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Long Noncoding RNA FGFR3-AS1 Promotes Hepatocellular Carcinoma Carcinogenesis via Modulating the PI3K/AKT Pathway

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Hepatocellular carcinoma (HCC) as one of the most refractory cancers leads to high mortality worldwide. Long noncoding RNAs have been widely acknowledged as important biomarkers and therapeutic targets in HCC. In this study, we investigated the effects of long noncoding RNA FGFR3-AS1 on tumor growth and metastasis in HCC. First, we found that the expression of FGFR3-AS1 was upregulated about threefold in HCC samples and cell lines. We knocked down FGFR3-AS1 in Huh7 and Hep3B cells and found that FGFR3-AS1 knockdown significantly inhibited cell proliferation but induced apoptosis. Moreover, FGFR3-AS1 knockdown led to more HCC cells arrested in the G_0 stage. FGFR3-AS1 knockdown significantly inhibited cell migration and invasion. Additionally, we found that FGFR3-AS1 silencing dramatically delayed tumor growth in vivo. We found that, mechanistically, FGFR3-AS1 silencing decreased the activation of the PI3K/AKT signaling pathway. Taken together, our data demonstrated the pro-oncogenic role of FGFR3-AS1 in HCC and suggested that FGFR3-AS1 may serve as a novel biomarker for the diagnosis and therapeutic target for HCC treatment.

Key words: Hepatocellular carcinoma (HCC); FGFR3-AS1; Proliferation; Migration; PI3K/AKT

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most refractory cancers and leads to a high mortality rate around the world[1,2]. Especially in China, there are about 700,000 new patients and deaths every year[3]. In recent decades, some advances in the methods for HCC treatment have been made. Nowadays, the main treatments include surgery, radiofrequency ablation, and chemotherapy[4]. However, because of tumor occurrence and metastasis, the 5-year survival rate of patients with HCC remains very low. Therefore, there is an urgent need to explore the underlying molecular mechanism and search for novel therapeutic targets.

Long noncoding RNAs (lncRNA) are a class of transcripts with a length longer than 200 nucleotides[5]. Many reports show that lncRNAs have no protein-coding potential[6]. Nevertheless, a few studies reveal that some lncRNAs may code short peptides[7]. For instance, long intergenic nonprotein-coding RNA 961 (LINC00961)-encoded small regulatory polypeptide of amino acid response (SPAAR) polypeptide could regulate muscle regeneration[8]. Increasing evidence has demonstrated that lncRNAs exert very important functions in various biological processes, including cell development, immune regulation, and tumorigenesis[9–11]. More and more reports indicate that expression of lncRNAs was closely related to tumor development and progression in various cancers, such as HCC and gastric cancer[12,13]. lncRNAs could regulate cell proliferation, apoptosis, migration, and invasion by sponging microRNAs (miRNAs) or by interacting with specific proteins[14,15]. For example, Lai et al. reported that lncRNA myocardial infarction-associated transcript (MIAT) activates matrix metalloproteinase 9 (MMP9) to increase the proliferation and metastasis of non-small cell

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lung cancer[16]. In liver cancer, several lncRNAs have been demonstrated to participate in tumor development and progression. For instance, lncRNA AB209630 suppresses cell proliferation and metastasis in human HCC[17]. Also, Li and colleagues showed that lncRNA taurine upregulated 1 (TUG1) sponged miR-132 to regulate HCC[18]. However, the functions of most lncRNAs still remain unknown, and the relationship between lncRNA dysregulation and HCC needs to be further investigated.

lncRNA fibroblast growth factor receptor 3-antisense transcript 1 (FGFR3-AS1) has been shown to promote bladder cell proliferation and osteosarcoma growth[19–21]. In this study, we found that FGFR3-AS1 was highly expressed in HCC tissues and cell lines. Knockdown of FGFR3-AS1 in Huh7 and Hep3B cells inhibited cell proliferation, migration, and invasion while inducing cell apoptosis. Moreover, FGFR3-AS1 knockdown significantly delayed tumor growth in vivo. Finally, we showed that FGFR3-AS1 depletion could inhibit the activation of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway. Altogether, our data revealed the pro-oncogenic role of FGFR3-AS1 in HCC and indicated that FGFR3-AS1 might serve as a novel therapeutic target for HCC treatment.

MATERIALS AND METHODS

Patient Samples

Forty-nine HCC tissues and 15 paired peritumor tissues in this study were collected from The Seventh People's Hospital, Shanghai University of Traditional Chinese Medicine (Shanghai, P.R. China). Written consent approving the use of their tissues for this research was obtained from all patients. The study protocol was approved by The Seventh People's Hospital, Shanghai University of Traditional Chinese Medicine.

Cell Lines and Cell Culture

SMMC-7721, BEL-7404 (7404), Huh7, Hep3B, and HepG2 human liver cancer cell lines and HL-7702 (7702) normal liver cell line were purchased from Shanghai Cancer Institute (P.R. China). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher, Waltham, MA, USA) and maintained at 37°C in a humidified incubator containing 5% CO_2. For inhibition of the AKT pathway, API-1 (SML1342; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added into the culture medium at indicated concentrations.

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from tissue samples and cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Total RNA was reverse transcribed into cDNA using the PrimeScript™ RT reagent kit with gDNA Eraser (Takara Biotechnology Co., Ltd., Dalian, P.R. China). RT reaction mixtures contained 1 µg of total RNA, 1 µl of RT Enzyme Mix 1, 1 µl of RT Primer Mix, 4 µl of 5× PrimeScript Buffer 2, and 4 µl of ddH_2O. The temperature protocol was as follows: at 37°C for 15 min and at 85°C for 5 s. qPCR was performed on cDNA using the QuantiFast SYBR-Green PCR kit (Qiagen GmbH, Hilden, Germany) on a StepOnePlus™ Real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). PCR mixtures contained 10 µl of 2× PCR Master Mix, 4 µl of sense and antisense primers, 2 µl of cDNA, and 4 µl of ddH_2O. The specificity of each PCR was confirmed using a melting curve analysis. PCR primers were synthesized by Invitrogen; Thermo Fisher Scientific, Inc. The results were normalized to the expression of GAPDH. All samples were assessed in triplicate.

Short Hairpin RNA (shRNA)-Mediated Interference

For small interfering (si)RNA transfection to avoid off-target effects, siRNA against FGFR3-AS1 consisted of three target-specific siRNAs, which were designed and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, P.R. China). For FGFR3-AS1 overexpression, FGFR3-AS1 was constructed into a pcDNA3.1 vector. Huh7 and Hep3B cells at a density of 3×10^5 cells/ml were seeded into six-well plates and transfected with siRNAs using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) as the transfection reagent, according to the manufacturer's protocol. After 48 h of transfection, cells were harvested for RT-qPCR to verify the silencing of FGFR3-AS1 expression.

In Vivo Xenograft Experiments

Six-week-old male BALB/c nude mice were purchased from HFK Biosciences and maintained under pathogen-free conditions. For tumor propagation analysis, 2×10^6 WT and siFGFR3-AS1 Hep3B cells were subcutaneously injected into the flanks of BALB/c nude mice. Eight nude mice were used for this experiment. Tumor volume was calculated by the formula $V=\pi ab^2/6$ (a is the tumor length, and b is the tumor width) at indicative time points. Tumor weight was measured on week 5 after injection. Animal experiments were performed in accordance with relevant guidelines and the regulations of the Institutional Animal Care and Use Committees at The Seventh People's Hospital, Shanghai University of Traditional Chinese Medicine, and protocols were approved by the Institutional Animal Care and Use Committees at The Seventh People's Hospital, Shanghai University of Traditional Chinese Medicine.

Cellular Proliferation Assays

Cells were seeded into 96-well plates at a density of 1×10^3 cells/well. After 24, 48, and 72 h of incubation at 37°C in DMEM, cellular viability was evaluated using a cell counting kit-8 assay (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's protocol. The absorbance was measured at a wavelength of 450 nm using a Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific, Inc.).

A colony formation assay was also performed. Cells were seeded into six-well plates with DMEM supplemented with 10% FBS and cultured at 37°C with 5% CO_2 for 14 days. Colonies were fixed with methanol at room temperature for 20 min and stained with 0.1% crystal violet (Sigma-Aldrich; Merck KGaA). The total number of visible colonies was determined under an optical microscope (Olympus Corporation, Tokyo, Japan). All experiments were repeated three times.

Cellular Migration and Invasion Assays

Cellular migration was assessed using 6.5-mm Transwell inserts with 8.0-μm pore polycarbonate membranes (Costar; Corning Incorporated, Corning, NY, USA). A cell invasion assay was performed using Transwell inserts coated with Matrigel (BD Biosciences, San Jose, CA, USA). Briefly, 2×10^5 transfected and nontransfected cells were suspended in DMEM supplemented with 1% FBS and were seeded into the upper chambers of the inserts. Medium supplemented with 10% FBS was added into the lower chambers as a chemoattractant. Following 24 h of incubation at 37°C, cells on the upper surface of the membrane were removed; cells that had migrated to the lower membrane were fixed with 100% methanol at room temperature for 20 min and stained with crystal violet. Cells were observed using an optical microscope (Olympus Corporation). Cells were counted in five random fields from each well, and the average number of migrated or invaded cells was calculated. The assays were performed in triplicate.

In Situ Hybridization (ISH)

ISH was conducted based on a previously described protocol[22]. In brief, HCC and peritumor samples were fixed with formalin and embedded with paraffin, and sections were obtained. Then the samples were incubated in graded alcohols and incubated in 3% hydrogen peroxide (H_2O_2) for 30 min. Biotin-conjugated probes and streptavidin–horseradish peroxidase (HRP) conjugate were used for ISH. Then the samples were costained with hematoxylin, followed by dehydration in graded alcohols and xylene. Biotin-conjugated probes were designed using online tools (www.biosearchtech.com) and synthesized by Invitrogen.

Cell Cycle and Apoptosis Analysis

Cell cycle analysis was performed in HCC cells 48 h after transfection. Cells were harvested, washed twice with cold PBS, fixed in ice-cold 70% ethanol, incubated with propidium iodide and RNase A, and then analyzed by FACS. Apoptosis analysis was performed using an Annexin-V-FITC Apoptosis Detection Kit II (BD Biosciences) according to the manufacturer's instructions. Cells were analyzed by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences). The data were analyzed using FlowJo software.

Western Blot

Proteins were extracted from cultured cells and were analyzed by SDS-PAGE. After blocking with 5% nonfat milk in phosphate-buffered saline with Tween 20 (PBST) for 1 h at room temperature, the primary antibodies were incubated overnight at 4°C. Secondary antibody conjugated with HRP (1:3,000; Santa Cruz Biotechnology) was applied for 1 h at room temperature. Blots were developed using ECL (Bio-Rad).

Statistical Analysis

The statistical significance of the differences between groups was assessed using Student's *t*-test for pairwise comparisons or one-way analysis of variance followed by Fisher's least significant difference post hoc test for multiple comparisons. A value of $p<0.05$ was considered to indicate a statistically significant difference. Data are expressed as the mean ± standard deviation of three independent experiments. Statistical analysis was performed using the SPSS software version 20.0 (IBM Corp., Armonk, NY, USA).

RESULTS

FGFR3-AS1 Is Overexpressed in HCC

To investigate the regulatory role of FGFR3-AS1 in HCC, we analyzed the expression levels in HCC tissues and adjacent peritumor tissues by RT-qPCR. The results showed that FGFR3-AS1 expression was significantly upregulated in HCC tissues compared to peritumor tissues (Fig. 1A). Additionally, we analyzed 15 pairs of HCC tissues and peritumors using RT-qPCR and found that most HCC tissues displayed higher levels of FGFR3-AS1 than peritumor (Fig. 1B). To further verify this observation, we performed ISH with pairs of HCC samples. The results also indicated that FGFR3-AS1 was overexpressed in HCC samples (Fig. 1C). Besides, RT-qPCR analysis showed that FGFR3-AS1 was highly expressed in HCC cell lines (Huh7, HepG2, SMMC7721, BEL-7404, and Hep3B) compared with the normal liver cell line HL-7702 (Fig. 1D). These findings indicated that

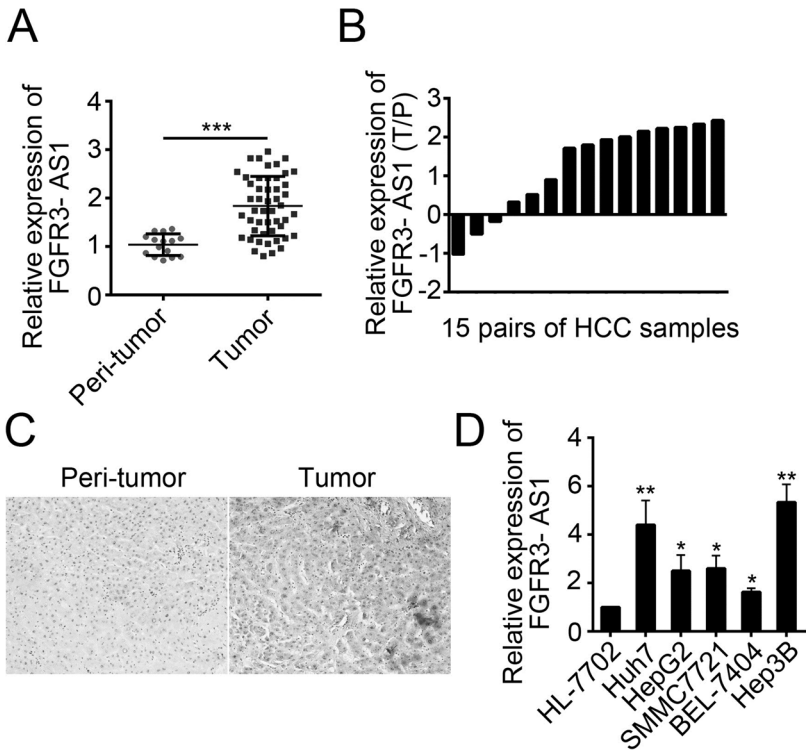

Figure 1. Fibroblast growth factor receptor 3-antisense 1 (FGFR3-AS1) is overexpressed in hepatocellular carcinoma (HCC). (A, B) Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was used to examine FGFR3-AS1 expression in HCC tissues compared with adjacent peritumor tissues. T, tumor; P, peritumor. (C) In situ hybridization was used to determine FGFR3-AS1 expression in pairs of HCC samples. (D) qRT-PCR was used to examine FGFR3-AS1 expression in HCC cell lines compared with normal liver cell line. $*p<0.05$, $**p<0.01$ and $***p<0.001$ by two-tailed Student's *t*-test. All data presented are shown as means ± SD collected from three independent experiments.

FGFR3-AS1 was upregulated in HCC samples and cell lines, which suggested that FGFR3-AS1 might contribute to the development of HCC.

Effect of FGFR3-AS1 Knockdown on Cell Proliferation and Apoptosis in HCC

To further explore the effect of FGFR3-AS1 expression on HCC cells, we knocked down FGFR3-AS1 in liver cancer cell lines (Huh7 and Hep3B) and performed in vitro experiments. RT-qPCR data showed that FGFR3-AS1 was significantly decreased in Huh7 and Hep3B cells after transduction with siFGFR3-AS1 (Fig. 2A). Through CCK-8 and colony formation assays, we found that FGFR3-AS1 knockdown significantly inhibited the proliferation of Huh7 and Hep3B cells (Fig. 2B and C). Furthermore, we assessed the effect of FGFR3-AS1 on cell apoptosis. By annexin V/PI staining and FACS analysis, we found that FGFR3-AS1 knockdown remarkably promoted cell apoptosis (Fig. 2D).

Knockdown of FGFR3-AS1 Arrests Cells in G_0 Phase

To explore the mechanism that FGFR3-AS1 regulates cell proliferation, we checked the cell cycle status of control and siFGFR3-AS1 Huh7 and Hep3B cells. The FACS results showed that FGFR3-AS1 knockdown significantly arrested more cells in the G_0 phase (Fig. 3A and B). Furthermore, Western blot (WB) results indicated that FGFR3-AS1 knockdown promoted the expression of p21 but inhibited cyclin D1 expression (Fig. 3C).

Effect of FGFR3-AS1 Knockdown on Cell Migration and Invasion in HCC

Next step, we assessed the effect of FGFR3-AS1 on tumor metastasis. We used Transwell migration and invasion assays with Huh7 and Hep3B cells. As shown, after FGFR3-AS1 depletion, fewer cells migrated or invaded (Fig. 4A and B). Moreover, WB results indicated that FGFR3-AS1 knockdown decreased the protein levels of metastasis-related genes, such as SNAI1 and TWIST (Fig. 4C).

FGFR3-AS1 Knockdown Inhibits Tumor Growth In Vivo

To further determine the physiological role of FGFR3-AS1 in vivo, we performed xenograft experiments with nude mice. SiFGFR3-AS1 and siNC Hep3B cells were injected into the recipient mice subcutaneously. At indicative time points, we measured the tumor volumes, and

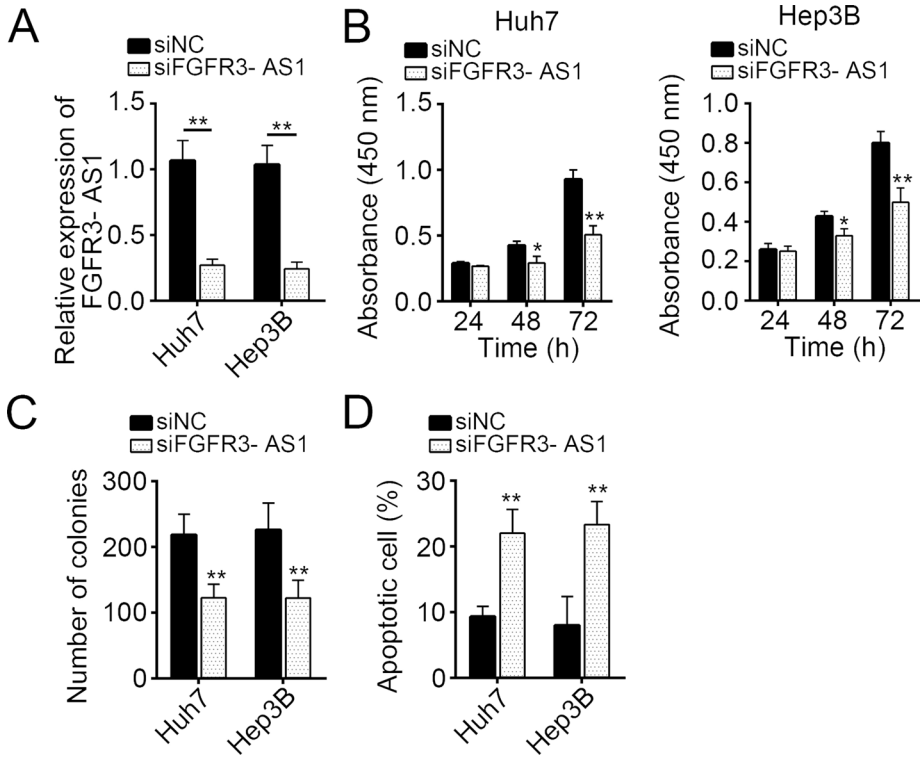

Figure 2. Effect of FGFR3-AS1 knockdown on cell proliferation and apoptosis in HCC. (A) qRT-PCR was used to determine the knockdown efficiency of FGFR3-AS1 in Huh7 and Hep3B cells. (B, C) Cell counting kit-8 (CCK-8) and colony formation assays were used for the detection of cell proliferation potential in Huh7 and Hep3B cells. (D) Huh7 and Hep3B cells were stained with annexin V/PI, followed by FACS analysis. *$p<0.05$ and **$p<0.01$ by two-tailed Student's *t*-test. All data presented are shown as means ± SD collected from three independent experiments.

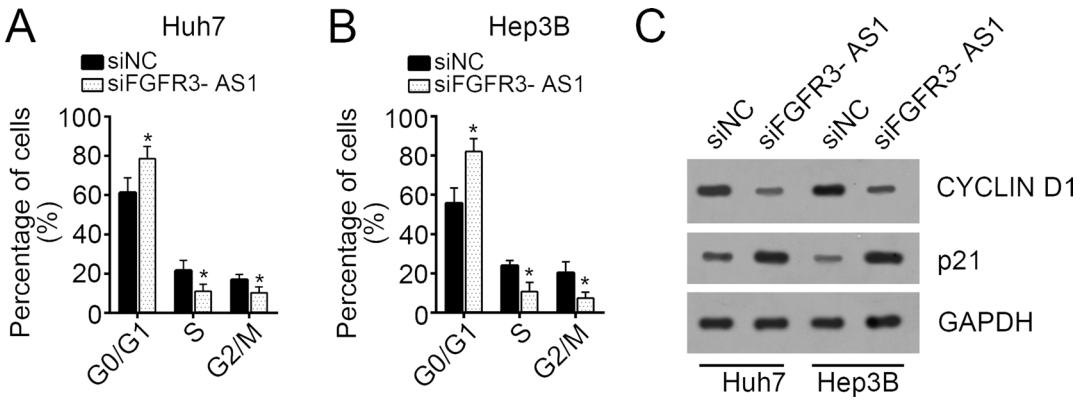

Figure 3. Knockdown of FGFR3-AS1 arrests cells in the G_0 phase. (A, B) Cell cycle was determined by FACS in Huh7 and Hep3B cells. (C) Western blot was used to check the protein levels of P21 and cyclin D1 in Huh7 and Hep3B cells. *$p<0.05$ by two-tailed Student's *t*-test . All data presented are shown as means ± SD collected from three independent experiments.

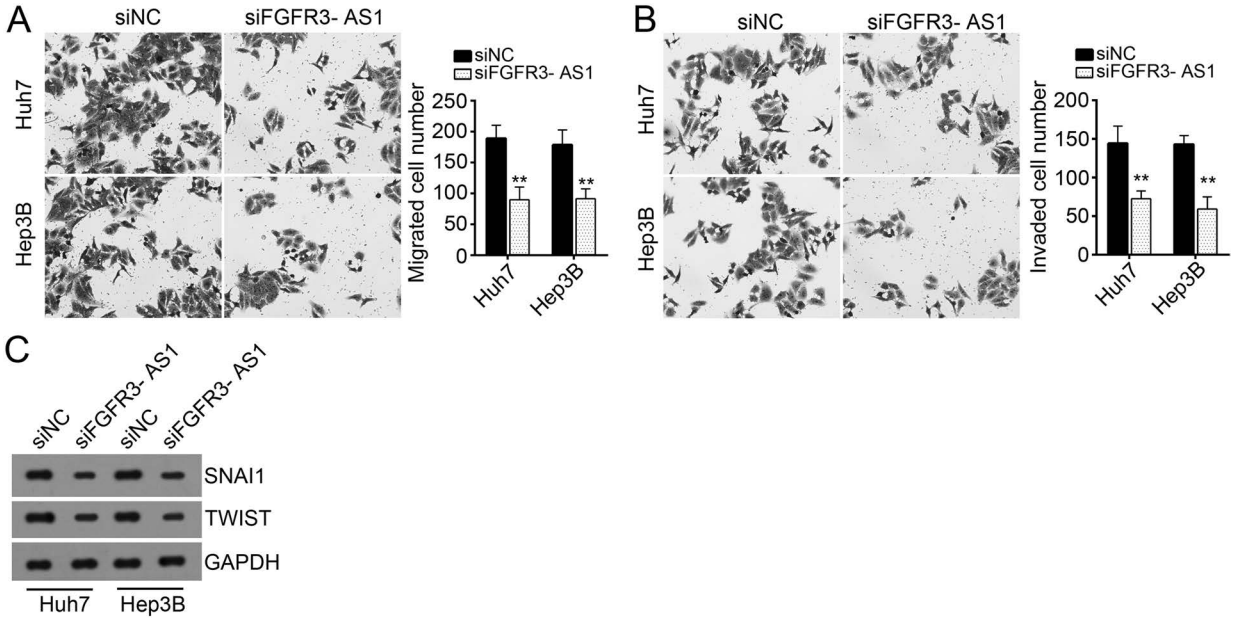

Figure 4. Effect of FGFR3-AS1 knockdown on cell migration and invasion in HCC. (A) Transwell migration and (B) invasion assays were used to assess the migration and invasion ability of Huh7 and Hep3B cells. (C) The protein levels of SNAI1 and TWIST were checked by Western blot in Huh7 and Hep3B cells. GAPDH acted as loading control. $^{**}p<0.01$ by two-tailed Student's *t*-test. All data presented are shown as means ± SD collected from three independent experiments.

at the end time point of experiments, we determined the tumor weights. As shown, FGFR3-AS1 knockdown significantly inhibited tumor growth in vivo (Fig. 5A–C). From all of the above data, we demonstrated that FGFR3-AS1 depletion remarkably inhibited cell proliferation, migration, and invasion while inducing cell apoptosis in HCC.

FGFR3-AS1 Knockdown Suppresses the PI3K/AKT Pathway in HCC

Finally, we wanted to determine the molecular mechanism by which FGFR3-AS1 exerted functions in HCC. Previous studies reported that the PI3K/AKT signaling pathway is involved in HCC development and progression[23,24]. Therefore, we checked the effect of FGFR3-AS1 on the PI3K/AKT pathway. As shown by WB, FGFR3-AS1 knockdown significantly inhibited the phosphorylation of PI3K and AKT in Huh7 and Hep3B cells (Fig. 6A–C). Moreover, we used API-1 (SML1342; Sigma-Aldrich), a specific inhibitor of AKT, to assess the effect of the PI3K/AKT signal on FGFR3-AS1-mediated HCC progression. First, we measured the toxic dose of API-1 and found that administration with 5 μM API-1 has no effect on cell viability (Fig. 6D). Then we

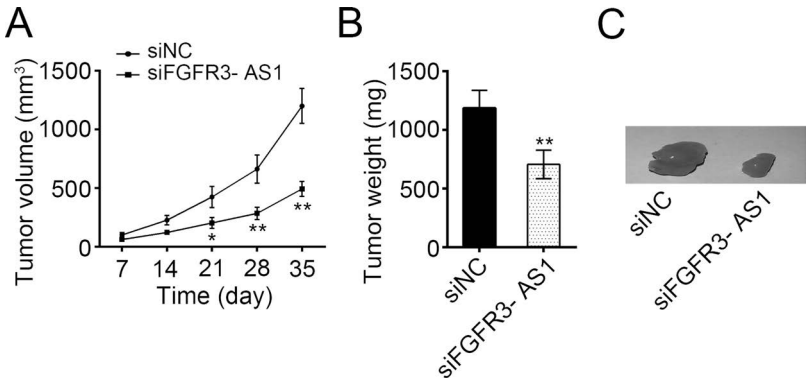

Figure 5. FGFR3-AS1 knockdown inhibits tumor growth in vivo. Hep3B cells were injected into nude mice subcutaneously. Tumor volumes (A) and tumor sizes (B, C) were measured at indicative time points. $^{*}p<0.05$ and $^{**}p<0.01$ by two-tailed Student's *t*-test. All data presented are shown as means ± SD collected from three independent experiments.

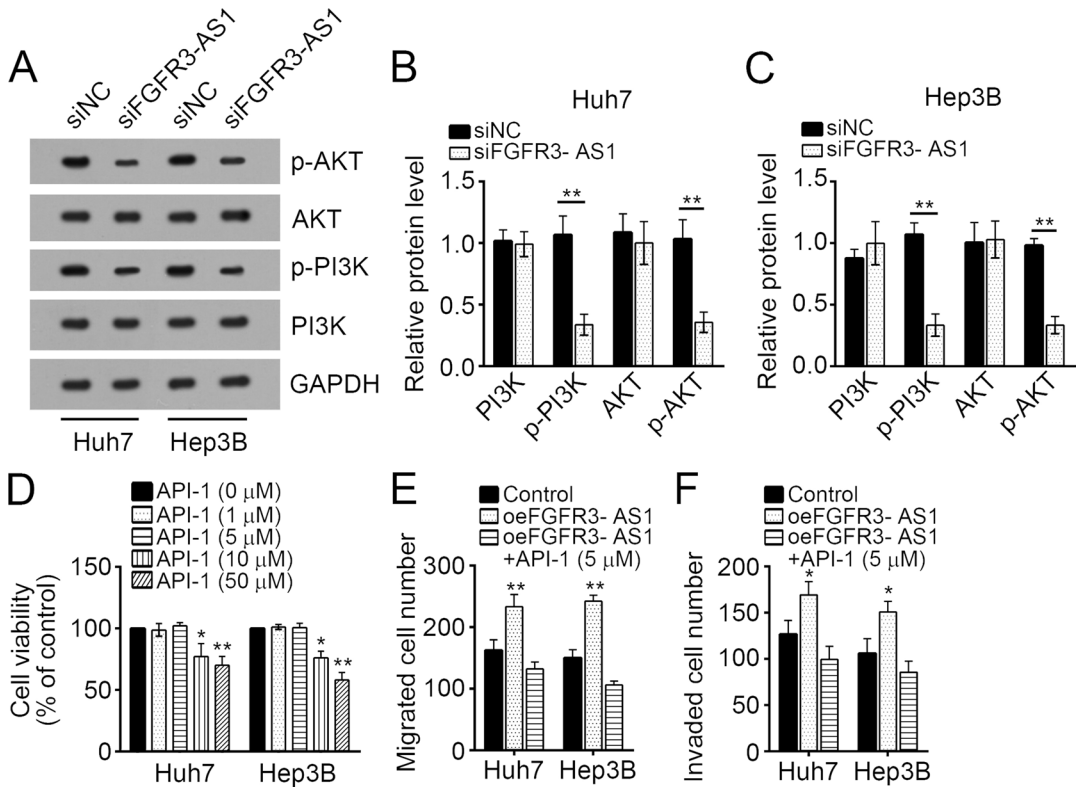

Figure 6. FGFR3-AS1 knockdown suppresses PI3K/AKT pathway in HCC. (A–C) Knockdown of FGFR3-AS1 significantly inhibited the phosphorylation of PI3K and AKT as shown by Western blot. (D) Cell viability examination indicated that API-1 (5 μM) has no toxic effect on Huh7 and Hep3B cells. (E, F) Transwell assays indicated that overexpression of FGFR3-AS1 promoted cell migration and invasion, while addition of API-1 reversed it. *$p<0.05$ and **$p<0.01$ by two-tailed Student's *t*-test. All data presented are shown as means ± SD collected from three independent experiments.

overexpressed FGFR3-AS1 and/or added API-1 in the medium. We found that overexpression of FGFR3-AS1 promoted cell migration and invasion, while addition of API-1 in the meantime abrogated it (Fig. 6E and F). Altogether, these findings suggested that FGFR3-AS1 promoted cell proliferation, migration, and invasion, at least partially, by activation of the PI3K/AKT pathway.

DISCUSSION

HCC is one of the most refractory cancers and leads to a high mortality rate around the world[1,2]. However, there is currently no effective method to fully heal HCC. Therefore, there is an urgent need to find novel biomarkers for the early diagnoses of HCC and design of therapeutic targets. The regulatory mechanism of FGFR3-AS1 on HCC growth and metastasis remains largely unknown. In the present study, we found that FGFR3-AS1 was highly expressed in HCC tissues and cell lines. Knockdown of FGFR3-AS1 significantly inhibited HCC cell proliferation, migration, and invasion while promoting cell apoptosis. Moreover, we found that knockdown of FGFR3-AS1 impaired the activation of the PI3K/AKT pathway in HCC.

Dysregulation of lncRNAs was related to diverse human cancers including HCC, gastric cancer, and colon cancer[1,13,25]. The relationship of FGFR3-AS1 with human cancers has not been widely explained. Previous studies showed that FGFR3-AS1 takes part in the regulation of bladder cancer and osteosarcoma. For instance, Liao et al. reported that FGFR3-AS1 regulates cell proliferation and apoptosis in bladder cancer[19]. They found that FGFR3-AS1 was overexpressed in bladder cancer tissues compared to normal tissues, and they showed that knockdown of FGFR3-AS1 inhibited cell proliferation but promoted cell apoptosis. In addition, Sun and colleagues reported that FGFR3-AS1 could promote osteosarcoma growth[20]. They showed that FGFR3-AS1 was upregulated in osteosarcoma, and the expression level of FGFR3-AS1 was positively correlated with tumor size, metastasis, and prognosis. Moreover, by in vitro experiments, they found that knockdown of FGFR3-AS1 inhibited the proliferation and cell cycle progression of osteosarcoma cells. However, the role of FGFR3-AS1 in HCC remains unclear. In the present study, we also found that FGFR3-AS1 was upregulated in HCC cells. These previous studies and our present data suggested

that FGFR3-AS1 might be a potential biomarker for the diagnosis of various cancers.

Furthermore, we showed that FGFR3-AS1 was upregulated in HCC cell lines compared to normal liver cells. Knockdown of FGFR3-AS1 reduced the proliferation and cell cycle in Huh7 and Hep3B cells. Previous reports also showed that FGFR3-AS1 knockdown suppressed the proliferation in bladder cancer and osteosarcoma. These results suggested that FGFR3-AS1 is a regulator of the cell cycle. Additionally, for the first time, our data indicated that FGFR3-AS1 regulated cell migration and invasion in HCC, which suggested the functional heterogeneity of FGFR3-AS1 in different cancers.

In this study, we showed that FGFR3-AS1 promoted the activation of the PI3K/AKT signaling pathway. Previous evidence indicated that the PI3K/AKT pathway plays essential functions in HCC. For example, endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor 2 (EDG2) could promote the progression of HCC through activation of PI3K/AKT/mTOR signaling[24]. Resveratrol inhibits proliferation and migration through modification of PI3K/AKT signaling in HCC[26]. In addition, Yang et al. reported that Ras-related protein Rab-27B (RAB27B) regulates HCC progression via PI3K/AKT/p21 signaling[27]. Our data also showed that FGFR3-AS1 knockdown significantly inhibited the activation of PI3K/AKT signaling. Therefore, the results of the current study expand on the importance of FGFR3-AS1/PI3K/AKT in human cancers.

In summary, our study shows that FGFR3-AS1 is able to promote cell proliferation, migration, invasion, and tumor propagation, at least partially, through PI3K/AKT signaling in HCC. Our findings highlight the importance of the FGFR3-AS1/PI3K/AKT axis in HCC progression.

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