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LncRNA SNHG6 regulating Hedgehog signaling pathway and affecting the biological function of gallbladder carcinoma cells through targeting miR-26b-5p

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Abstract. – OBJECTIVE: Long-chain non-coding RNA (LncRNA) is abnormally expressed in various malignant tumors. In recent years, it has been found that the expression of LncRNA SNHG6 is upregulated in gallbladder carcinoma tissues, which participated in the occurrence and development of gallbladder carcinoma. However, the clinical value of SNHG6 in gallbladder cancer serum is not clear, and there are few studies regulating the biological function of gallbladder carcinoma cells. This study aimed to investigate LncRNA SNHG6 and miR-26b-5p in gallbladder carcinoma and its related mechanisms.

PATIENTS AND METHODS: From February 2017 to February 2019, altogether 68 cases of gallbladder cancer patients admitted to the Yantai Yeda Hospital were collected as a study group, 70 healthy people as a control group. Gallbladder cancer cells and human colorectal mucosa cells were purchased. Sh-SNHG6, si-SNHG6, NC, miR-26b-5p-inhibitor, and miR-26b-5p-mimics were transfected into GBC-SD and NOZ cells. For the detection of SNHG6 and miR-26b-5p in samples we used qRT-PCR, WB was applied for the decreased protein expression of Gli1, Gli2, Shh, Smo, N-cadherin, vimentin, Snail, E-Cadherin, and Gli3 in cells. MTT assay was applied for the detection of cell proliferation, transwell assay for cell invasion, and flow cytometry assay for apoptosis.

RESULTS: SNHG6 was highly expressed in gallbladder carcinoma, miR-26b-5p was downregulated, and the area under curve (AUC) of LncRNA SNHG6 and miR-26b-5p was more than 0.8. LncRNA SNHG6 and miR-26b-5p were related to age, sex, tumor invasion, differentiation degree, tumor location, and tumor-node-metastasis (TNM) staging of gallbladder cancer patients. Silencing of SNHG6 and upregulation of miR-26b-5p could promote cell apoptosis, inhibit cell growth, and epithelial-mesenchymal transition (ETM). Silencing of SNHG6 and upregulation of miR-26b-5p could inhibit Gli1, Gli2, Shh, Smo, N-cadherin, vimentin and Snail proteins, and promote upregulation of Gli3 and E-Cadherin expression. Dual-Luciferase report confirmed that SNHG6 and miR-26b-5p have targeted relationship. Rescue experiments showed that after co-transfecting sh-SNHG6+miR-26b-5p-mimics, and si-SNHG6+miR-26b-5p-inhibitor into GBC-SD and NOZ, the proliferation, invasion and apoptosis of cells were not different from those of miR-NC group without transfection sequence.

CONCLUSIONS: Inhibition of LncRNA SNHG6 expression can upregulate miR-26b-5p mediated Hedgehog signaling pathway, affect epithelial-mesenchymal transition, proliferation and invasion of cells, so LncRNA SNHG6is hoped to be a latent therapeutic target for gallbladder carcinoma.

Key Words:
Gallbladder carcinoma, LncRNA SNHG6, Diagnosis, Prognosis, Hedgehog signaling pathway, Biological function.

Introduction

Gallbladder cancer is a kind of highly invasive malignant tumor of biliary tract system and is one of the main factors causing human cancer death[1]. The diagnosis rate of early gallbladder cancer is relatively low, which is usually diagnosed in the late stage and the surgical resection rate is relatively low. However, the poor clinical effect of traditional radiotherapy and chemotherapy results in poor prognosis of patients, and the survival rate of gallbladder cancer patients within five years is less than 10[2]. Despite the continuous development of molecular diagnosis and targeted therapy

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in recent years, gallbladder cancer, as a highly invasive disease, still lacks effective biomarkers for prevention, diagnosis, metastasis, and prognosis[3]. Therefore, determining the occurrence, development, prognosis, and potential mechanism of gallbladder cancer is helpful for clinicians to explore a more appropriate treatment scheme for gallbladder cancer[4]. In recent years, some studies have found that the occurrence and development of gallbladder carcinoma are relevant to long-chain non-coding RNA (10 ng non-coding RNA, LncRNA). LncRNA affects EMT of gallbladder cancer cells and the biological function of cancer cells. Research on targeted diagnosis or treatment of gallbladder cancer by LncRNA shows broad development prospects[5].

LncRNA genome position is closely related to protein coding genes and participates in various biological processes of tumors, having effects in cell cycle, cell differentiation, proliferation, and other life activities[6-8]. Many studies showed that LncRNA is abnormally expressed in various cancers, which has diagnostic and prognostic value for tumors[9,10]. LncRNA SNHG6 is upregulated in tumors, such as liver cancer and gastric cancer, and can further regulate the occurrence and development of tumors through various molecular mechanisms[11,12]. However, the relationship between serum SNHG6 and the diagnosis and prognosis of gallbladder cancer and the possible molecular mechanism is still unclear. At present, studies[13,14] have found that Hedgehog signals are reactivated in various types of cancers and are potential therapeutic targets. Wang et al[15] found that LncRNA SNHG6 activates TGF-β/ Smad signaling pathway through targeted miRNA, induces EMT, and promotes the growth of gallbladder cancer cells. There are not many researches about the biological significance of Hedgehog signaling in human gallbladder cancer.

Therefore, by detecting the expression of SNHG6 in gallbladder carcinoma, we explored the clinical value and possible molecular mechanism of SNHG6 in gallbladder carcinoma, in order to find reliable diagnosis and prognosis for clinical gallbladder carcinoma, tumor markers, and potential drug targets.

Patients and Methods

From February 2017 to February 2019, altogether 68 patients with gallbladder cancer who visited YantaiYeda Hospital were collected as the research group, containing 39 men and 29 women, with the average age of (54.8± 3.52) years. A total of 70 healthy people were collected as a control group, containing 34 men and 36 women, with the average age of (55.1± 3.70) years ($p > 0.05$). Exclusion criteria were as follows: patients confirmed as gallbladder carcinoma[16] by pathology, cytology and imaging; gallbladder cancer patients who did not receive relevant preoperative chemotherapy, immunotherapy, radiation and other anti-tumor treatment; patients complicated with liver cirrhosis and coagulation dysfunction; the general clinical data were incomplete; patients did not cooperate with follow-up, the expected survival time was less than 1 month; patients lost to follow-up. The study was approved by the Ethics Committee of Jiyang District People's Hospital and the patients signed informed consent forms in advance.

Main Instruments and Reagents

GBC-SD, NOZ, human gallbladder carcinoma cell line and HIBEpiC normal human intrahepatic bile duct epithelial cells (BNCC341817, BNCC342126, BNCC339778) were purchased from BeNa Biotechnology (Inchon, South Korea). ABI StepOne Plus Real Time Fluorescence quantitative PCR, Lipofectamine™ 2000 transfection kit, TRIzol extraction kit, Annexin V/ PI apoptosis detection kit were purchased from Invitrogen, (Carlsbad, CA, USA). SYBR Green PCR Master Mix was purchased from Applied Biosystems (Foster City, CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) kit was purchased from (Beyotime Biotechnology Co., Ltd. (Songjiang, Shanghai, China. Product Number: C0009). 10% of fetal bovine serum (FBS), penicillin-streptomycin mixed solution (100× double antibody), Dulbecco's Modified Eagle's Medium (DMEM), and transwell kit were purchased from Gibco™ BRL (Gaithersburg, MD, USA). β-catenin polyclonal goat IgG, cyclin D1 polyclonal goat IgG, c-myc polyclonal goat IgG, β-actin, goat anti-mouse secondary antibody were purchased from R&D System (Minneapolis, MN, USA). Enhanced chemiluminescence (ECL) kit, BCA protein kit, Multiskan™ GO Full Wavelength Enzyme Labeling Instrument were purchased from Thermo Fisher Technology Co., Ltd., (Pudong New Area, Shanghai, China). FACSCanto flow cytometer was purchased from Becton Dickinson (Franklin Lakes, NJ, USA). DR5000 ultraviolet-visible spectrophotometer was purchased from (BioRad, Hercules, CA, USA). All primer sequences were

synthesized by Sangon Biotechnology Co., Ltd. (Shanghai, China).

Detection Methods

Sample collection: A 5 mL of elbow venous blood from gallbladder cancer patients and healthy people were collected, centrifuged at 3000×g for 10 min, and serum was placed for later use.

Cell Culture and Transfection Experiments

Gallbladder cancer cell lines were transfected into DMEM medium (including 10 % fetal bovine serum and penicillin-streptomycin mixed solution) and put in a cell incubator with 5% CO_2 at 37°C with constant temperature and humidity. SNHG6-siRNA (si-SNHG6), SNHG6-shRNA (sh-FOXF2), and empty plasmid (siRNA-NC) were transfected respectively according to the instructions of Lipofectamine™ 2000 transfection kit. Primers were transfected into the cells with the greatest difference in SNHG6 expression, and 6 hours later, the samples were cultured in a culture solution (including 10% fetal bovine serum). The detection of the transfection efficiency of cells used qRT-PCR.

qRT-PCR Detection

The detection of mRNA in serum and cells used Real Time-Reverse Transcription Polymerase Chain Reaction (qRT-PCR). Total RNA in serum was dissolved in 20 μL DEPC water according to TRIzol reagent operation instructions, and transcribed by a reverse transcription kit, of which the steps were as follows: 1 μl of M-MLV, 1 μl of olig (dT), 0.5 μl of RNA enzyme inhibitor, 1 μl of d NTPs, and RNase free water was used to make up to 15 μl. The sample was incubated at 38°C for 60 min. The PCR reaction steps were as follows: 2.5 μl of 10×PCR buffer, 1 μl of d NTPs, 1 μl of upstream and downstream primers, 0.25 μl of Taq DNA polymerase, dd H_2O was supplemented to 25 μl. Reaction steps were as follows: 95°C for 15 min, 95°C for 15 s, 58°C for 30 s, for a total of 35 cycles. Finally, the sample was extended for 15 min at 72°C. U6 was the internal reference of miR-26b-9p and GAPDH was the internal reference of SNHG6 (Table I).

Western Blot Test

The lysed cells were collected and transferred to a centrifuge tube at 12000×g for 10 min at 4°C. Bicinchoninic Acid (BCA) method was applied for the detection of the protein concentration. Lysis buffer was added to dilute the protein sample to prepare 20 mg/ml protein, 8.00% separation gel and 5.00% lamination gel. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis and polyvinylidene difluoride (PVDF) membrane transfer were performed. β-catenin, cyclin D1, and c-myc (1:1000) primary antibody were added, β-actin (1:3000) was used as internal reference and sealed overnight at 4°C. Horseradish peroxidase (HRP) labeled sheep anti-mouse secondary antibody (1:5000) was added, incubated at 37°C for 1 h, and washed with Tris-Buffered Saline and Tween-20 (TBST) 3 times, for 5 min/time. Then, it was developed in dark room, the excess liquid on the membrane was absorbed, enhanced chemiluminescence (ECL) was performed. The protein bands were scanned, and the gray values were analyzed using Quantity One (Molecular Devices Corp, The Bay Area, CA, USA).

Cell Proliferation Experiment

MTT assay was applied for the detection of cell viability. Cells harvested 24 hours after transfection were collected. Cell density was adjusted to 5×10^3 cells/well. Cells were inoculated on 96-well plates at 37°C for 24, 48, and 72 hours respectively. A total of 20 μL of MTT solution (5 μM g/ml) was put in at each time point. Culture was continued at 37°C for 4 hours. Altogether 200 μL of dimethyl sulfoxide (DMSO) was put in per well. The OD value was detected by spectrophotometer at 570 mm wavelength.

Table I. MiR-26b-9p, SNHG6 and their internal reference primer sequences.

Genes	Forward primer	Reverse primer
MiR-26b-5p	5′-GGCTTCAAGTAATTCAGGATAGG-3′	5’-GTGCAGGGTCCGAGGT-3’
U6	5’-CTCGCTTCGGCAGCACA-3’	5’-AACGCTTCACGAATTTGCGT-3’
SNHG6	5’-TGCCAGCAGTGACAGCAGCA-3’	5’-TACGGAGGTGGAGTGCCAT-3’
GAPDH	5’-CAAAGGTGGATCAGATTCAAG-3’	5’-GGTGAGCATTATCACCCAGAA-3’

Transwell Invasion Experiment

Matrigel glue was coated in transwell chamber and placed at 37°C for 30 min. The cells were collected in a serum-free DMEM culture with 4×10^5 cells/mL. A total of 200 μL of cell suspension was put in the upper chamber and 800 μL of DMEM medium (10% fetal bovine serum) in the lower chamber. After culturing for 24-48 h, the chamber was taken out, the cells were fixed with 4% paraformaldehyde, and then, 0.1 % crystal violet was used for staining. Finally, the cells in the upper chamber were wiped. Ten high power fields were selected under an optical microscope to count the cells passing through the basement membrane of the small chamber, which indicates the cell invasion ability.

Apoptosis Experiment

Cells transfected for 48 h were digested with 0.25% trypsin, washed with PBS twice, resuspended with 100 μL of AnnexinV-binding buffer, configured as 1×10^6 cells/mL suspension, added with 5 μL of Annexin-V/FITC solution, and incubated at 4°C for 15 min. A total of 5 μl of propidium iodide (PI) staining solution was added and incubated at 4°C for 5 min. Flow cytometry was used for detection. The average value was applied after repeating the experiment for 3 times.

Xenotransplantation Tumor Model

Female BALB/c nude mice aged 4 weeks were fed in sterile environment, then, 3×10^6 GBC-SD cells of stable si-SNHG6 and NC plasmids were subcutaneously injected into the left abdomen of nude mice, 4 nude mice in each group. Tumor growth was detected 7 days once. The rats were executed by cervical dislocation after 28 days of injection, and tumor size and *in vivo* quality were accurately measured. (The nude mouse experiment was approved by the Animal Ethics Committee of the Medical Department of our hospital)

Statistical Analysis

SPSS 20.0 (IBM Corp., Armonk, NY, USA) was applied for statistical analysis. Normal distribution data was expressed by mean±standard deviation (mean±SD). The data at multiple time

Table II. Relationship between serum SNHG6 and pathological parameters of gallbladder carcinoma (meas±SD).

Clinicopathological parameters	No.	SNHG6	*t* /*F*	*p*
Gender			4.183	<0.001
Male	47	0.76±0.17		
Female	21	0.92±0.06		
Age			4.046	<0.001
<60	39	0.74±0.19		
≥60	29	0.89±0.07		
Tumor size (cm)			3.619	<0.001
<2	33	0.74±0.2		
≥2	35	0.61±0.07		
T staging			4.028	<0.001
T1/T2	43	0.75±0.18		
T3/T4	25	0.9±0.06		
N staging			4.028	<0.001
N0	43	0.75±0.18		
N1	25	0.9±0.06		
M staging			3.659	<0.001
M0	32	0.74±0.2		
M1	36	0.87±0.07		
Degree of differentiation			4.018	<0.001
High/medium	53	0.77±0.16		
Low	15	0.94±0.06		
Tumor invasion			7.897	<0.001
Liver invasion	20	0.78±0.24		
Lymphatic invasion	10	0.67±0.06		
Extrahepatic bile duct	28	0.83±0.04		
Vascular invasion	10	0.96±0.06		
Tumor location			9.472	<0.001
Neck	8	0.81±0.21		
Body part	39	0.75±0.16		
Bottom	21	0.92±0.06		

points were compared by repeated measurement analysis of variance. Bonferroni method was applied for back testing, One-way ANOVA for comparison among mean values of multiple groups, and LSD-t test for afterwards. The diagnostic value was evaluated by receiver operating characteristic (ROC) curve. Pearson test was applied to correlation. The *p*-value less than 0.05 was regarded as statistical significance.

Results

Expression and Clinical Value of SNHG6 and MiR-26b-5p

By detecting SNHG6 and miR-26b-5p, we found that SNHG6 in the study group was higher than that in the control group, while miR-26b-5p in the study group was lower than that in the control group ($p < 0.001$). Pearson test indicated that SNHG6 was negatively correlated with miR-26b-5p ($p < 0.001$). The AUC of SNHG6 and miR-26b-5p were 0.883 and 0.850 respectively by visualizing ROC curves. Further analysis of the relationship between the two indexes and the pathological data of patients showed that SNHG6 and miR-26b-5p were closely related to age, sex, differentiation degree, TNM stage, tumor invasion and location ($p < 0.05$). Tables II, III, and Figure 1.

SNHG6 and MiR-26b-5p in Cells and Their Effects on Cell Biological Functions

qRT-PCR was applied for the detection of SNHG6 in various cell lines. Compared with HIBEpiC normal human pancreatic duct epithelial

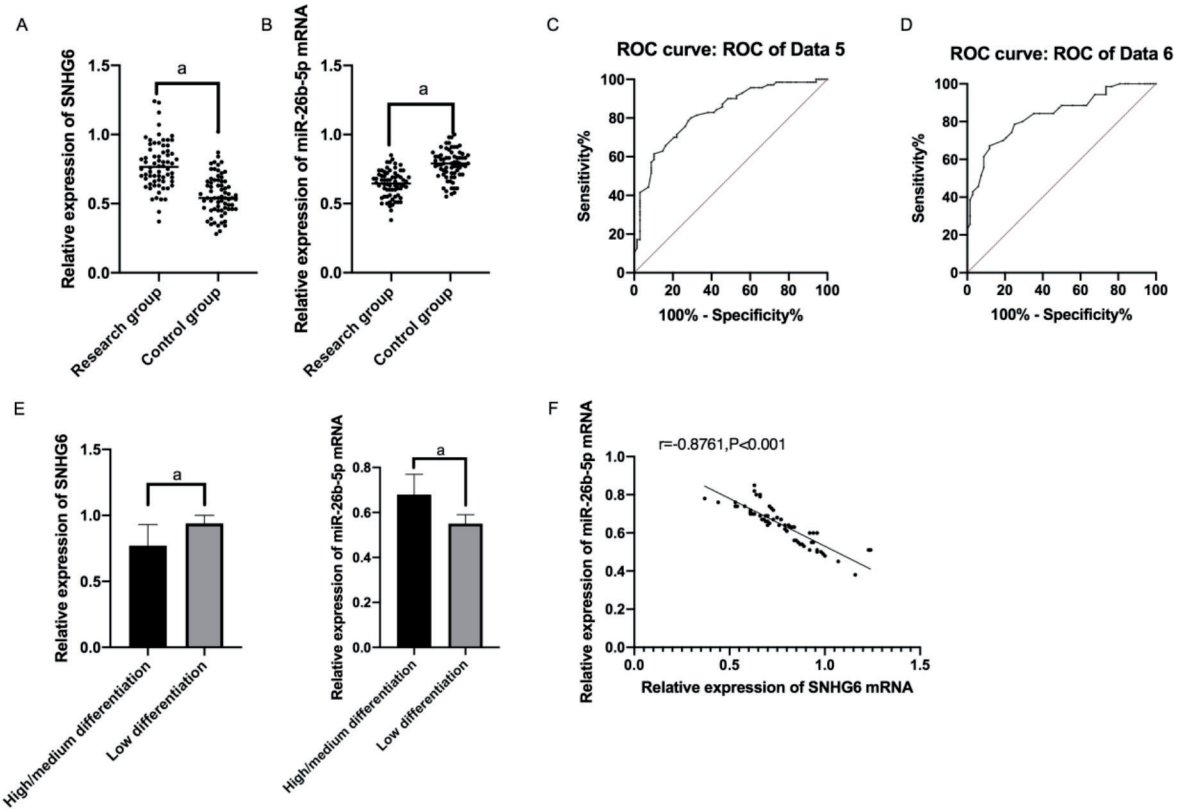

Figure 1. Expression and clinical value of SNHG6 and miR-26b-5p in serum of gallbladder cancer patients. **A,** The expression level of SNHG6 in the study group was significantly higher than that in the control group. SNHG6 was highly expressed in the serum of gallbladder cancer patients. Note: a indicates that $p < 0.001$. **B,** The expression level of miR-26b-5p in the study group was significantly lower than that in the control group, and miR-26b-5p was low in gallbladder cancer patients. Note: a indicates that $p < 0.001$. **C,** AUC of SNHG6 was 0.883. **D,** AUC of miR-26b-5p was 0.850. **E,** The expression level of SNHG6 in the low-differentiated patients in the study group was significantly higher than that in the medium-differentiated and high-differentiated patients, and the expression level of miR-26b-5p in the low-differentiated patients in the study group was significantly lower than that in the medium-differentiated and high-differentiated patients. Note: a indicates that $p < 0.001$. **F,** The expression of SNHG6 and miR-26b-5p in serum of gallbladder cancer patients was negatively correlated ($r=-0.8761$, $p < 0.001$).

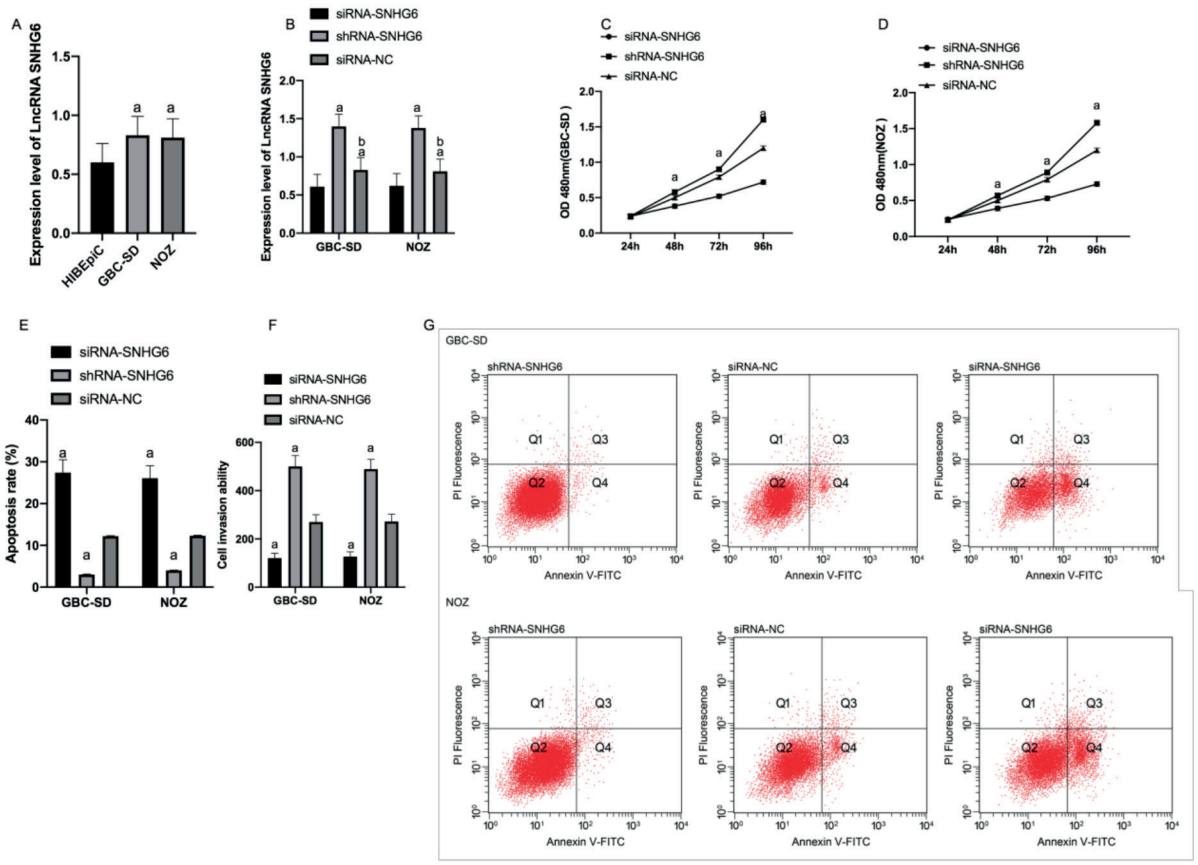

Figure 2. Expression of SNHG6 in cells and its effect on cell biological function. Expression of SNHG6 in various cell lines **(A)**. Expression of SNHG6 after transfection of GBC-SD and NOZ cells **(B)**. Proliferation of GBC-SD **(C)** and NOZ **(D)** cells after transfection. Apoptosis of GBC-SD and NOZ cells after transfection **(E)**. Invasion of GBC-SD and NOZ cells after transfection **(F)**. Apoptosis map **(G)**. Note: a/b indicates that $p < 0.001$.

cells, SNHG6 in GBC-SD and NOZ cells was significantly increased ($p < 0.05$). GBC-SD and NOZ cells with the greatest expression difference were selected for transfection. Compared with HIBEpiC normal human pancreatic duct epithelial cells, SNHG6 in GBC-SD and NOZ cells remarkably increased ($p < 0.05$). SNHG6 in siRNA-SNHG6 group was lower ($p < 0.01$). SNHG6 in shRNA-SNHG6 group was higher ($p < 0.01$). MTT results showed that the proliferation ability of siRNA-SNHG6 group was lower ($p < 0.05$), and the proliferation ability of shRNA-SNHG6 group was upregulated ($p < 0.05$). Flow cytometry indicated that the apoptosis rate of siRNA-SNHG6 group was lower ($p < 0.001$), and that of shRNA-SNHG6 group was also lower ($p < 0.001$). Transwell experiment results indicated that the cell invasion ability of siRNA-SNHG6 group was lower ($p < 0.001$), and the cell invasion ability of shRNA-SNHG6 group was higher ($p < 0.001$) (Figure 2).

qRT-PCR was applied for the detection of miR-26b-5p in each group of cell lines. Compared with HIBEpiC normal human pancreatic duct epithelial cells, GBC-SD and NOZ cells showed downregulated miR-26b-5p ($p < 0.05$). GBC-SD and NOZ cells with the greatest expression difference were selected for transfection. MiR-26b-5p in miR-26b-5p-inhibitor group was lower ($p < 0.01$), and miR-26b-5p in miR-26b-5p-mimics group was higher ($p < 0.01$). CCK-8 results indicated that the proliferation ability of miR-26b-5p-mimics group was lower ($p < 0.05$), and the proliferation ability of miR-26b-5p-inhibitor group was higher ($p< 0.05$). Flow cytometry results indicated that the apoptosis rate of miR-26b-5p-mimics group was higher ($p < 0.001$), and the apoptosis rate of miR-26b-5p-inhibitor group was lower ($p < 0.001$). Transwell experiment results indicated

that the cell invasion ability of miR-26b-5p-mimics group was lower ($p < 0.001$), and the cell invasion ability of miR-26b-5p-inhibitor group was higher ($p < 0.001$) (Figure 3).

Effects of SNHG6 and MiR-26b-5p Expression on Hedgehog Signaling Pathway and EMT

Western Blot results indicated that Gli1, Gli2, Shh, Smo, N-cadherin, vimentin, and Snail proteins in cells transfected with sh-SNHG6 were significantly upregulated compared with cells transfacted with miR-NC, while E-Cadherin and Gli3 in cells transfected with sh-SNHG6 was significantly downregulated compared with cells transfected with miR-NC. However, compared with siRNA-NC cells, the expression of Gli1, Gli2, Shh, Smo, N-cadherin, vimentin and Snail was significantly downregulated after transfection of si-SNHG6, and E-Cadherin and Gli3 was significantly upregulated ($p < 0.05$).

Compared with Gli1, Gli2, Shh, Smo, N-cadherin, vimentin, and Snail proteins in transfected miR-NC cells, miR-26b-5p-inhibitor was upregulated, and E-Cadherin and Gli3 were downregulated. However, Gli1, Gli2, Shh, Smo, N-cadherin, vimentin and Snail in transfected miR-26b-5p-mimics were lower, and E-Cadherin and Gli3 were higher ($p < 0.05$) (Figure 4).

Identification of SNHG6 Target Gene

In order to verify the correlation between SNHG6 and miR-26b-5p, firstly, a target-binding site between miR-26b-5p and SNHG6 was found by predicting a target downstream gene of SNHG6 through TargetScan7.2. For this reason, we carried out Dual-Luciferase activity detection and found that pmirGLO-SNHG6-3'UT Wt Luciferase activity was significantly reduced after overexpression of miR-26b-5p ($p < 0.001$), but it had no effect on pmirGLO-SNHG6-3'UTR Mut Luciferase activity ($p > 0.05$). PCR detection found that miR-26b-5p expression in GBC-SD and NOZ cells was downregulated after Sh-SNHG6 transfection, and miR-26b-5p expression in

Table III. Relationship of miR-26b-5p with pathological data of gallbladder cancer patients.

Clinicopathological parameters	No.	SNHG6	t /F	p
Gender			5.714	<0.001
Male	47	0.69±0.09		
Female	21	0.57±0.05		
Age			4.778	<0.001
<60	39	0.69±0.1		
≥60	29	0.59±0.06		
Tumor size (cm)			3.599	<0.001
<2	33	0.69±0.11		
≥2	35	0.61±0.07		
T staging			6.185	<0.001
T1/T2	43	0.69±0.1		
T3/T4	25	0.58±0.06		
N staging			6.185	<0.001
N0	43	0.69±0.1		
N1	25	0.58±0.06		
M staging			3.618	<0.001
M0	32	0.69±0.11		
M1	36	0.61±0.07		
Degree of differentiation			5.422	<0.001
High/medium	53	0.68±0.09		
Low	15	0.55±0.04		
Tumor invasion			14.300	<0.001
Liver invasion	20	0.64±0.11		
Lymphatic invasion	10	0.78±0.06		
Extrahepatic bile duct	28	0.66±0.05		
Vascular invasion	10	0.53±0.04		
Tumor location			44.080	<0.001
Neck	8	0.55±0.06		
Body part	39	0.71±0.07		
Bottom	21	0.57±0.05		

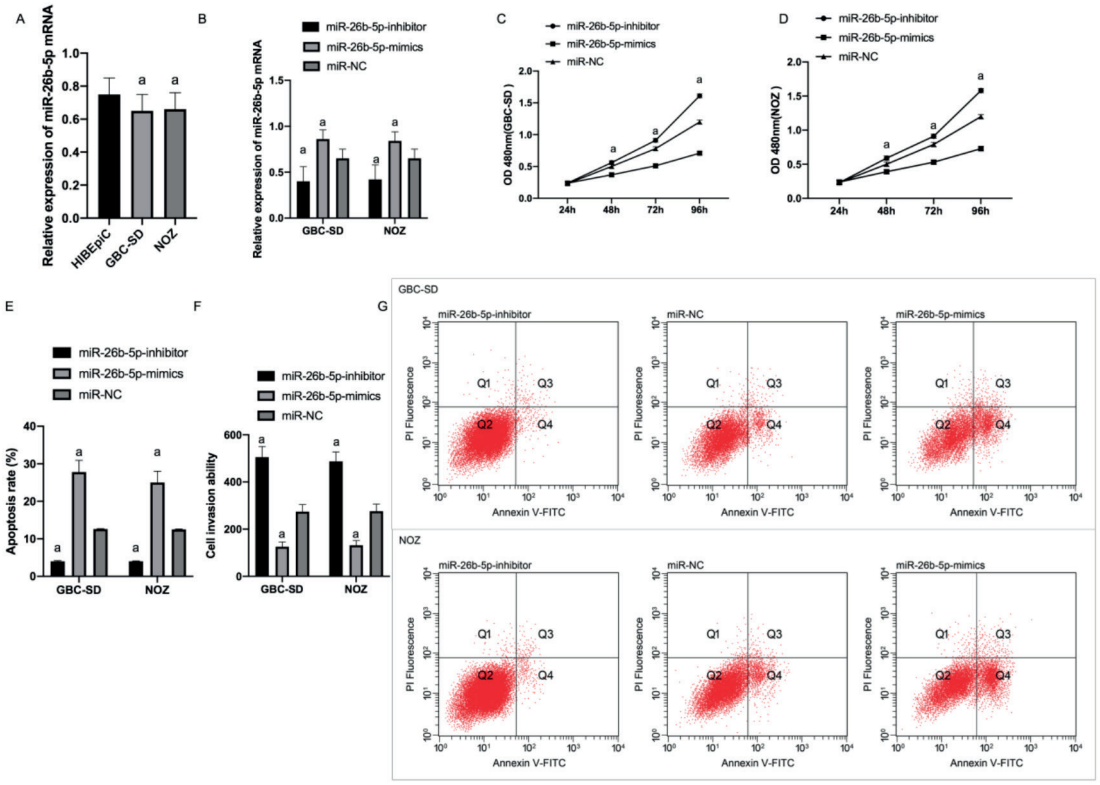

Figure 3. Expression of miR-26b-5p in cells and its effect on cell biological function. Expression of miR-26b-5p in various cell lines **(A)**. Expression of SNHG6 after transfection of GBC-SD and NOZ cells **(B)**. Proliferation of GBC-SD **(C)** and NOZ **(D)** cells after transfection. Apoptosis of GBC-SD and NOZ cells after transfection **(E)**. Invasion of GBC-SD and NOZ cells after transfection **(F)**. Apoptosis map **(G)**. Note: a/b indicates that $p < 0.001$.

GBC-SD and NOZ cells was upregulated after Si-SNHG6 transfection ($p< 0.05$) (Figure 5).

Rescue Experiment

Sh-SNHG6+miR-26b-5p-mimics and Si-SNHG6+miR-26b-5p-inhibitor were further transfected into GBC-SD and NOZ cells for the detection of the biological functions. It was found that the proliferation, invasion, and migration of transfected Sh-SNHG6+miR-26b-5p-mimics and Si-SNHG6+miR-26b-5p-inhibitor have no difference with siRNA-NC group ($p > 0.05$). However, compared with Sh-SNHG6, Sh-SNHG6+miR-26b-5p-mimics, Si-SNHG6or+mir-26b-5p-inhibitor had significantly higher proliferation, invasion, and migration capabilities ($p< 0.05$), while their proliferation, invasion, and migration capabilities are lower than those of Si-SNHG6 ($p < 0.05$). EMT-related protein detection showed that E-cadherin, N-cadherin, vimentin, and Snail proteins in Sh-SNHG6+miR-26b-5p-mimics and Si-SNHG6+miR-26b-5p-inhibitor had no difference with siRNA group ($p > 0.05$). However, by comparingSh-SNHG6+miR-26b-5p-mimics and Si-SNHG6+miR-26b-5p-inhibitor with Sh-SNHG6, E-Cadherin protein was remarkably reduced, and N-cadherin, vimentin and Snail protein weresignificantly increased. However, compared with Si-SNHG6, E-Cadherin protein was higher, and N-cadherin, vimentin and Snail protein was lower ($p < 0.05$) as shown in Figure 6.

SNHG6 Silencing Expression Inhibiting Tumor Growth in Nude Mice

We established a tumor formation model by injecting GBC-SD cells transfected with Si-SNHG6 and siRNA-NC into the abdomen of nude mice. The above results indicated that the tumor growth rate of nude mice in Si-SNHG6 group was lower, the tumor size and tumor weight obtained after killing nude mice were lower ($p < 0.001$), and SNHG6 in tumor tissues of nude mice in Si-SNHG6 group was lower ($p < 0.001$ (Figure 7).

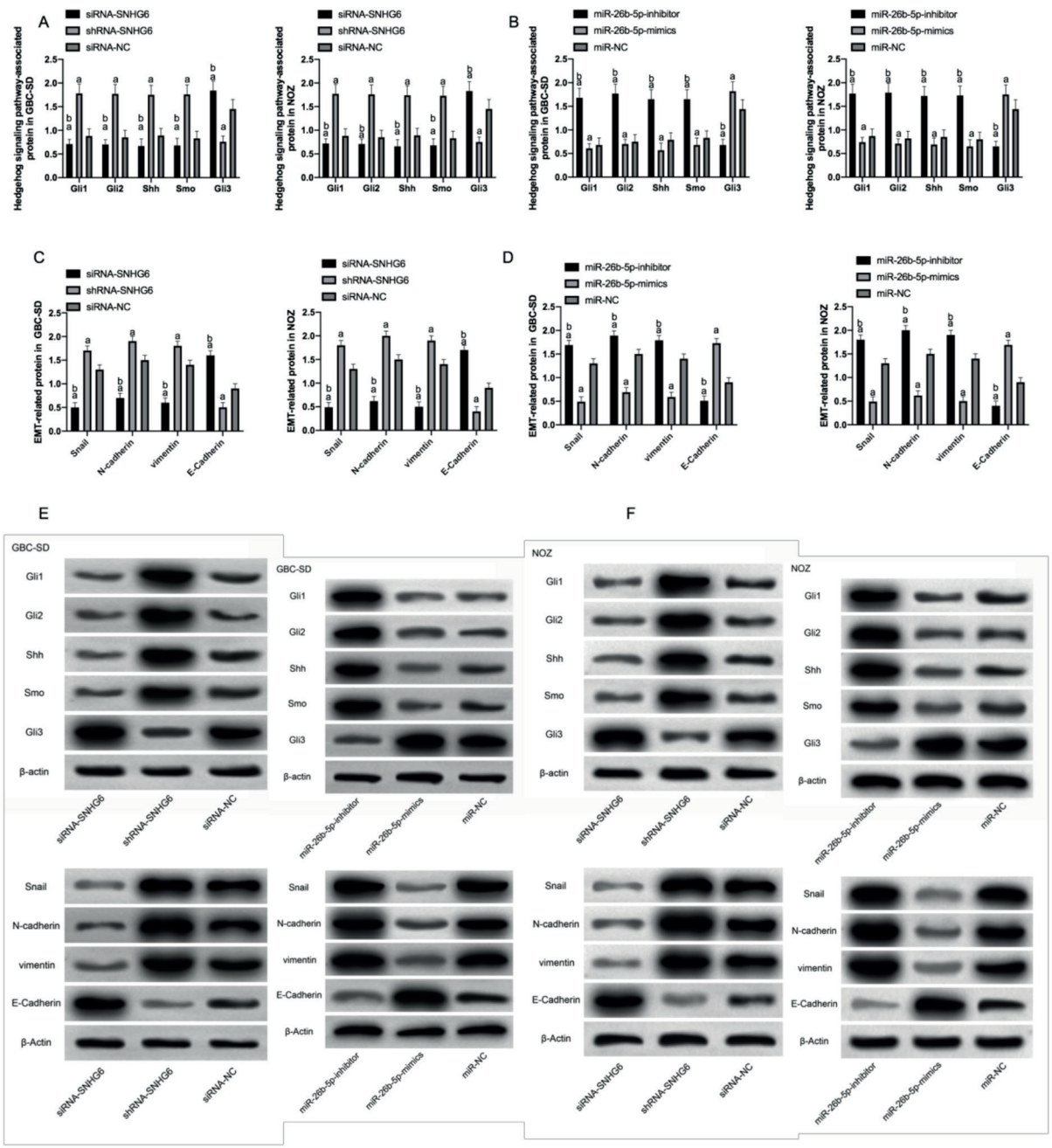

Figure 4. Effect of SNHG6 and miR-26b-5p expression on Hedgehog signaling pathway and EMT. **A,** Compared with siRNA-NC, the expression of Gli1, Gli2, Shh and Smo proteins in transfected Sh-SNHG6 cells was significantly up-regulated, and the expression of Gli3 was significantly down-regulated, while the expression of Gli1, Gli2, Shh and Smo proteins in transfected Si-SNHG6 cells was significantly down-regulated, and the expression of Gli3 was significantly up-regulated. **B,** Compared with miR-NC, the expressions of Gli1, Gli2, Shh and Smo proteins in transfected miR-26b-5p-inhibitor cells were significantly up-regulated and Gli3 was significantly downregulated, while the expressions of Gli1, Gli2, Shh and Smo proteins in transfected miR-26b-5p-mimics cells were significantly down-regulated and Gli3 was significantly up-regulated. **C,** Compared with siRNA-NC, the expression of N-cadherin, vimentin and Snail in transfected sh-SNHG6 cells was significantly upregulated, and the expression of E-Cadherin was significantly down-regulated, while the expression of N-cadherin, vimentin and Snail in transfected Si-SNHG6 cells was significantly down-regulated, and the expression of E-Cadherin was significantly up-regulated. **D,** Compared with miR-NC, the expression of N-cadherin, vimentin and Snail in transfected miR-26b-5p-inhibitor cells was significantly upregulated, and the expression of E-Cadherin was significantly down-regulated, while the expression of N-cadherin, vimentin and Snail in transfected miR-26b-5p-mimics cells was significantly downregulated, and the expression of E-Cadherin was significantly up-regulated. E, Western Blot map. **F,** Western Blot map. Note: a indicates that compared with NC group, $p < 0.001$; b indicates that compared with miR-26b-5p-mimics group, $p < 0.001$.

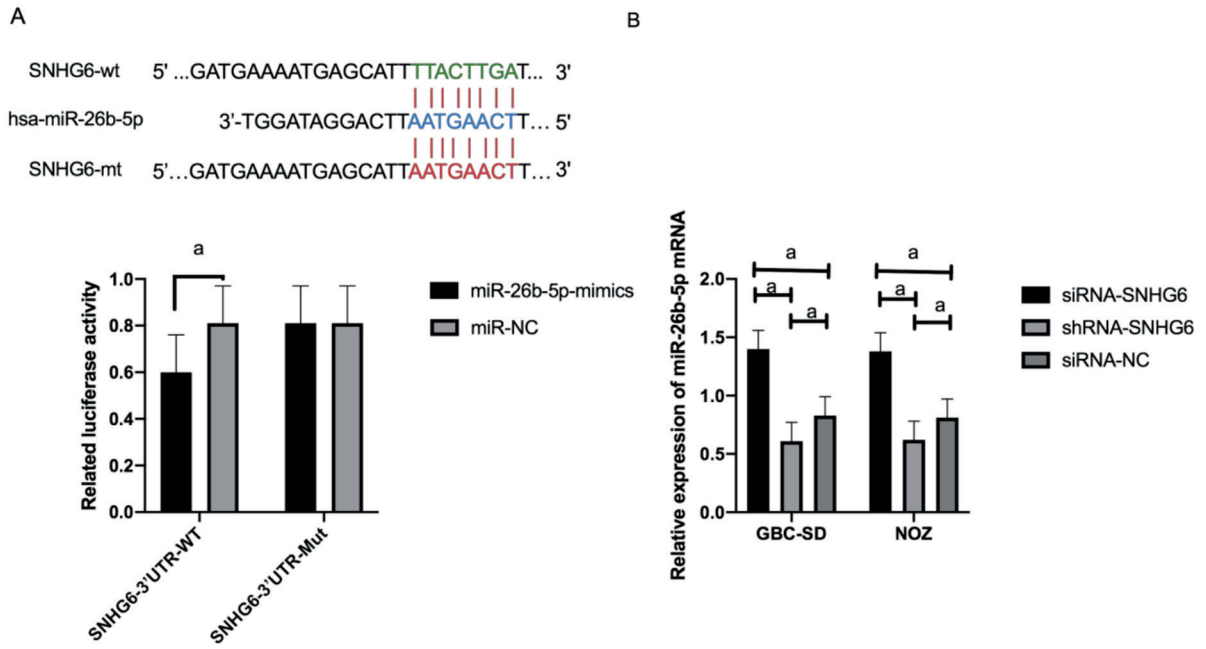

Figure 5. Detection of double Luciferase activity. **A,** There is a binding site between SNHG6 and miR-26b-5p (relative Luciferase activity-double Luciferase reporting test). **B,** The expression of miR-26b-5p in transfected GBC-SD and NOZ cells. Note: a indicates that $p < 0.001$.

Discussion

Some studies showed that LncRNA is related to various tumors. LncRNA has effects in carcinogenesis and tumor progression. LncRNA expression affects the growth and decline of tumor cells[17]. LncRNA shows a unique expression state in different cancer types. Related cellular biology studies have found that cytoplasmic miRNA is affected by LncRNA, which in turn regulates cell signaling function[18]. Scholars[19,20] have confirmed that miR-26b-5p in the serum or tissue cells of breast cancer and other patients is downregulated, and the changes of miR-26b-5p can affect the EMT phenomenon of cancer cells, but the specific mechanism has not been elaborated in detail. We tend to study SNHG6 on EMT and biological function of gallbladder cancer cells by regulating miR-26b-5p mediated Hedgehog signaling pathway, providing a new theoretical basis for gallbladder cancer in molecular biology.

In this study, qRT-PCR technology was applied for exploration of SNHG6 in the serum of gallbladder cancer patients and normal people and found that SNHG6 was abnormally increased in gallbladder cancer patients. We further found that the high expression of SNHG6 was correlated with the age, sex, tumor invasion, tumor location, differentiation degree, and TNM stage of gallbladder cancer patients through correlation analysis of the clinical and pathological characteristics of patients, and the AUC of SNHG6 was found to be more than 0.8 by visualizing ROC curve. At present, reports[21,22] have shown that LncRNA can target downstream gene pairs to regulate cell biological functions. We further found the targeted correlation of SNHG6 with miR-26b-5p through TargetScan database. The clinical and pathological features of miR-26b-5p and gallbladder cancer patients were also analyzed. The low miR-26b-5p has a correlation with the age, sex, tumor invasion, tumor location, differentiation degree, and TNM staging of gallbladder cancer patients. The AUC found by ROC curve was more than 0.8. MiR-26b-5p participates in the change process of biological functions of various tumor cells and has an important regulatory effect in the process of tumor development and differentiation[23]. SNHG6 can regulate the gene and affect the cell cycle of gallbladder cancer[24]. In this research, We detected the expression of SNHG6 in The Cancer Genome Atlas (TCGA) database and in gallbladder cancer cells in this research. The results indicated that SNHG6 was high and miR-26b-5p was

low in gallbladder cancer cells. This is consistent with the results of this study, which suggests that SNHG6 and miR-26b-5p may play regulatory roles in gallbladder cancer.

In cell experiments, we compared human gallbladder carcinoma cell lines GBC-SD and NOZ with HIBEpiC normal human intrahepatic bile duct epithelial cells and found that SNHG6 was high and miR-26b-5p was low in gallbladder carcinoma cell lines. Subsequently, we upregulated and downregulated SNHG6 and miR-26b-5p in GBC-SD and NOZ cells. We further transfected sh-SNHG6, si-SNHG6, miR-26b-5p-mimics, miR-26b-5p-inhibitor sequences into GBC-SD and NOZ to observe the discovery of cell biological functions. The results showed that SNHG6 and the proliferation and invasion ability of cells after miR-26b-5p over-expression are signficantly inhibited, and N-cadherin, vimentin, and Snail in the EMT process are significantly reduced, and E-cadherin is significantly increased, but GBC-SD and NOZ cells transfected with miR-26b-5p-mimics and Si-SNHG6 are decreased. The occurrence and development of tumor have a close relationship with EMT. The occurrence of EMT will promote the biological function changes of tumor cells. This suggests that miR-26b-5p can be applied as a latent target for gallbladder cancer. Inhibition of SNHG6 expression can inhibit the proliferation, invasion and EMT of gallbladder cancer cells, and inhibition of SNHG6 can significantly inhibit the formation and growth of tumors through *invitro* nude mouse tumorigenesis experiments, which further indicates the influ-

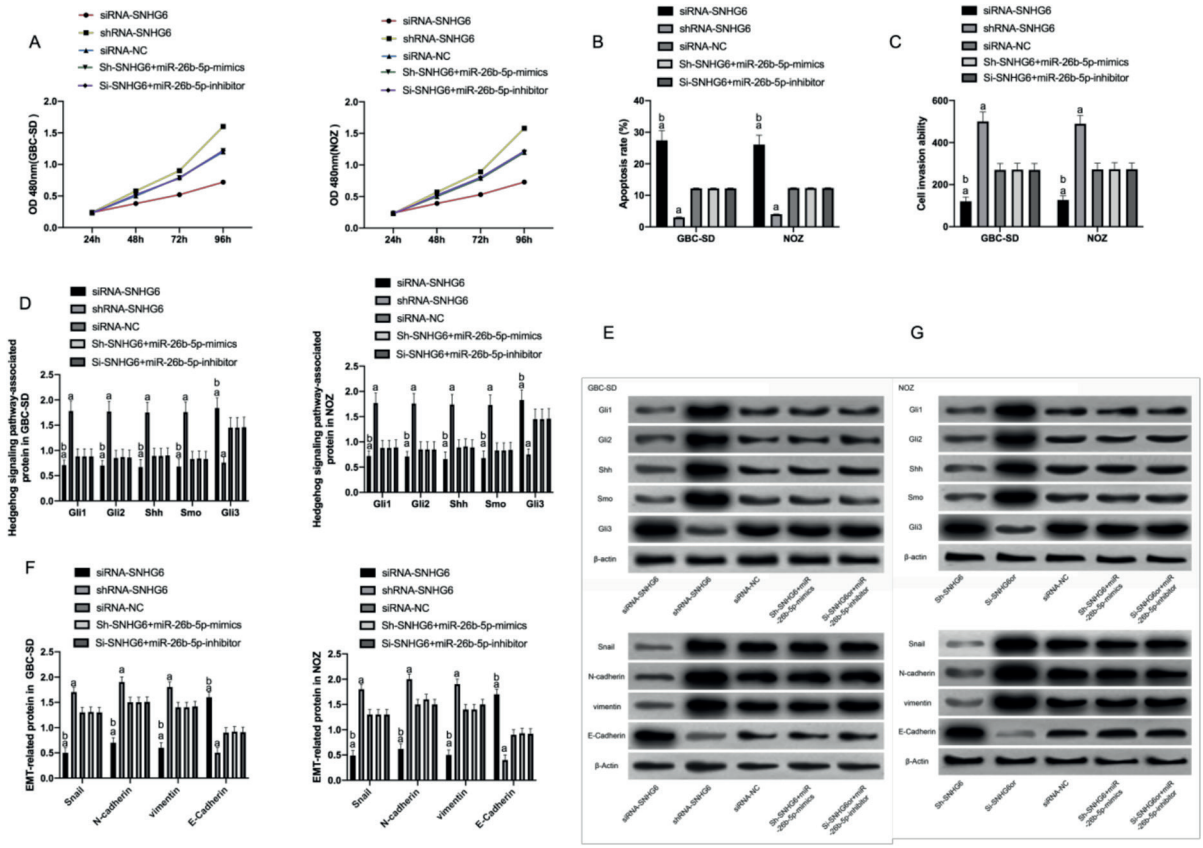

Figure 6. Rescue experiment. **A,** Cell proliferation ability after transfection of Sh-SNHG6+miR-26b-5p-mimics or Si-SNHG6+miR-26b-5p-inhibitor. **B,** Cell invasion capability after transfection of Sh-SNHG6+miR-26b-5p-mimics or Si-SNHG6+miR-26b-5p-inhibitor. **C,** Cell migration ability after transfection of Sh-SNHG6+miR-26b-5p-mimics or Si-SNHG6+miR-26b-5p-inhibitor. **D,** The expression of Gli1, Gli2, Shh, Smo and Gli3 proteins after transfection of sh-snhg6+mir-26b-5p-inhibitor. **E,** Western Blot map. **F,** The expression of E-cadherin, N-cadherin, vimentin, Snail and Snail proteins after transfection of Sh-SNHG6+miR-26b-5p-mimics or si-snhg6 or+mir-26b-5p-inhibitor. **G,** Western Blot map. Note: a indicates that compared with siRNA-NC, Sh-SNHG6+miR-26b-5p-mimics and Si-SNHG6+miR-26b-5p-inhibitor group, $p < 0.001$; b indicates that compared with Si-SNHG6, $p < 0.001$.

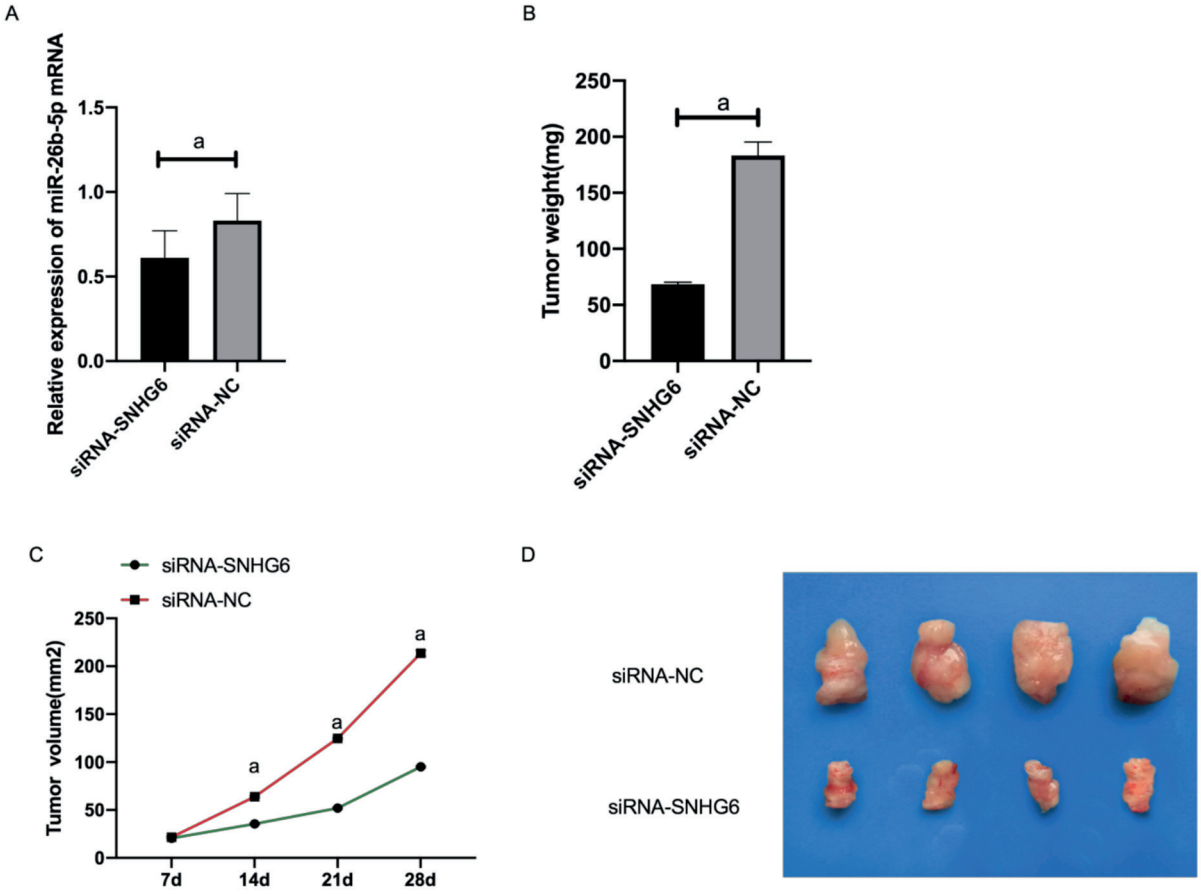

Figure 7. Effect of inhibition of SNHG6 expression on tumor growth in nude mice. **A,** After comparing the two groups, the expression of SNHG6 in Si-SNHG6 tumor tissue was lower. **B,** After comparing the two groups, the tumor weight of Si-SNHG6 group was significantly smaller. **C,** After comparing the two groups, the growth rate of tumor in Si-SNHG6 group was significantly slower. **D,** After comparing the two groups, the tumor size of Si-SNHG6 group was significantly smaller. Note: a indicates that $p < 0.001$.

ence of changes in SNHG6 on gallbladder cancer. However, it is not clear how SNHG6 influences the biological function of gallbladder cancer cells and EMT.

Takebe et al[25] indicated that the activation of Hedgehog signaling pathway has an effect in the EMT process of gallbladder cancer cells. In recent years, related researches have found that SNHG6 can activate TGF-β/Smad signaling pathway by targeting UPF1, thus promoting the development of colon cancer[15]. In this study, we observed Hedgehog signaling pathway-related proteins by inhibiting or overexpressing SNHG6 and miR-26b-5p in gallbladder cancer cells. The above data indicated that Gli1, Gli2, Shh, Smo, N-cadherin, vimentin and Snail proteins in cells with low SNHG6 expression or high miR-26b-5p expression were downregulated, while E-Cadherin and Gli3 were upregulated, whereas the results of high miR-26b-5p expression or low SNHG6 expression were downregulated, suggesting that SNHG6 can inhibit the activation of Hedgehog signaling pathway and the EMT of cells by regulating miR-26b-5p. There are also studies showing that the activation of Hedgehog signaling pathway can inhibit EMT and promote proliferation of pancreatic cancer and medulloblastoma cells[26,27].

At the end of the study, we found through rescue experiments that after co-transfecting miR-26b-5p-mimics+sh-SNHG6, miR-26b-5p-inhibitor+si-SNHG6 into GBC-SD, and NOZ, the biological function changes of the cells were not different from those of the miR-NC group, while compared with si-SNHG6, the proliferation, invasion, and migration abilities were en-

hanced, while sh-SNHG6 was decreased. This suggests that SNHG6 can target miR-26b-5p. Therefore, we verified the correlation between miR-19a-3 with SNHG6 by Dual-Luciferase report. The results showed that the activity of SNHG6-3'UT Wt Luciferase increased significantly after miR-26b-5p overexpression, but it had no effect on the activity of SNHG6-3'UTR Mut Luciferase. Moreover, miR-26b-5p is significantly increased in transfected si-SNHG6, which suggests that there is a targeted regulatory relationship between SNHG6 and miR-26b-5p. Therefore, we suggest that inhibiting SNHG6 expression can promote miR-26b-5p expression, thus affecting the biological function of gallbladder carcinoma cells.

In this study, we demonstrated that SNHG6 is highly expressed in gallbladder cancer and can regulate signal-related protein changes in the Hedgehog signaling pathway by inhibiting miR-26b-5p-mediated cell proliferation. However, in this study, there are still some limitations. The regulatory network of SNHG6 is still unclear, and the occurrence and development of tumors need further exploration through other ways. Therefore, we hope to explore SNHG6 regulatory network through bioinformatics analysis in future research, providing more basis for our experiments.

Conclusions

To sum up, SNHG6 can regulate the growth and apoptosis of gallbladder cancer cells by regulating miR-26b-5p and inhibiting Hedgehog signaling pathway, so SNHG6 is hoped to be a latent clinical therapeutic target for gallbladder carcinoma. Moreover, SNHG6 may be a useful marker for diagnosis and prognosis evaluation of the disease.

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