European Review for Medical and Pharmacological Sciences 2019; 23: 2768-2776

LncRNA SNHG1 promotes liver cancer development through inhibiting p53 expression via binding to DNMT1

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Abstract. – OBJECTIVE: This study aims to investigate whether long non-coding RNA (lncRNA) SNHG1 could regulate proliferative and invasive abilities of liver cancer (LC) cells via p53 and DNMT1, so as to regulate the occurrence and progression of LC.

PATIENTS AND METHODS: SNHG1 expression in LC tissues and paracancerous tissues was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Correlation between SNHG1 expression and tumor stage of LC patients was analyzed. The regulatory effects of SNHG1 and p53 on proliferative, invasive capacities and cell cycle were accessed by CCK-8 (cell counting kit-8), transwell assay and flow cytometry, respectively. The binding condition between SNHG1 and DNMT1 was determined by RNA binding protein immunoprecipitation (RIP) and chromatin immunoprecipitation (ChIP). Western blot was conducted to determine whether SNHG1 could regulate p53 in LC cells. Finally, rescue experiments were carried out to evaluate whether SNHG1 regulates proliferative and invasive abilities of LC cells through p53.

RESULTS: SNHG1 expression was higher in LC tissues than that of paracancerous tissues. LC patients with stage III-IV presented higher expression level of SNHG1 than those with stage I-II. Similarly, SNHG1 was highly expressed in LC cells than that of normal liver cells. LC cell lines SMMC-7721 and SK-HEP-1 were selected for this study. SNHG1 knockdown inhibited the proliferative and invasive abilities, and arrested the cell cycle in the G0/G1 phase of SMMC-7721 and SK-HEP-1 cells. RIP and ChIP results demonstrated that SNHG1 could bind to DNMT1 and inhibit p53 expression. Overexpression of p53 partially reversed the inhibitory effects of SNHG1 on proliferative and invasive abilities of LC cells.

CONCLUSIONS: High expression of SNHG1 could promote proliferative and invasive abilities of LC cells through targeting inhibition of p53 expression by binding to DNMT1.

Key Words:
SNHG1, Liver cancer (LC), Proliferation, Invasion.

Introduction

Liver cancer (LC) is the third major fatal cancer in the world, with 80% of LC cases occurring in East Asia and sub-Saharan Africa[1]. Although diagnostic and treatment approaches have been greatly advanced, the 5-year survival rate of LC is still only 7%. The mechanisms of LC pathogenesis have not been fully elucidated[2,3]. LC results from multiple pathogenic factors[4]. Currently, it is believed that oncogene activation and tumor-suppressor gene inactivation lead to LC development[5,6]. Hence, explorations on LC pathogenesis contribute to develop novel treatment strategies for LC.

P53 gene is located at 17p13.1 with about 20 kb in length. It consists of 11 exons and 10 introns, encoding 393 amino acids[7]. P53 mediates the normal cell growth that exerts an important regulatory role in the proliferation, differentiation, senescence, and deterioration of cells[8]. P53 inhibits the activities Bcl-2, survivin, IGFR, Mcl-1, and PIK3CA at the transcriptional level, leading to cell growth arrest. Previous studies have shown that mutant p53 can form a complex with wild-

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type p53, which greatly reduces its physiological regulatory function. Key factors in p53 pathway are dysregulated, including p21, WAF/CIP1, and MDM2, thus resulting in cell transformation and tumorigenesis. P53 mainly functions as a cell killer, while it could be served as a lifeguard under some circumstances[9,10].

Long non-coding RNA (lncRNA) is a type of RNA molecule with a transcript of over 200 nt in length, which is distributed in the nucleus or cytoplasm[11]. LncRNA function is multidimensional[12]. Highly converted sequences and stable cleavage sites allow lncRNA to regulate DNA replication, chromosomal modification at multiple levels. LncRNA also serves as a key regulator in various biological processes, which is well concerned in mediating tumor development[13-15].

SNHG1 (small nucleolar RNA host gene 1) is a potential therapeutic target in various tumors such as esophageal cancer, glioma, and prostate cancer. It is reported that SNHG1 could accelerate proliferation and inhibit apoptosis of tumor cells[16-18]. SNHG1 is a predictive hallmark in liver cancer and utilized as a therapeutic target[19]. However, the specific mechanism of SNHG1 remains to be further investigated.

Patients and Methods

Sample Collection

74 cases of LC tissues and paracancerous tissues surgically resected from July 2016 to September 2017 in The Affiliated Yantai Yuhuangding Hospital of Qingdao University were collected and preserved in liquid nitrogen. LC patients did not receive preoperative treatments and were pathologically confirmed without any family history. Informed consent was signed before the study. The Affiliated Yantai Yuhuangding Hospital of Qingdao University Ethics Committee approved this study.

Cell Culture and Transfection

Normal liver cell line (THLE-2) and LC cell lines (HepG, PLC, SMMC-7721, and SK-HEP-1) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium) containing 10% FBS (fetal bovine serum), 100 U/mL penicillin and 100 μg/mL streptomycin (Hyclone, South Logan, UT, USA). Cells were incubated in a 5% CO_2 incubator at 37°C. Cell passage was performed using trypsin until 80%-90% of confluence.

One day prior to cell transfection, LC cells in good growth condition were seeded into 6-well plates at a density of 3×10^5 per well. Cells were transfected with corresponding plasmids when the confluence was up to 50%, following the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Culture medium was replaced 4 h later. Transfection plasmids were constructed by GenePharma (Shanghai, China).

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA in treated cells was extracted using TRIzol method (Invitrogen, Carlsbad, CA, USA) for reverse transcription according to the instructions of PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). RNA concentration was detected using a spectrometer and those samples with A260/A280 ratio of 1.8-2.0 were selected for the following qRT-PCR reaction. QRT-PCR was then performed based on the instructions of SYBR Premix Ex Taq TM (TaKaRa, Otsu, Shiga, Japan). The relative gene expression was calculated using the $2^{-\Delta Ct}$ method. Primers used in the study were as follows: SNHG1, F: 5'-TAACCTGCTTGGCTCAAAGGG-3', R: 5'-CAGCCTGGAGTGAACACAGA-3'; p15, F: 5'-CGGCGGTCAACCTGGAGGACTCC-3', R: 5'-CCAGTGCAGGGTCCGAGGTAT-3'; p21, F: 5'-CGACGCGTCGTTGTAATAAAGCCTCCAG-3', R: 5'-GACTAGTCGTTTTCAT TTCAATCGTAG-3'; p27, F: 5'-TGGAAGACTAGTGATTTTGTTGT-3', R: 5'-TACTGGCACCACTGGAAACC-3'; p53, F: 5'-GGACTCTGCCCTGCCACCATTTA-3', R: 5'-CTTGTGCCCTGTGAGGTCGTTGA-3'; DNMT1, F: 5'-TGGGAACTATATCTCTCGCTTGC-3', R: 5'-GGGTGAGACAGAACCGTCT-3'; GAPDH, F: 5'-AGCCACATCGCTCAGACAC-3', R: 5'-GCCCAATACGACCAAATCC-3'.

CCK-8 (Cell Counting Kit-8) Assay

LC cells were seeded into 96-well plates at a density of 1×10^6/mL. 10 μL of the CCK-8 solution (cell counting kit-8, Dojindo, Kumamoto, Japan) was added in each well. 4 hours later, fresh medium was replaced and cells were incubated for 1 h. The absorbance at 450 nm of each sample was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

Transwell Assay

Matrigel dose was adjusted to 1 mg/mL with pre-cooled serum-free DMEM. Transwell chamber was pre-coated with diluted Matrigel 3-5 h prior to the assay. LC cells were adjusted to 2×10^5/ml, and 100 μL of cell suspension was added in the upper chamber. 600 μL of DMEM containing 10% FBS was added in the lower chamber. After cell culture for 24 h, cells were fixed and stained with crystal violet for 15-30 min. Invasive cells were observed and captured using a microscope.

Cell Cycle Detection

LC cells were diluted to 1×10^5/mL and fixed with 75% ethanol at -4°C overnight. After PBS wash twice, cells were incubated with 100 μL of RNaseA at 37°C water bath in the dark. Subsequently, cells were incubated with 400 μL of Propidium Iodide (PI) at 4°C in the dark. 30 minutes later, cell cycle was detected using flow cytometry at the wavelength of 488 nm.

Western Blot

Cells were lysed for protein extraction. The concentration of each protein sample was determined by a BCA (bicinchoninic acid) kit (Abcam, Cambridge, MA, USA). The protein sample was separated by gel electrophoresis and transferred to PVDF (polyvinylidene difluoride) membranes (Roche, Basel, Switzerland). After incubation with primary and secondary antibody, immunoreactive bands were exposed by enhanced chemiluminescence (ECL) method.

Chromatin Fractionation

Cells were fully lysed in 200 μL of Lysis Buffer J. After centrifugation, the supernatant contained cytoplasmic RNA. The remaining was centrifuged again and the supernatant containing nuclear RNA was collected. Buffer SK and absolute ethyl alcohol were added to the supernatant containing cytoplasmic or nuclear RNA, respectively. Finally, cytoplasmic and nuclear RNA were obtained by column centrifugation.

RNA Binding Protein Immunoprecipitation (RIP)

Cells were washed and cross-linked with 0.01% formaldehyde for 15 min. After centrifugation and cell lysis, cell extracts were incubated with RIP buffer containing protein A/G magnetic beads coated with anti-Ago2 or negative control anti-IgG antibody. After overnight incubation at 4°C, cells were incubated with Protein A Agarose for 1 h at 4°C, followed by the isolation and quantification of RNA.

Chromatin Immunoprecipitation (ChIP)

The cells were fixed in 1% formaldehyde for 10 min. Anti-RNA polymerase, normal mouse IgG, and BCL6 antibodies were immunoprecipitated overnight at 4°C. Protein G agarose (Cell Signaling Technology, Danvers, MA, USA) was added to collect immune complexes. The beads were resuspended in elution buffer and incubated overnight at 65°C before DNA was extracted. The DNA was purified using a spin column and quantified using qRT-PCR.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 statistical software (IBM, Armonk, NY, USA) was used for data analysis. GraphPad Prism 5 (La Jolla, CA, USA) was used for figure editing. Data were expressed as mean ± standard deviation ($\bar{x}\pm s$). Measurement data were compared using the *t*-test. $p<0.05$ considered the difference was statistically significant.

Results

SNHG1 Was Highly Expressed in LC

QRT-PCR results showed that SNHG1 was highly expressed in LC tissues than that of paracancerous tissues (Figure 1A). Besides, patients with advanced LC presented higher expression of SNHG1 than those with early-stage LC (Figure 1B). Similarly, SNHG1 was also highly expressed in LC cells than that of controls, especially in SMMC-7721 and SK-HEP-1 cells (Figure 1C). We first constructed two lines of si-SNHG1 and tested their transfection efficacies in LC cells. QRT-PCR data showed that si-SNHG1#1 presents a better transfection efficacy, which was selected for the following experiments (Figure 1D).

SNHG1 Knockdown Inhibited Proliferative and Invasive Abilities, but Induced Cell Cycle Arrest in LC

Decreased proliferative ability in LC cells was observed after the SNHG1 knockdown in SMMC-7721 and SK-HEP-1 cells (Figure 2A). Flow cytometry data indicated that SNHG1 knockdown in SMMC-7721 and SK-HEP-1 cells arrests the cell cycle in the G0/G1 phase (Figure 2B). Meanwhile, inhibited invasive ability was shown after

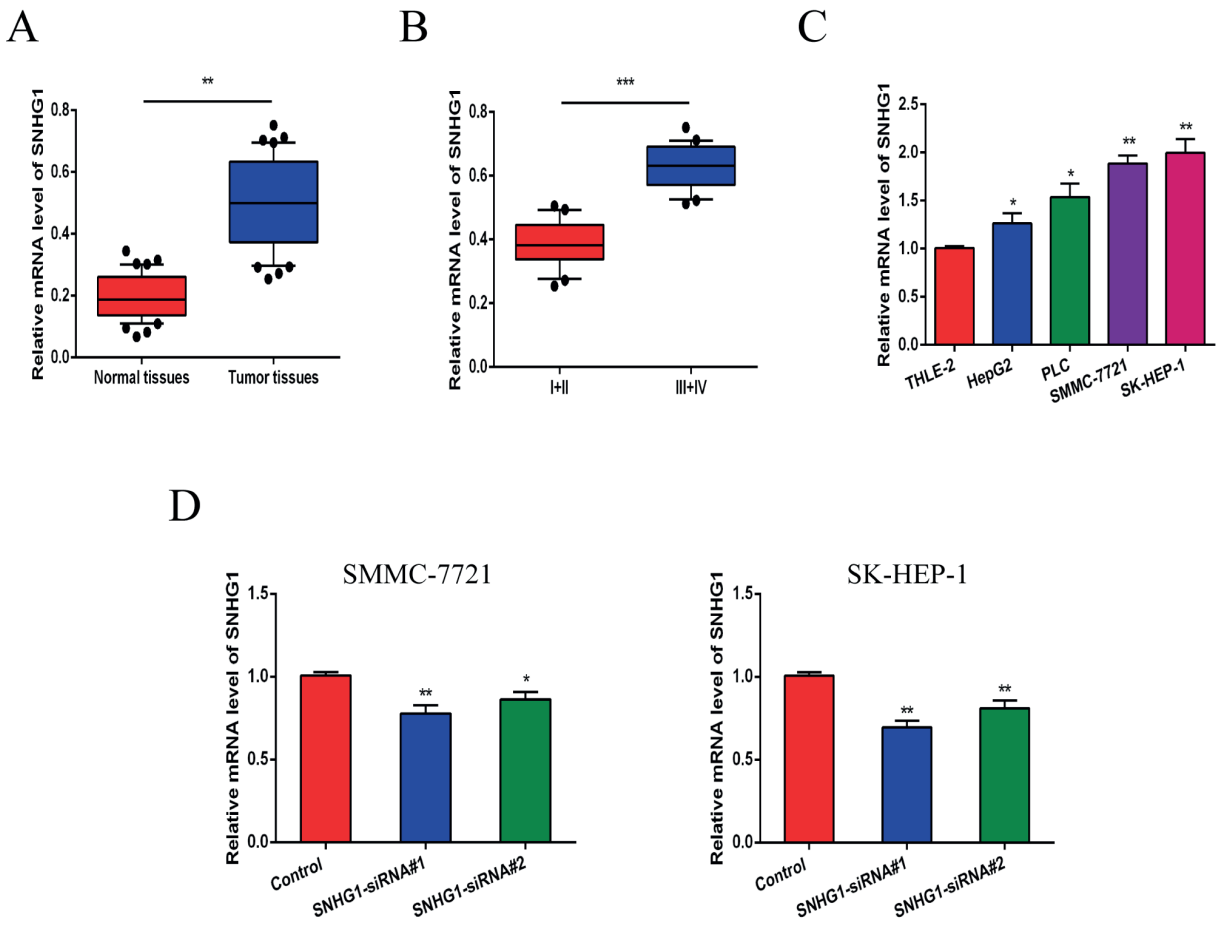

Figure 1. SNHG1 was highly expressed in LC. ***A,*** SNHG1 was highly expressed in LC tissues than that of paracancerous tissues. ***B,*** SNHG1 expression was higher in advanced LC patients than those with early-stage LC. ***C,*** SNHG1 was highly expressed in LC cell lines than that of normal liver cell line. ***D,*** Transfection efficacy of si-SNHG1 in SMMC-7721 and SK-HEP-1 cells.

the SNHG1 knockdown in SMMC-7721 and SK-HEP-1 cells (Figure 2C).

SNHG1 Inhibited p53 Expression by Binding to DNMT1

The mRNA levels of oncogenes p15, p21, p27, and p53 in SMMC-7721 and SK-HEP-1 cells were upregulated after SNHG1 knockdown (Figure 3A). Western blot results found that protein expression of p53 increased in LC cells after transfection of si-SNHG1 (Figure 3B). To further explore the regulatory mechanism of SNHG1 in regulating LC development, subcellular localization of SNHG1 in SMMC-7721 cells was determined. It is shown that SNHG1 is mainly distributed in the nucleus of SMMC-7721 cells, indicating that SNHG1 may exert its regulatory function at the transcriptional level (Figure 3C). Previous studies have found that DNMT1 can inhibit p53 expression by binding to its promoter region. We hypothesized that SNHG1 can bind to DNMT1 and stabilize DNMT1 expression, so as to downregulate p53 expression. RIP and ChIP were then conducted in SMMC-7721 cells. RIP results demonstrated that SNHG1 binds to DNMT1 (Figure 3D). ChIP results elucidated that DNMT1 binds to DNAs in the p53 promoter region (Figure 3E). Furthermore, after knocking down SNHG1 in SMMC-7721 cells, we found that the binding level of DNMT1 and p53 promoter was downregulated (Figure 3F). After interfering with DNMT1 in SMMC-7721 cells, DNMT1 expression was significantly downregulated (Figure 3G) and p53 expression significantly increased (Figure 3H). The above experimental results illustrated that SNHG1 can inhibit p53 expression by binding to DNMT1.

P53 Reversed the Anti-tumor Effect of SNHG1 on LC

After transfection of pcDNA-p53 in SMMC-7721 and SK-HEP-1 cells, both mRNA and protein levels of p53 were significantly upregulated (Figure 4A and 4B). Cell proliferative ability was significantly enhanced after overexpression of SNHG1, which was reversed by overexpression of p53 (Figure 4C). The promoted cell cycle and invasive ability of LC cells by SNHG1 overexpression were both reversed by p53 overexpression (Figure 4D and 4E).

Discussion

LC is the third fatal malignancy of the digestive system second only to gastric cancer and esophageal cancer. The epigenetic modification exerts a vital role in the pathogenesis of LC[20]. LncRNAs are a class of non-coding RNAs without open reading frame[21]. In recent years, with the rapid development of high-throughput sequencing, large-scale lncRNA cDNA library and gene chip technology, lncRNA functions have been well concerned[22,23]. It is believed that lncRNAs

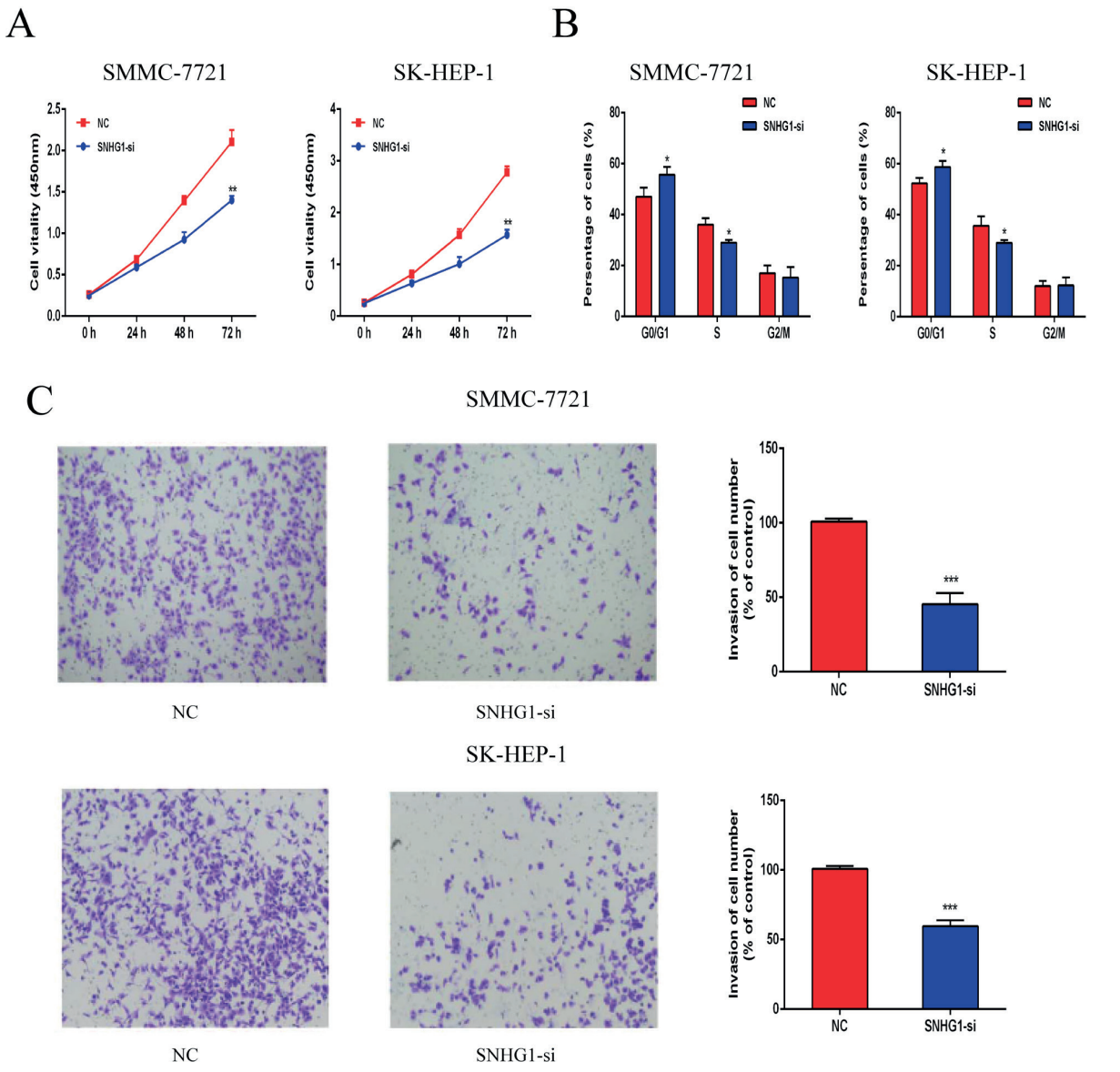

Figure 2. SNHG1 knockdown inhibited proliferative and invasive abilities, but induced cell cycle arrest in LC. ***A,*** Cell proliferation decreased after SNHG1 knockdown in SMMC-7721 and SK-HEP-1 cells. ***B,*** LC cells were arrested in G0/G1 phase after SNHG1 knockdown. ***C,*** Cell invasion decreased after SNHG1 knockdown in SMMC-7721 and SK-HEP-1 cells.

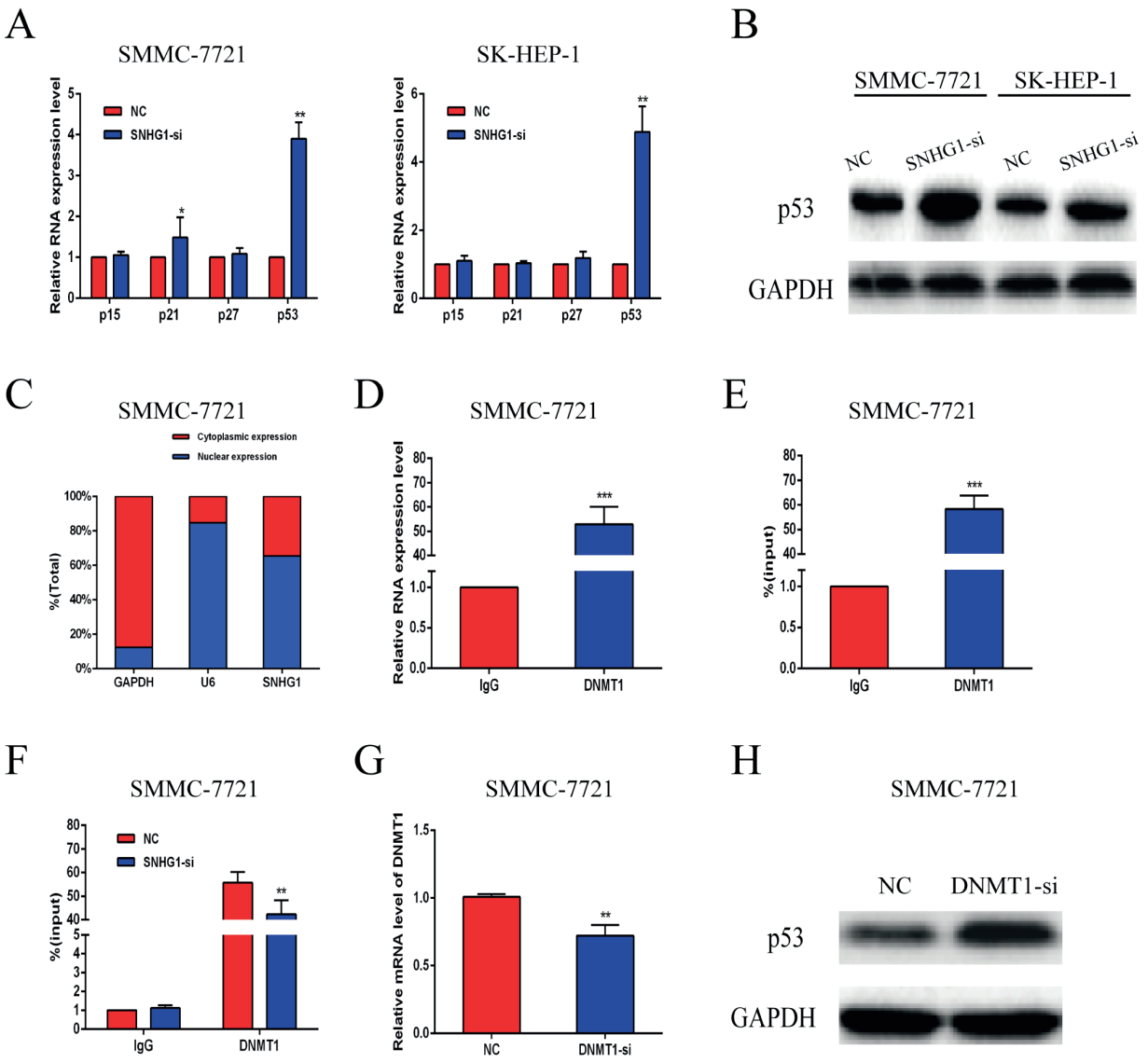

Figure 3. SNHG1 inhibited p53 expression by binding to DNMT1. ***A,*** SNHG1 knockdown upregulated mRNA expression of p53 in SMMC-7721 and SK-HEP-1 cells. ***B,*** SNHG1 knockdown upregulated protein expression of p53 in SMMC-7721 and SK-HEP-1 cells. ***C,*** SNHG1 was mainly distributed in nucleus of SMMC-7721 cells. ***D,*** RIP results demonstrated that SNHG1 binds to DNMT1. ***E,*** ChIP results elucidated that DNMT1 binds to DNAs in the p53 promoter region. ***F,*** Knockdown of SNHG1 in SMMC-7721 cells downregulated the binding level of DNMT1 and p53 promoter. ***G,*** After interfering with DNMT1 in SMMC-7721 cells, DNMT1 expression was significantly downregulated. ***H,*** After interfering with DNMT1 in SMMC-7721 cells, p53 expression significantly increased.

are involved in the occurrence, progression, and metastasis of tumors.

Some studies have identified the role of lncRNA in the disease progression of LC. Many lncRNAs are differentially expressed in LC patients, which may serve as tumor hallmarks. For example, high expression of H19 may lead to LC development by interaction with angiopoietins and fibroblast growth factors. The expression level of H19 in LC patients is higher than that of alpha-fetoprotein (AFP). As a result, the combined detection of H19 with AFP may improve the diagnosis of early-stage LC[24]. HOTAIR expression is related to the recurrence and the survival time of LC patients. HOTAIR knockdown in LC cells decreases cell invasive and increases sensitive to cisplatin and doxorubicin[25]. MALAT1 knockdown promotes proliferative and invasive capacities of HepG2 cells at the transcriptional and post-transcriptional levels[26].

Immunohistochemical methods are difficult to detect wild-type p53 due to its short half-life. On the contrary, mutant-type p53 has a longer half-life (2-12 h) and could be accumulated in cells. Previous researches have pointed out that p53 detection by immunohistochemistry can better reflect the level of mutant-type p53[27]. The mutated p53 can form an oligomeric protein complex with the wild-type p53, which reduces the ability of wild-type p53 to bind to DNA, thereby preventing the transcription of genes at these binding sites. Cell growth and reproduction are then out of control, further leading to cell transformation and canceration. The mutation of codon 249 in

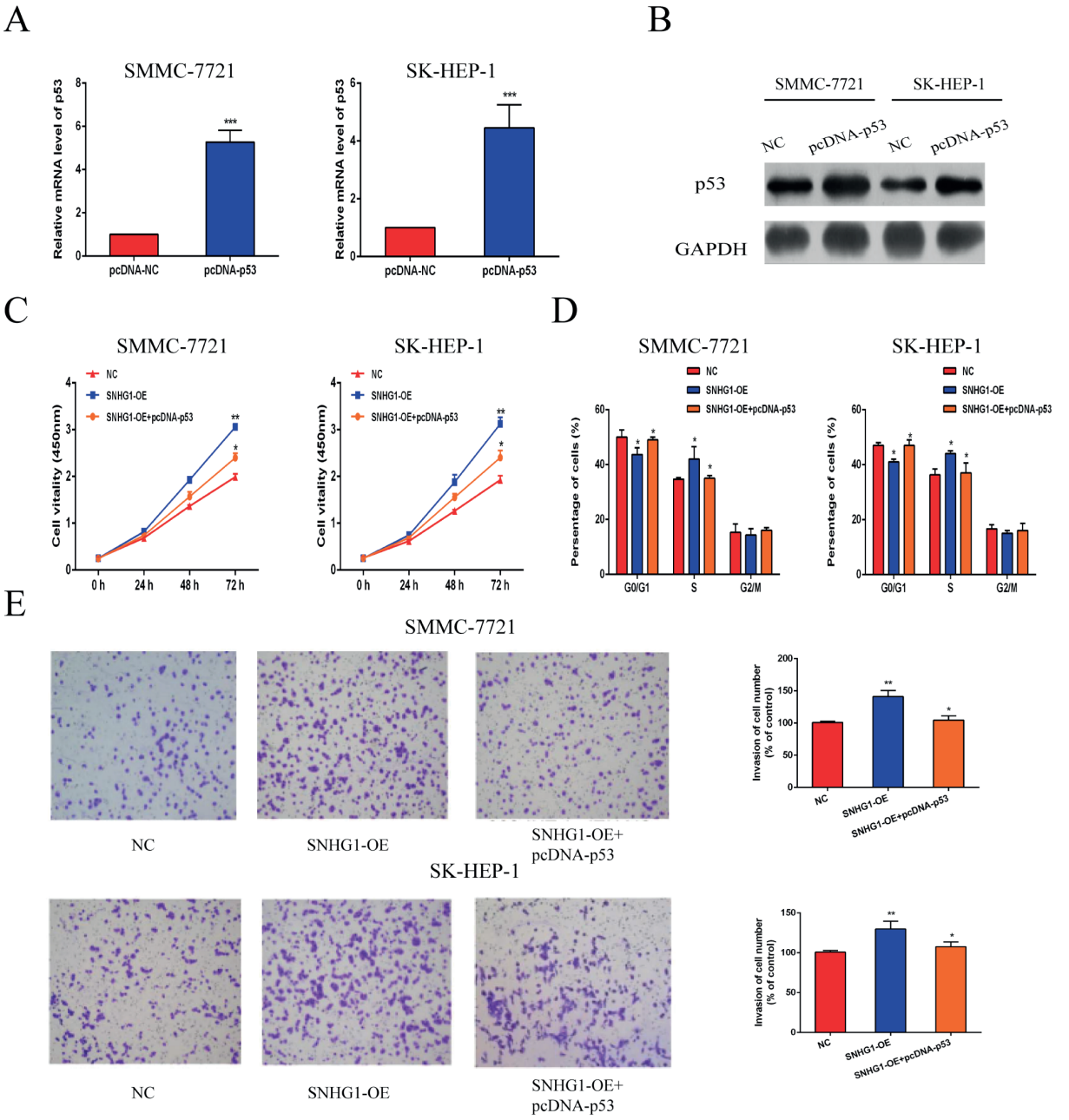

Figure 4. P53 reversed the anti-tumor effect of SNHG1 on LC. ***A-B,*** After transfection of pcDNA-p53 in SMMC-7721 and SK-HEP-1 cells, both mRNA (***A***) and protein (***B***) levels of p53 were significantly upregulated. ***C,*** Cell proliferative ability was significantly enhanced after overexpression of SNHG1, which was reversed by overexpression of p53. ***D,*** Cell cycle was significantly enhanced after overexpression of SNHG1, which was reversed by overexpression of p53. ***E,*** Cell invasive ability was significantly enhanced after overexpression of SNHG1, which was reversed by overexpression of p53.

p53 gene caused by aflatoxin and hepatitis B virus is considered to be an intermediate link in LC tumorigenesis[28].

In the present study, we found that SNHG1 is highly expressed in LC tissues and cells. SNHG1 promoted the proliferative and invasive abilities of MMC-7721 and SK-HEP-1 cells by inhibiting p53 expression. It is speculated that p53 might be a target gene of SNHG1.

Accumulating evidence has proved that lncRNA can form a regulatory network with transcription factors to participate in target gene regulation. RIP experiments elucidated that DNMT1 binds to SNHG1, and SNHG1 stabilizes DNMT1 expression. DNMT1 is the most important methyltransferase in the human body, maintaining the methyl group of the newly synthesized DNA[29]. In tumor cells, high methylation of tumor-suppressor genes and abnormal cell proliferation and differentiation are associated with increased DNMT1 activity[30]. Subsequently, ChIP verified that DNMT1 can bind to the p53 promoter region and inhibit its expression. SNHG1 can downregulate p53 expression by binding to DNMT1.

Conclusions

We provided evidence that high expression of SNHG1 promoted proliferative and invasive abilities of LC cells through targeting inhibition of p53 expression by binding to DNMT1.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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