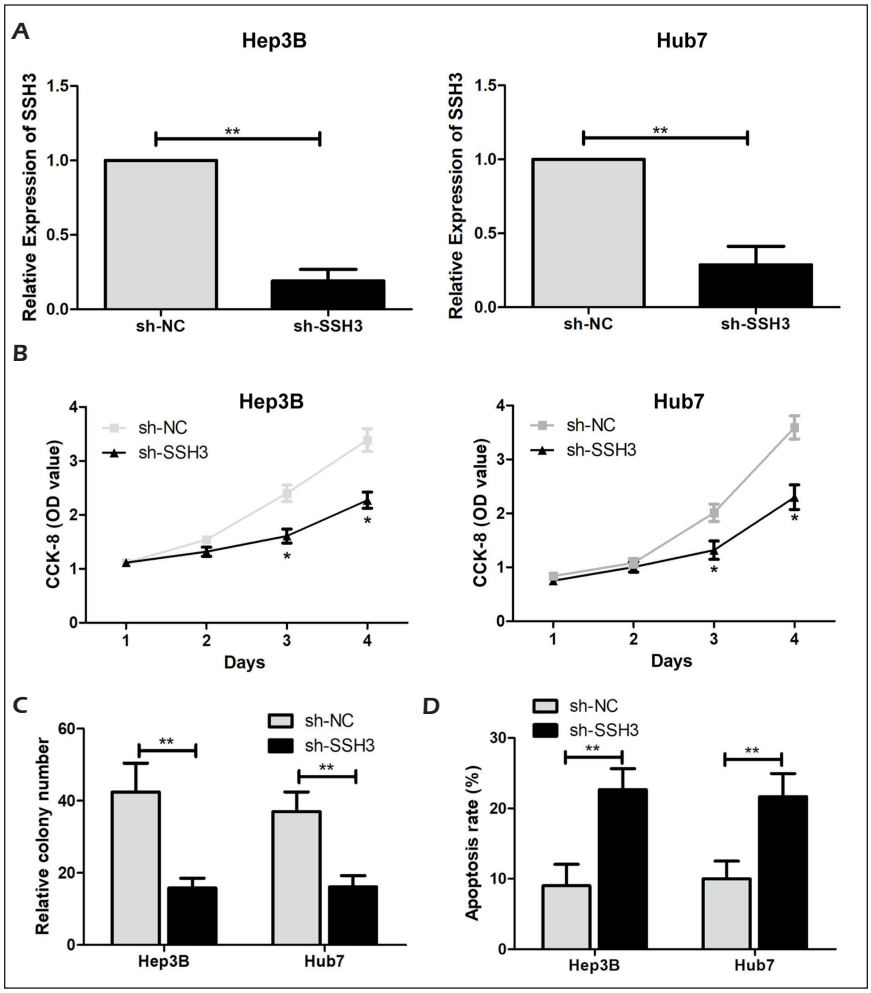

Figure 2. Silencing SSH3 inhibits HCC cell proliferation and promotes its apoptosis ability. **A**, qRT-PCR verified transfection efficiency after lentiviral transfection of SSH3 silencing vector in Hub7 and HepG2 cell lines. **B**, CCK-8 assay determines the effect of transfection of SSH3 silencing vector on hepatocellular carcinoma cell proliferation in Hub7 and HepG2 cell lines. **C**, Plate cloning assay was performed to detect the number of hepatocellular carcinoma positive cells after transfection of SSH3 silencing vector. **D**, Cell flow assay was used to estimate the apoptosis ability of hepatocellular carcinoma cells after transfection of SSH3 silencing vector. Data are mean ± SD, *p<0.05, **p<0.01.

of HCC patients. *In vitro*, we also verified that SSH3 could promote the proliferation and inhibit the apoptosis of HCC cells and thus plays a vital part in HCC progression.

The FGF family consists of 22 members and functions through paracrine and autocrine forms[18-21]. Tumor cells can change themselves and the extracellular matrix through high expression of FGF, and the activation of FGF/FGFR pathway has great relevance to tumor cell proliferation and apoptosis[22,23]. In this study, we detected a significant decrease in the protein expression of FGF/FGFR pathway related genes FGF1, FGFR1, and FGFR2 in HCC cells after SSH3 downregulation. The above results suggest that SSH3 can induce changes in the microenvironment of liver tumor cells through the FGF/FGFR pathway, leading to further malignant progression of HCC. Studies[22,23] have shown that increased FGF1 protein levels are closely relevant to the occurrence of a variety of human malignant tumors. FGF1 pathway plays a significant part in primary invasion and secondary metastasis of ovarian cancer, lung cancer, and other cancers[24-26]. Therefore, we per-

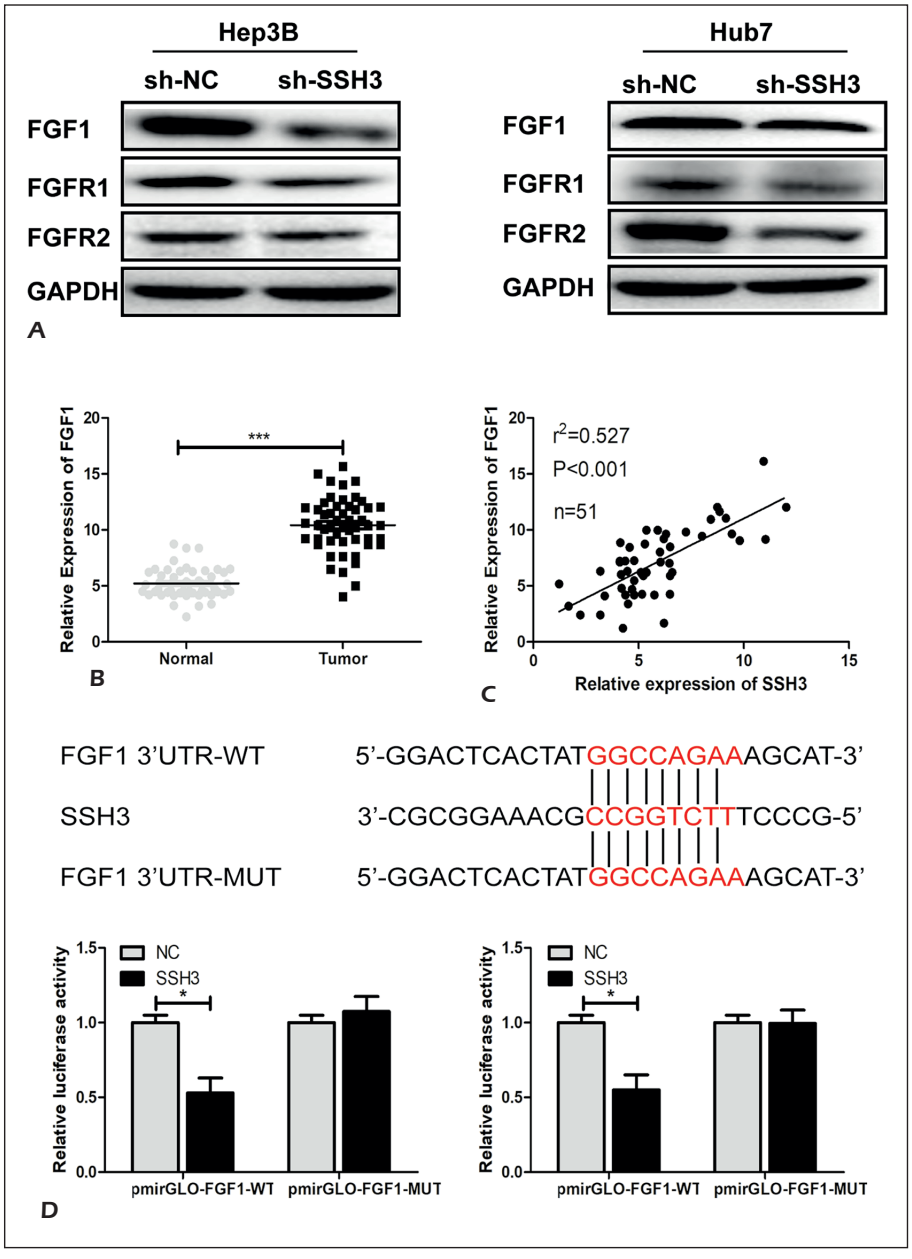

Figure 3. SSH3 regulates FGF1 expression to mediate the FGF/FGFR pathway. **A**, Western Blotting was used to detect the expression levels of FGF/FGFR pathway related genes FGF1, FGFR1 and FGFR2 in Hub7 and HepG2 cell lines. **B**, qRT-PCR was used to detect the difference in expression of FGF1 in tumor tissues and paracancerous tissues of patients with hepatocellular carcinoma. **C**, qRT-PCR was used to detect the difference in expression of SSH3 and FGF1 in hepatocellular carcinoma tumor tissues. **D**, Luciferase reporter assay suggests that SSH3 can bind to FGF1-specific sites. Data are mean ± SD, $^{**}p<0.01$, $^{***}p<0.001$.

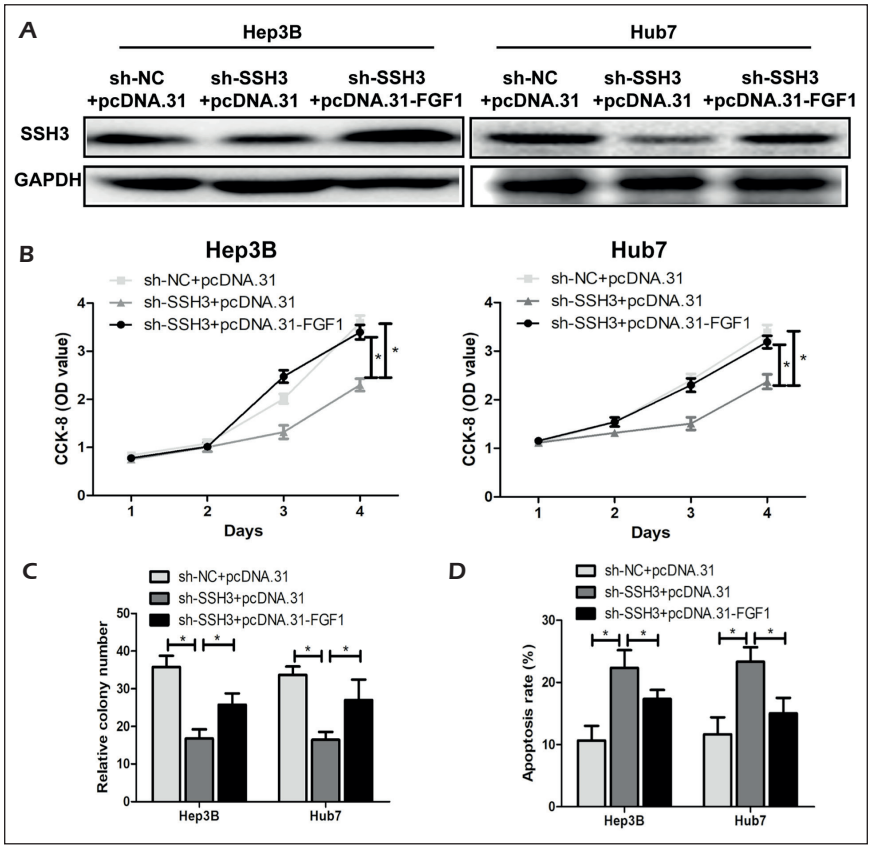

Figure 4. SSH3 regulates the mechanism of action of FGF1 in hepatocellular carcinoma cells. **A**, SSH3 expression levels were detected by Western blotting after co-transfection of SSH3 and FGF1 vector in HCC cell lines. **B**, CCK-8 detects the proliferation of hepatocellular carcinoma cells after transfection of SSH3 and FGF1 vector HCC cell lines. **C**, Plate cloning experiments detects the number of hepatocellular carcinoma positive cells after co-transfection of SSH3 and FGF1 vector in HCC cell lines. **D**, Cell-flow experiment detects cell apoptosis rate after co-transfection of SSH3 and FGF1 vector in HCC cell lines.

formed further Luciferase reporter gene assay to verify that SSH3 can bind to FGF1 specific sites. In addition, FGF1 overexpression remarkably reversed the reduced proliferation and elevated apoptosis rate of HCC cells induced by silencing SSH3, suggesting that SSH3 may accelerate malignant progression of HCC by regulating FGF1 expression.

Conclusions

The results in this study demonstrated that SSH3 promotes malignant progression of HCC by activating FGF1-mediated FGF/FGFR pathways and thus becomes a new target for HCC therapy.

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Conflict of Interests

The authors declare that they have no conflict of interest.

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