

# lncRNA LOXL1-AS1 promotes liver cancer cell proliferation and migration by regulating the miR-377-3p/NFIB axis

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**Abstract.** Liver cancer is becoming one of the most lethal malignancies due to its high incidence and mortality. Accumulating studies have indicated that long non-coding RNAs (lncRNAs) are critical regulators of the tumorigenesis and development of various types of cancer, including liver cancer. lncRNA LOXL1-antisense RNA 1 (LOXL1-AS1) has been identified as an oncogene in some types of human cancer; however, its role in liver cancer remains obscure. Reverse transcription-quantitative PCR was used to measure LOXL1-AS1 expression in liver cancer tissues and cells. Western blot, MTT, colony formation, glucose uptake and wound healing assays were used to explore the biological function of LOXL1-AS1 in liver cancer cells. Bioinformatics analysis and RNA pull-down and luciferase reporter assays were used to explore the molecular mechanism of LOXL1-AS1 in liver cancer cells. Statistical analysis was used to compare the experimental results of different groups. In the present study, LOXL1-AS1 expression was significantly upregulated in liver cancer tissues and cells compared with in normal liver tissues and cells, respectively. High LOXL1-AS1 expression was associated with poor clinical outcomes in patients with liver cancer. Furthermore, LOXL1-AS1-knockdown suppressed glucose metabolism, proliferation, migration and epithelial-mesenchymal transition (EMT) of liver cancer cells. Subsequently, LOXL1-AS1 acted as a microRNA (miR)-377-3p sponge, and nuclear factor I B (NFIB) was confirmed as the downstream target of miR-377-3p in liver cancer cells. Additionally, rescue assays suggested that NFIB overexpression counteracted the inhibitory influence of LOXL1-AS1 silencing on liver cancer cellular processes. The present study demonstrated that LOXL1-AS1 promoted glucose metabolism, proliferation, migration and EMT of liver cancer cells by sponging miR-377-3p and modulating

NFIB, which may provide a novel insight for the treatment of liver cancer.

## Introduction

Liver cancer, divided into primary and secondary hepatic carcinoma, is one of the most common malignant tumors and the second leading cause of cancer-associated mortality worldwide based on the GLOBOCAN 2008 estimates (1). It is believed that the pathogenesis of primary liver cancer is a complicated process involving multiple factors (2). Epidemiological research has shown that hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, aflatoxin, alcoholism and nitrosamines are the main risk factors that lead to the incidence of primary hepatic carcinoma (3). Secondary hepatic carcinoma may develop in different ways, such as with blood and lymph metastasis and direct invasion of the liver (4). Surgical resection, including liver transplantation, is the major liver cancer therapy and results in survival benefits for patients with liver cancer (5); however, the overall survival and prognosis of patients remain unsatisfactory on account of the high relapse rate (6). Approximately 20% of patients survive for  $\geq 5$  years after diagnosis (7). Therefore, further exploration of crucial liver cancer molecular mechanisms is of paramount importance to seek effective therapeutic strategies.

Long non-coding RNAs (lncRNAs), a novel type of RNAs comprising  $>200$  nucleotides in length, are known to be involved in the tumorigenesis and progression of numerous human malignancies (8). For example, lncRNA-ATB promotes cell proliferation and migration in papillary thyroid cancer (9), while lncRNA SOCS2-AS1 promotes the proliferation and suppresses apoptosis of prostate cancer cells (10). lncRNA HEIH serves as a marker of poor prognosis and facilitates colorectal cancer tumorigenesis (11). Moreover, the pivotal roles of lncRNAs in liver cancer progression have been previously clarified (12-14). Increasing research has demonstrated that lncRNA LOXL1-antisense RNA 1 (LOXL1-AS1) serves an oncogenic role in multiple types of cancer. For instance, LOXL1-AS1 downregulation suppresses the proliferation and induces apoptosis of breast cancer cells (15). Additionally, LOXL1-AS1 exerts oncogenic effects on the tumorigenesis of lung adenocarcinoma (16). lncRNA LOXL1-AS1 facilitates pancreatic cancer progression by

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promoting pancreatic cancer cell proliferation and migration (17). Moreover, LOXL1-AS1 facilitates gastric cancer progression by modulating the malignant phenotypes of gastric cancer cells (18) and facilitates the proliferation and invasion of non-small cell lung cancer cells (19). However, its role in liver cancer remains unclear.

The present study focused on the role of LOXL1-AS1 in liver cancer, which may provide potential novel insights for the treatment of liver cancer.

## Materials and methods

**Patients and samples.** Liver cancer tissues (n=38) and paired adjacent non-tumor tissues (n=38) ( $\geq 3$  cm from the tumor margin) were collected from 24 male and 14 female patients with liver cancer (age range, 33-71 years; mean age  $\pm$  SD, 53.1 $\pm$ 5.4 years) of the Affiliated Hospital of Yangzhou University (Yangzhou, China) between August 2017 and August 2019. The collected samples were stored at -80°C. The inclusion criteria were as follows: i) Patients diagnosed with hepatocellular carcinoma based on imaging and pathological biopsy; ii) patients meeting the TNM staging criteria for hepatocellular carcinoma by the American Joint Committee on Cancer in 2009 (20); iii) patients who had not taken part in any previous targeted tumor research (surgery, radiotherapy or chemotherapy), understood the study and signed an informed consent form (including their families); and iv) patients whose expected survival time was  $>3$  months. The exclusion criteria were as follows: Patients with other combined tumors, renal function diseases or infection before admission. The present study received approval from the Ethics Committee of the Affiliated Hospital of Yangzhou University.

**Cell lines.** Liver cancer cell lines (SK-HEP-1, Hep3B and SNU1) and a normal human hepatic cell line (THLE-2) were obtained from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. All cells were cultured in DMEM/F-12 (cat no. 31330095; Gibco; Thermo Fisher Scientific, Inc.) mixed with high glucose containing 10% FBS (Invitrogen; Thermo Fisher Scientific, Inc.) and were grown in a humid atmosphere with 5% CO<sub>2</sub> at 37°C.

**Transfection.** Short hairpin RNA (shRNA) against LOXL1-AS1 (sh-LOXL1-AS1) was utilized to knock down LOXL1-AS1, and sh-NC was used as the negative non-targeting control. To overexpress nuclear factor I B (NFIB), the sequences of NFIB were subcloned into the pcDNA3.1 vector to produce pcDNA3.1/NFIB. The empty pcDNA3.1 vector served as the control. The microRNA (miRNA/miR)-377-3p mimics vector was used to overexpress miR-377-3p, and NC mimics (cat. no. B01001) were used as the scrambled control. All vectors were obtained from Shanghai GenePharma Co., Ltd. SK-HEP-1 and Hep3B cells (1 $\times 10^6$  cells/well) were seeded in 24-well plates and 500  $\mu$ l DMEM was added to each well. When the cells reached 40-60% confluence, the aforementioned vectors were transfected into cells at a final concentration of 50 nM using Lipofectamine<sup>®</sup> 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C with 5% CO<sub>2</sub>. Transfection was conducted for 48 h according to the manufacturer's instructions.

**Reverse transcription-quantitative PCR (RT-qPCR).** Total RNA was extracted from tissues and cells using TRIzol<sup>®</sup> reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The extracted RNA was reverse transcribed into cDNA using a PrimeScript RT reagent kit (Takara Bio, Inc.) according to the manufacturer's protocol. qPCR was conducted using a SYBR Premix Ex Taq II kit (Takara Bio, Inc.) with the ABI7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR amplification conditions were as follows: A pre-denaturation step of 1 min at 94°C, followed by 40 cycles at 95°C for 30 sec and 60°C for 1 min. The relative quantitation of gene expression was calculated using the 2<sup>- $\Delta\Delta C_q$</sup>  method (21). GAPDH and RNU6 (U6) were used as internal controls for lncRNAs (or mRNAs) and miRNAs, respectively. The PCR primers used are shown in Table I.

**MTT assay.** An MTT assay was performed to examine cell proliferation. SK-HEP-1 and Hep3B cells were plated in 96-well plates at a density of 4 $\times 10^3$  cells/well and cultured at room temperature for 0, 24, 48 and 72 h at a density of 60-70% after adherence. At every transfection time point, 20  $\mu$ l of MTT solution (5 mg/ml) was added to each well of the culture plate, followed by another 4 h of incubation at room temperature. Subsequently, the precipitated formazan was dissolved in dimethyl sulfoxide. The absorbance at 490 nm was detected using an ELX-800 spectrometer reader (Bio-Tek Instruments, Inc.; Agilent Technologies, Inc.).

**Colony formation assay.** In brief, the transfected liver cancer cells were seeded in 6-well plates (1 $\times 10^3$  cells/well) and grown in DMEM mixed with high glucose containing 10% FBS at 37°C. The culture medium was replaced every 3 days. After incubation for 2 weeks, the colonies were fixed with methanol at room temperature for 10 min, followed by staining using crystal violet at room temperature for 5 min. The visible colonies with  $>50$  cells were counted manually with a light microscope.

**Glucose uptake assay.** A 2-Deoxyglucose Glucose Uptake Assay kit (Abcam) was used to perform the glucose uptake assay. A total of 1 $\times 10^6$  transfected SK-HEP-1 and Hep3B cells cultured in DMEM/F-12 mixed with glucose (0, 5 and 20 mM; cat no. 31330095; Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Invitrogen; Thermo Fisher Scientific, Inc.) were grown in 96-well plates. After overnight incubation, the cells were incubated with 2-deoxyglucose in the dark for 30 min in a humid atmosphere with 5% CO<sub>2</sub> at 37°C. The detection of 2-deoxyglucose uptake was observed under a fluorescence microplate reader.

**Western blot analysis.** Liver cancer cells were lysed in RIPA lysis buffer (Beyotime Institute of Biotechnology), and the protein concentration was measured utilizing a bicinchoninic acid assay. Equal amounts of protein (20  $\mu$ g/lane) were separated via 12% SDS-PAGE for 2 h and transferred onto polyvinylidene fluoride membranes (EMD Millipore). Subsequently, the membranes were blocked with 5% skim milk for 1 h at room temperature, followed by incubation with diluted primary antibodies, including anti-E-cadherin (1:1,000; cat. no. ab194982; Abcam), anti-N-cadherin

Table I. Primer sequences used for reverse transcription-quantitative PCR.

Target	Primer sequences (5'-3')
LOXL1-AS1	F: TTCCCATTACCTGCCCCGAAG R: GTCAGCAAACACATGGCAAC
miR-377-3p	F: ATCACACAAAGGCAACTTTTGT R: ATCACACAAAGGCAACTTTTGT
miR-524-5p	F: GCTGTGACCCTACAAAGGGA R: ACCGTAACACTCCAAAGGGA
miR-526b-5p	F: ACCCTCTTGAGGGAAGCACT R: GACAGTAAGCCTCTAAAAGGAAGC
miR-3614-5p	F: TTCAAAACACCAAGATCTGAAGGC R: CTTGGGCCACTTGGATCTGA
miR-18b-5p	F: TGTGCAAATCCATGCAAAACTGA R: GTGCAGGGTCCGAGGT
NFIB	F: ACAAGTCTGGCGTCTGGAT R: GGCTGGACACAAAGTGCTG
NR3C1	F: GAATGAACCTGGAAGCTCG R: AGGTTTCTTGAGACTCTCT
GRSF1	F: AAGCTGATGTGCACTTTGAG R: TATACCTATGATGAACGTGGGAC
PRR7	F: ACCACCGTGTTACGAAGAG R: GTGAGCACCTCGCTATAGG
MADD	F: AAAGCATTAAAGCAGGCCT R: TGGCCCTCATGAAATTTCTC
ARHGAP29	F: CTAACAAATTTGTGCAGCCTC R: GCTCCTGTTTCCAAAGCTC
SSFA2	F: ATATGGCTCAAGGACTGCC R: TTTCTCACAAAGCACACCCT
KDM6B	F: AGGAAGTCCTGTGATTGGC R: CTTTCACAGCCAATTCCGG
XIAP	F: GGAGGGCTAACTGATTGGA R: TAACAGATATTTGCACCCTGGA
FMR1	F: CATGCACTTTCGGAGTCTG R: CAAGCTGCCTTGAACCTCTC
NELFA	F: ATCTTCCCATCTCAGCCTC R: ACTCACCCACTGTAATCCC
GAPDH	F: CGGAGTCAACGGATTTGGTCGTAT R: AGCCTTCTCCATGGTGGTGAAGAC
U6	F: GCTTCGGCAGCACATATACTAAAAT R: CGCTTCACGAATTTGCGTGTTCAT-3

F, forward; R, reverse; miR, microRNA; LOXL1-AS1, LOXL1-antisense RNA 1; NFIB, nuclear factor I B.

(1:4,000; cat. no. ab18203; Abcam), anti-LOXL1 (1:200; cat. no. ab238152; Abcam) and anti-GAPDH (1:500; cat. no. ab181602; Abcam) at 4°C overnight. The membranes were further incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (cat. no. sc-2357; 1:5,000; Santa Cruz Biotechnology, Inc.) for 1 h at 37°C. The protein bands were visualized using an electrochemiluminescence assay (EMD Millipore) and analyzed using

the Odyssey Infrared Imaging system version 2.1 (LI-COR Biosciences).

**Wound healing assay.** Horizontal cell migration was examined using a wound healing assay. Liver cancer cells were treated with 3  $\mu$ M of mitomycin C (Roche Diagnostics) for 1 h at 37°C to prevent further cell proliferation. After transfection for 48 h, cells were subjected to serum starvation for 4 h and incubated in 6-well plates overnight. When cells reached 80% confluence, a wound was created using a sterile pipette tip. Subsequently, the cells were washed twice with PBS and cultured for 12 and 24 h at room temperature. The wound was photographed at 0, 12 and 24 h under a light microscope (magnification, x100) and quantified using Image-Pro Plus Analysis software (National Institutes of Health).

**Bioinformatics analysis.** The potential downstream genes of LOXL1-AS1 were predicted using the starBase database (<http://starbase.sysu.edu.cn/>) with the condition of 4 cancer types. The putative downstream genes of miR-377-3p were predicted using the starBase database (<http://starbase.sysu.edu.cn/>) overlapped by microT, miRanda, miRmap, PicTar and PITA (CLIP Data: Strict stringency; Degradome data: High stringency). The interaction between LOXL1-AS1 and miR-377-3p or the interaction between NFIB and miR-377-3p was also predicted using the starBase database.

**RNA pull-down assay.** For the RNA pull-down assay, Bio-LOXL1-AS1 (or Bio-miR-377-3p) and Bio-NC (Guangzhou RiboBio Co., Ltd.) were transcribed by the Biotin RNA Labeling Mix (Roche Diagnostics) and T7 RNA polymerase (Promega Corporation), and liver cancer cells were transfected with 50 nM Biotin-labeled RNAs using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h at room temperature. Subsequently, the cells were lysed using RIPA lysis buffer (Sangon Biotech Co., Ltd.) containing RNase inhibitors (Invitrogen; Thermo Fisher Scientific, Inc.). After 1 ml of cell lysates were collected, 20  $\mu$ l of biotin-labeled RNA and 40  $\mu$ l of streptavidin magnetic beads were added and incubated with cell lysates for 2 h, forming RNA complexes. Proteinase K (Sigma-Aldrich; Merck KGaA) was added and incubated with the supernatants overnight at 4°C to isolate the RNA. Beads were isolated from the supernatant after centrifugation (2,500 x g, 5 min, 4°C) and washed with washing buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 2 M NaCl and 0.1% Tween-20) followed by another centrifugation step (2,500 x g, 5 min, 4°C). The pellet was then collected, and the RNA-RNA complexes were eluted using Tris-EDTA buffer (Invitrogen; Thermo Fisher Scientific, Inc.) and purified using ethanol. Finally, miR-377-3p, miR-524-5p, miR-526b-5p, miR-3614-5p and miR-18b-5p expression was measured by RT-qPCR, as aforementioned.

**Luciferase reporter assays.** The full-length LOXL1-AS1 [wild-type (Wt)/mutant (Mut)] or NFIB 3'-untranslated region (UTR) (Wt/Mut) was subcloned into pmirGLO vectors (Promega Corporation) to construct luciferase reporter vectors (LOXL1-AS1-Wt/Mut and NFIB-Wt/Mut). The LOXL1-AS1-Wt vectors or LOXL1-AS1-Mut vectors were co-transfected with the miR-377-3p mimics or NC mimics into

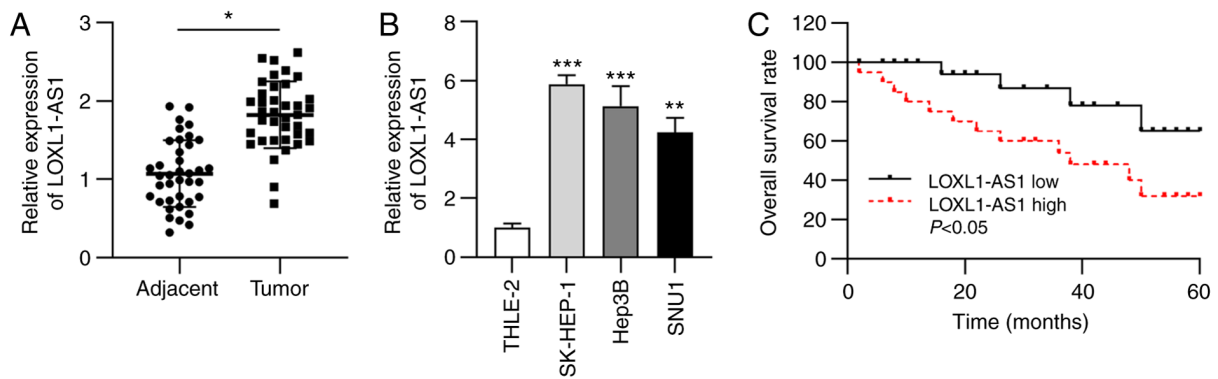


Figure 1. LOXL1-AS1 expression is upregulated in liver cancer tissues and cells. (A) LOXL1-AS1 expression in liver cancer tissues and matched adjacent non-tumor liver tissues was measured through RT-qPCR. \* $P < 0.05$ . (B) LOXL1-AS1 expression in liver cancer cell lines (SK-HEP-1, Hep3B and SNU1) and a normal human hepatic cell line (THLE-2) was determined by RT-qPCR. \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. THLE-2. (C) Overall survival rate of patients with liver cancer with high and low LOXL1-AS1 expression was analyzed by Kaplan-Meier analysis. RT-qPCR, reverse transcription-quantitative PCR; LOXL1-AS1, LOXL1-antisense RNA 1.

liver cancer cells, respectively. In addition, NFIB-Wt vectors or NFIB-Mut vectors were co-transfected with the miR-377-3p mimics or NC mimics into liver cancer cells. Transfection was conducted using Lipofectamine<sup>®</sup> 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h, after which the relative luciferase activity was detected using a luciferase reporter assay system (E1910; Promega Corporation) in comparison with *Renilla* luciferase activity.

**Statistical analysis.** All assays were repeated  $\geq 3$  times. Statistical data, presented as the mean  $\pm$  SD, were analyzed using SPSS 22.0 software (IBM Corp.). For data conforming to normal distribution and homogeneity of variance, the paired t-test was used to compare paired samples, while the unpaired t-test was used for comparisons between two groups, and the differences among more than two groups were compared by one-way ANOVA followed by Tukey's post-hoc test. Patients with liver cancer were classified into either a low expression group or a high expression group based on the median expression value of LOXL1-AS1 (median expression value, 1.5) in tumor samples. Kaplan-Meier survival plots were generated using the log-rank test. Spearman's correlation analysis was utilized to analyze the correlations among genes in liver cancer tissues.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**LOXL1-AS1 expression is upregulated in liver cancer tissues and cells.** Abnormal expression of LOXL1-AS1 has been previously reported in breast cancer and lung adenocarcinoma (15,16); therefore, the present study aimed to test the expression status of LOXL1-AS1 in liver cancer. Initially, RT-qPCR analysis was performed to determine LOXL1-AS1 expression in liver cancer tissues. The data revealed that LOXL1-AS1 expression in liver cancer tissues was significantly upregulated compared with that in corresponding normal tissues (Fig. 1A). Furthermore, LOXL1-AS1 expression was measured in liver cancer cell lines. RT-qPCR analysis indicated significant upregulation of LOXL1-AS1 expression in liver cancer cell lines

(SK-HEP-1, Hep3B and SNU1) compared with that in the normal human hepatic cell line (THLE-2) (Fig. 1B). Subsequently, liver cancer samples were grouped into a high LOXL1-AS1 expression group and a low expression group. Kaplan-Meier analysis indicated that higher LOXL1-AS1 expression was associated with a lower overall survival rate in patients with liver cancer (Fig. 1C).

**LOXL1-AS1 mediates glucose metabolism, proliferation, migration and epithelial-mesenchymal transition (EMT) in liver cancer cells.** To probe the biological function of LOXL1-AS1 in liver cancer, liver cancer cells (SK-HEP-1 and Hep3B, which showed higher LOXL1-AS1 expression) were transfected with sh-LOXL1-AS1 or sh-NC. The results revealed that LOXL1-AS1 expression was significantly downregulated after transfection with sh-LOXL1-AS1 compared with sh-NC (Fig. 2A). Additionally, lncRNA LOXL1-AS1 is the antisense strand of LOXL1. RT-qPCR and western blot results suggested that LOXL1 mRNA and protein expression in liver cancer cells were not affected by LOXL1-AS1 silencing (Fig. S1A). Previous evidence has demonstrated that the metabolism of cancer cells is reprogrammed to maintain cell survival and growth (22). Therefore, the present study investigated whether LOXL1-AS1 influenced glucose metabolism in liver cancer cells. SK-HEP-1 and Hep3B cells were treated with different concentrations of glucose (0, 5 and 20 mM). Through glucose uptake assays, it was revealed that LOXL1-AS1 silencing significantly inhibited the glucose uptake of liver cancer cells treated with 5 and 20 mM glucose (Fig. 2B), indicating that LOXL1-AS1 participated in the progression of glucose metabolism in liver cancer. Subsequently, the effects of LOXL1-AS1-knockdown on liver cancer progression were investigated. LOXL1-AS1-knockdown triggered significant decreases in cell viability and proliferation, as demonstrated by MTT and colony formation assays (Fig. 2C-E). Moreover, the results from the wound healing assay indicated that the migration of cells was significantly suppressed after LOXL1-AS1 silencing (Fig. 2F). Since EMT serves a key role in tumor metastasis, western blot analysis was conducted to assess the effects of LOXL1-AS1



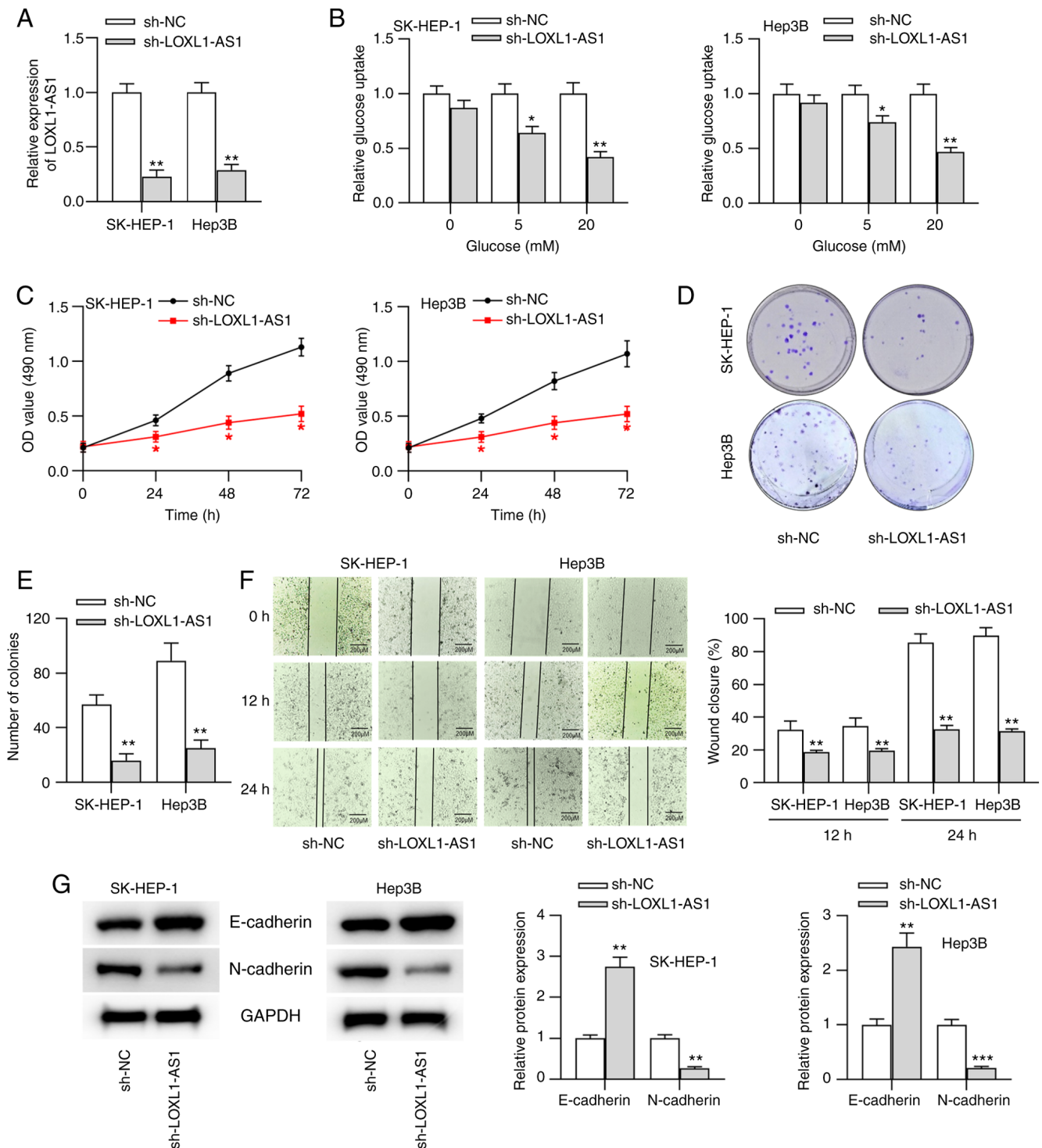


Figure 2. LOXL1-AS1 mediates glucose metabolism, proliferation, migration and EMT in liver cancer cells. (A) sh-NC or sh-LOXL1-AS1 were transfected into SK-HEP-1 and Hep3B cells, and the transfection efficacy was tested by reverse transcription-quantitative PCR analysis. (B) Glucose uptake ability in transfected liver cancer cells was examined by a glucose uptake assay. Cell viability and proliferation were detected through (C) MTT and (D and E) colony formation assays, respectively. (F) Cell migration was evaluated using a wound healing assay (scale bar, 200  $\mu$ m). (G) Protein expression levels of EMT markers (E-cadherin and N-cadherin) were measured by western blot analysis. \* $P$ <0.05, \*\* $P$ <0.01 and \*\*\* $P$ <0.001 vs. sh-NC. sh, short hairpin RNA; NC, negative control; LOXL1-AS1, LOXL1-antisense RNA 1; OD, optical density.

on the expression levels of EMT markers (E-cadherin and N-cadherin) in liver cancer cells. The results revealed that LOXL1-AS1-knockdown significantly increased E-cadherin expression but impaired N-cadherin expression (Fig. 2G), suggesting the regulatory role of LOXL1-AS1 on cell migration.

*LOXL1-AS1 functions as a sponge of miR-377-3p.* As lncRNAs have been reported to act as competitive endogenous RNAs (ceRNAs) to sponge miRNAs in numerous types of cancer, such as oral squamous cell carcinoma and bladder urothelial carcinoma (23,24), the present study aimed to explore whether LOXL1-AS1 interacted with miRNA to affect liver cancer

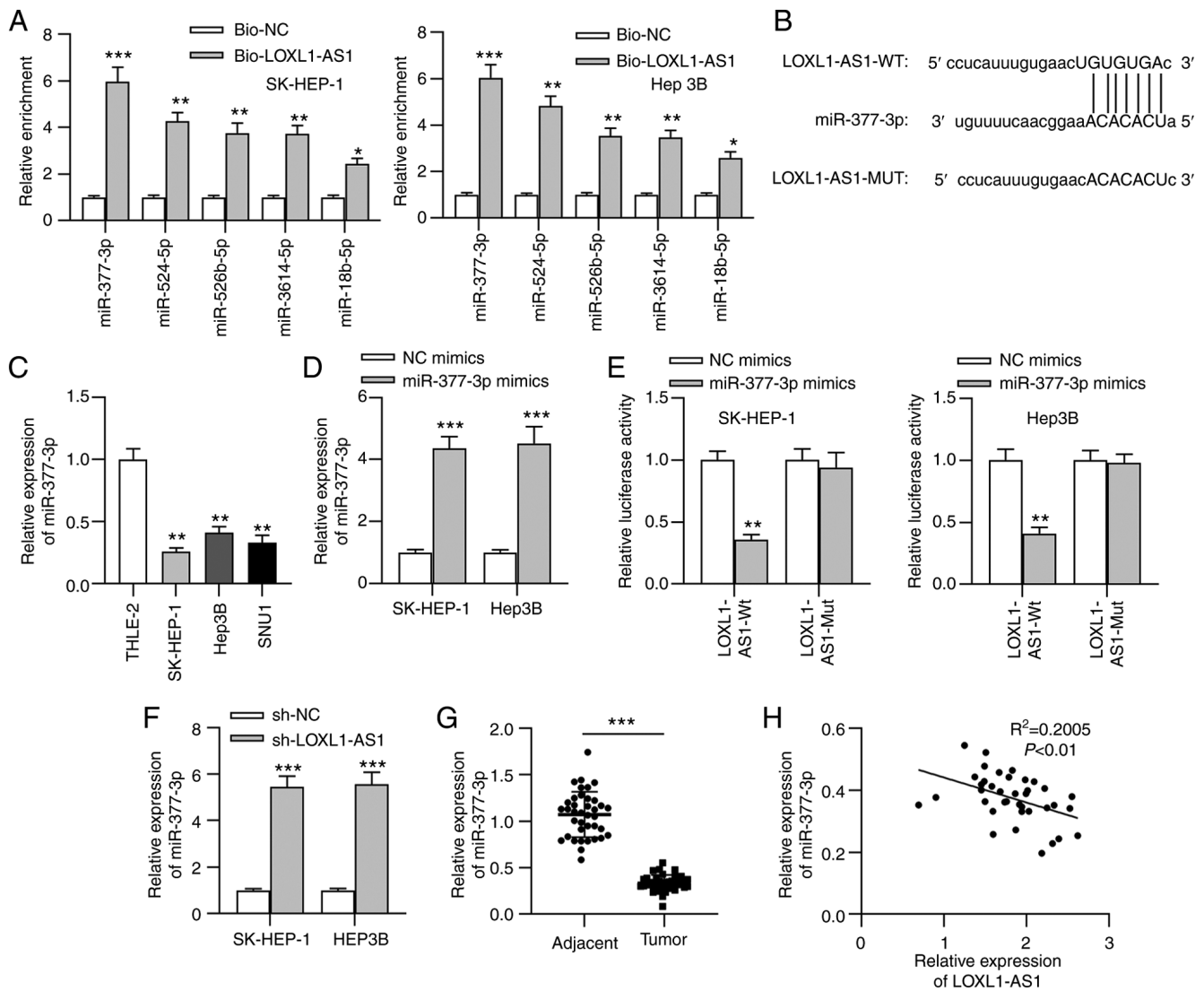


Figure 3. LOXL1-AS1 functions as a sponge of miR-377-3p. (A) A total of 29 miRNAs that may bind to LOXL1-AS1 were obtained from StarBase, and only 5 miRNAs showed enrichment, with miR-377-3p exhibiting the most significant binding capacity to LOXL1-AS1 through an RNA pull-down assay. \* $P<0.05$ , \*\* $P<0.01$  and \*\*\* $P<0.001$  vs. Bio-NC. (B) Bioinformatics analysis predicted the binding site between LOXL1-AS1 and miR-377-3p. (C) miR-377-3p expression in liver cancer cell lines and a normal liver cell line was measured by RT-qPCR. \*\* $P<0.01$  vs. THLE-2. (D) SK-HEP-1 and Hep3B cells were transfected with miR-377-3p mimics or NC mimics, and RT-qPCR analysis was used to test the transfection efficacy. (E) Binding ability between LOXL1-AS1 and miR-377-3p in liver cancer cells was assessed using a luciferase reporter assay. \*\* $P<0.01$  and \*\*\* $P<0.001$  vs. NC mimics. (F) RT-qPCR analysis measured miR-377-3p expression in liver cancer cells transfected with sh-LOXL1-AS1 or sh-NC. \*\*\* $P<0.001$  vs. sh-NC. (G) RT-qPCR data of miR-377-3p expression in liver cancer tissues and adjacent non-tumor tissues. \*\*\* $P<0.001$ . (H) Spearman's correlation analysis assessed the correlation between LOXL1-AS1 and miR-377-3p expression in liver cancer tissues. RT-qPCR, reverse transcription-quantitative PCR; LOXL1-AS1, LOXL1-antisense RNA 1; sh, short hairpin RNA; NC, negative control; miR, microRNA; WT, wild-type; MUT, mutant; Bio, biotinylated.

progression. A total of 29 potential miRNAs were identified that may bind to LOXL1-AS1 using StarBase under pancancer conditions (4 cancer types). RNA pull-down assays indicated that miR-377-3p was the most significantly enriched in RNA complexes pulled down by the Bio-LOXL1-AS1 probe among all candidate miRNAs (the 5 genes that showed enrichment in pull-down products are shown in Fig. 3A). Thus, miR-377-3p was selected for subsequent assays. The predicted binding site between LOXL1-AS1 and miR-377-3p is shown in Fig. 3B. miR-377-3p expression was significantly downregulated in liver cancer cell lines compared with in normal liver cells (Fig. 3C). Additionally, miR-377-3p expression in cells transfected with miR-377-3p mimics was assessed by RT-qPCR (Fig. 3D). To further validate the binding capacity between

miR-377-3p and LOXL1-AS1 in liver cancer cells, a luciferase reporter assay was performed. The results revealed that the luciferase activity of LOXL1-AS1-Wt reporters was significantly weakened by miR-377-3p mimics in liver cancer cells, while that of LOXL1-AS1-Mut reporters remained unchanged (Fig. 3E), indicating that miR-377-3p bound to LOXL1-AS1 in liver cancer cells. In addition, LOXL1-AS1-knockdown significantly increased miR-377-3p expression in liver cancer cells (Fig. 3F). Furthermore, the RT-qPCR results suggested that miR-377-3p expression in liver cancer tissues was significantly downregulated compared with that in adjacent non-tumor tissues (Fig. 3G). Moreover, a negative correlation between miR-377-3p and LOXL1-AS1 expression in liver cancer tissues was observed using Spearman's correlation analysis (Fig. 3H).

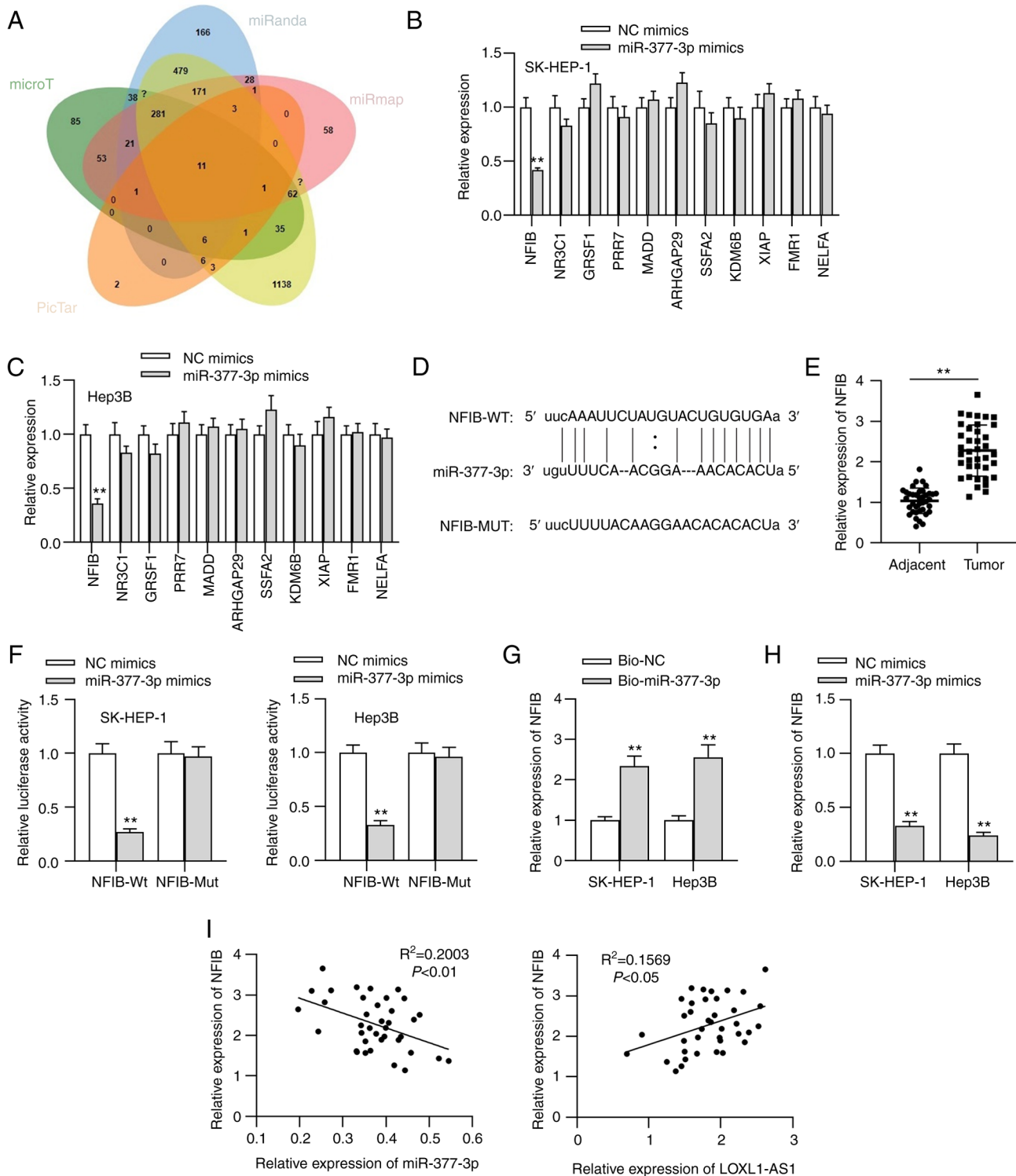


Figure 4. NFIB is targeted by miR-377-3p. (A) Venn diagram showing 11 mRNAs that may be target genes of miR-377-3p. Expression levels of candidate mRNAs in liver cancer (B) SK-HEP-1 and (C) Hep3B cells transfected with miR-377-3p mimics or NC mimics were evaluated by RT-qPCR. (D) Predicted binding site between NFIB and miR-377-3p. (E) NFIB expression in liver cancer tissues was measured through RT-qPCR. Binding capacity between miR-377-3p and NFIB in liver cancer cells was assessed by (F) luciferase reporter and (G) RNA pull-down assays. (H) RT-qPCR was used to measure the expression levels of NFIB in liver cancer cells transfected with miR-377-3p mimics or NC mimics. (I) Correlations among target gene expression in clinical liver cancer tissues were obtained by Spearman's correlation analysis. \*\*\*P<0.01 vs. NC mimics or Bio-NC. RT-qPCR, reverse transcription-quantitative PCR; LOXL1-AS1, LOXL1-antisense RNA 1; sh, short hairpin RNA; NC, negative control; miR, microRNA; WT, wild-type; MUT, mutant; Bio, biotinylated; NFIB, nuclear factor I B.

**NFIB is targeted by miR-377-3p.** To further investigate the regulatory mechanism of LOXL1-AS1 in liver cancer cells, 11 underlying target genes of miR-377-3p were identified using

online tools (Fig. 4A). As shown in Fig. 4B and C, the level of NFIB presented with a significant decrease compared with the levels of the other mRNAs upon miR-377-3p overexpression.

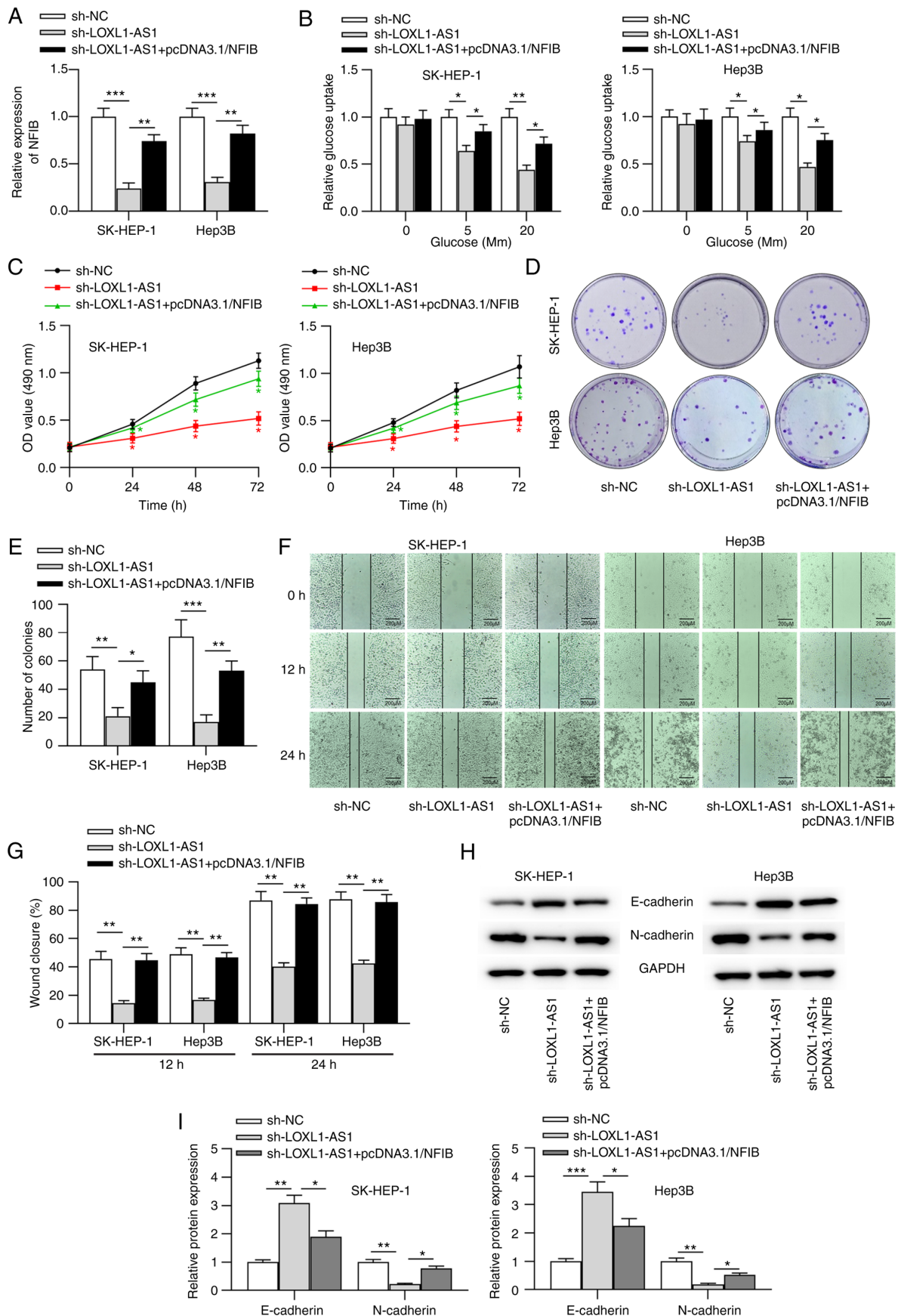


Figure 5. LOXL1-AS1 promotes liver cancer cellular malignancy via the miR-377-3p/NFIB axis. (A) NFIB expression in SK-HEP-1 and Hep3B cells transfected with sh-LOXL1-AS1 alone or co-transfected with sh-LOXL1-AS1 and pcDNA3.1/NFIB was detected by reverse transcription-quantitative PCR. (B) Glucose uptake ability was examined by a glucose uptake assay. Liver cancer cell viability and proliferation were assessed using (C) MTT and (D and E) colony formation assays, respectively. (F and G) Liver cancer cell migration was determined by a wound healing assay (scale bar, 200  $\mu$ m). (H and I) Protein expression levels of EMT markers (E-cadherin and N-cadherin) were measured through western blot analysis. \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001. LOXL1-AS1, LOXL1-antisense RNA 1; sh, short hairpin RNA; NC, negative control; NFIB, nuclear factor I B; OD, optical density.



NFIB is a transcription factor that has been implicated in driving a highly migratory phenotype by enhancing cell-cell adhesion and motility (25). Therefore, NFIB was chosen as the object of further study. The putative binding site between NFIB and miR-377-3p was predicted using StarBase (Fig. 4D). The upregulation of NFIB expression in liver cancer tissues was verified through RT-qPCR (Fig. 4E). The association between NFIB and miR-377-3p was further explored in liver cancer cells through luciferase reporter and RNA pull-down assays. As shown in Fig. 4F and G, the luciferase activity of NFIB-Wt reporters was significantly decreased by miR-377-3p overexpression, and NFIB was significantly enriched in the RNA complexes pulled down by the Bio-miR-377-3p probe, confirming the interaction between NFIB and miR-377-3p in liver cancer cells. Additionally, miR-377-3p mimics significantly decreased NFIB expression in liver cancer cells (Fig. 4H). Moreover, a negative correlation between NFIB and miR-377-3p expression, as well as a positive correlation between NFIB and LOXL1-AS1 expression, was observed in clinical liver cancer tissues (Fig. 4I).

*LOXL1-AS1 promotes liver cancer cellular malignancy via the miR-377-3p/NFIB axis.* To determine whether LOXL1-AS1 mediated the cellular processes of liver cancer via the regulation of the miR-377-3p/NFIB axis, follow-up rescue assays were performed. First, pcDNA3.1/NFIB was transfected into liver cancer cells. RT-qPCR results indicated that NFIB expression was significantly increased in liver cancer cells following transfection with pcDNA3.1/NFIB (Fig. S1B). Subsequently, sh-LOXL1-AS1 alone or sh-LOXL1-AS1 with pcDNA3.1/NFIB were transfected into liver cancer cells. RT-qPCR analysis revealed that sh-LOXL1-AS1 significantly inhibited NFIB expression, which was counteracted by NFIB overexpression in liver cancer cells (Fig. 5A). Moreover, the repression of glucose metabolism caused by sh-LOXL1-AS1 was reversed by NFIB overexpression in liver cancer cells (Fig. 5B). Moreover, overexpression of NFIB counteracted the inhibitory influence of LOXL1-AS1-knockdown on the viability, proliferation, and migration of liver cancer cells (Fig. 5C-G). The western blot analysis results suggested that NFIB overexpression abolished the LOXL1-AS1 silencing-mediated repression of the EMT process (Fig. 5H and I).

## Discussion

Previously, Liu *et al* (26) revealed that LOXL1-AS1 expression was upregulated in hepatocellular carcinoma tissues and promoted hepatocellular carcinoma cell proliferation, migration and invasion. In the present study, the current data confirmed the upregulation of LOXL1-AS1 expression in liver cancer tissues and cells, indicating that LOXL1 may act as an oncogene in liver cancer cellular processes. Additionally, the present study suggested that high LOXL1-AS1 expression predicts an adverse prognosis in patients with liver cancer. LOXL1-AS1 is an antisense strand of LOXL1. The present study revealed that LOXL1 mRNA and protein expression was not affected by lncRNA LOXL1-AS1 in liver cancer cells. Previous studies have demonstrated that LOXL1-AS1 promotes cancer progression by promoting cancer cell proliferation, migration and

invasion (27,28). In the current study, LOXL1-AS1 was revealed to promote liver cancer progression by facilitating glucose metabolism, proliferation, migration and EMT in liver cancer cells.

miRNAs, a class of small, single-stranded RNAs 20-22 nucleotides in length, are known to modulate the expression of target genes at the post-transcriptional level (29). Increasing studies have demonstrated that lncRNAs act as ceRNAs to regulate mRNAs by competitively binding to miRNAs, thus affecting the progression of various types of cancer, including liver cancer (23,24,30). There is evidence suggesting that LOXL1-AS1 serves as a ceRNA to mediate the development of cancer (31,32). In the present study, miR-377-3p was predicted as the downstream molecule of LOXL1-AS1 using online tools. miR-377-3p has been reported to be a tumor suppressor in several types of human cancer. For example, miR-377-3p inhibits the development of ovarian cancer by repressing cell proliferation and invasion (33). A high level of miR-377-3p is associated with longer overall survival of patients with breast cancer (34). The present study revealed that miR-377-3p expression was downregulated in liver cancer tissues and cells. The current mechanistic experimental results verified the binding capacity between miR-377-3p and LOXL1-AS1 in liver cancer cells and the negative correlation of miR-377-3p and LOXL1-AS1 expression in liver cancer tissues. In addition, miR-377-3p expression was upregulated by LOXL1-AS1 silencing. The present results suggested that LOXL1-AS1 served as a miR-377-3p sponge.

Multiple transcription factors have been reported to participate in the metastatic cascade (35). NFIB is a transcription factor that belongs to the NFI family, which regulates development and cellular differentiation in several types of tissue (36). Bioinformatics analysis predicted that NFIB was a potential target gene of miR-377-3p. Recent studies have suggested that NFIB exerts carcinogenic effects on the progression of diverse types of cancer. For example, NFIB-knockdown suppresses cell proliferation, migration and EMT in gastric cancer (37). Additionally, NFIB exhibits an oncogenic function in colorectal cancer (38). The present study revealed that the NFIB 3'-UTR was directly targeted by miR-377-3p in liver cancer. Furthermore, NFIB expression was negatively modulated by miR-377-3p in liver cancer cells. A positive correlation was identified between NFIB and LOXL1-AS1 expression, as well as a negative correlation between NFIB and miR-377-3p expression. LOXL1-AS1 upregulated NFIB by competitively binding to miR-377-3p in liver cancer cells. Rescue assays indicated that NFIB overexpression counteracted the inhibitory influence of LOXL1-AS1 silencing on liver cancer cellular processes.

In conclusion, to the best of our knowledge, the present study was the first to propose and verify that LOXL1-AS1 expression was upregulated in liver cancer tissues and cells, and that high LOXL1-AS1 expression predicted a poor prognosis for patients with liver cancer. The current study innovatively provided evidence that the LOXL1-AS1/miR-377-3p/NFIB axis may aggravate the cellular processes of liver cancer, which may provide novel insights for the clinical treatment of liver cancer.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

WY and YD participated in the literature search, analysis and interpretation of the data, and the writing of the manuscript. Both authors confirm the authenticity of the raw data and have read and approved the final manuscript.

## Ethics approval and consent to participate

The present study received approval from the Ethics Committee of the Affiliated Hospital of Yangzhou University (approval no. 2020-004). All patients provided written informed consent.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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