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Long non-coding RNA (lncRNA) small nucleolar RNA host gene 1 (SNHG1) promote cell proliferation in colorectal cancer by affecting P53

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Abstract. – OBJECTIVE: Colorectal cancer (CRC) is one of the most frequent malignant tumors worldwide. The connection between lncRNAs expression and CRC development has not been well identified in the recent literature. This study focuses on the role of lncRNA-SNHG1 on CRC progression and development. The quantitative Real-time PCR (qRT-PCR) assay was conducted to identify the expression level of small nucleolar RNA host gene 1 (SNHG1).

PATIENTS AND METHODS: Cell proliferation and viability were examined by 3-(4,5)-dimethylthiazol(-z-y1)-3,5-diphenyl tetrazoliumbromide (MTT assay) and colony formation assay. Cell apoptosis and cell cycle distribution were detected by flow cytometry.

RESULTS: Expressions of p53, p21, BAX were assessed by Western blotting. CRC cells transfected with lncRNA-shRNA were injected into nude mice to identify the role of SNHG1 on tumorigenesis *in vivo*. SNHG1 expression level was elevated in CRC tissues when compared to adjacent tissues (n=86). SNHG1 knockdown significantly suppressed cell proliferation and viability, while SNHG1 overexpression had the opposite effect. Decreased SNHG1 expression enhanced cell apoptosis and triggered cell cycle arrest in G0/G1 phase, while elevated SNHG1 expression done the opposite. Besides, downregulation of SNHG1 impeded tumorigenesis *in vivo*. Protein levels of p53 and p53 target genes were affected by SNHG1 *in vitro*.

CONCLUSIONS: Our research demonstrated that SNHG1 may participate in controlling CRC proliferation, viability, and apoptosis via modulating p53 partially, which provides potential therapeutic targets for CRC.

Key Words:
LncRNA, SNHG1, CRC, Proliferation, p53.

Introduction

Colorectal cancer (CRC), as the most frequent type of malignant tumor, had a high degree of malignancy and poor prognosis[1]. It ranked third in the number of cancer-related death in the world, accounting for 693,900 deaths every year approximately[2]. It can onset primarily *in situ* as well as be transferred from distance, or caused by infringement of adjacent tissue lesions[3]. Great progress has been made in the diagnosis and treatment of CRC recently. The mortality remains high; the 5-year survival rate is less than 65%[4]. CRC cell invasion and metastasis may be attributed to the high mortality of CRC patients[2]. Therefore, further comprehension of the molecular mechanisms of CRC occurrence and development is quite supportive for developing new therapeutic targets for CRC patients.

Long noncoding RNAs (lncRNAs) are out of protein-coding capacity that are longer than 200 nucleotides in length. lncRNA is known as a type of noncoding RNA transcript[5]. Several studies discovered that aberrant expressions of lncRNAs could play a vital role in various cell progression[6], especially cancers such as lung cancer[7], gastric cancer[8], colorectal cancer[9], and so on. Therefore,

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a good investigation of lncRNAs may be of great value in explaining the occurrence and development of tumors.

Small nucleolar RNA host gene 1 (SNHG1), a new-found lncRNA located at chromosome 11q12.3, has been demonstrated to be upregulated; it plays a tumor-promoting role in some cancer, such as lung cancer[10] and hepatocellular carcinoma (HCC)[11]. However, until now, no research had shown the effect of SNHG1 in the progression and development of CRC.

In this work, we revealed that SNHG1 was significantly overexpressed in CRC tissues compared to para-carcinoma tissues. Then, aberrant expression of SNHG1 accelerated CRC cell's viability and proliferation, inhibited apoptosis, and triggered cell cycle arrest in G0/G1 phase.

Patients and Methods

Clinical Samples

86 pairs of CRC tissues and para-carcinoma normal tissues used in this work were obtained from patients undergoing routine surgery in Huai'an First People's Hospital from 2015-2016. All surgical specimens were collected and, then, frozen immediately in liquid nitrogen until use. The tumor tissues were diagnosed and confirmed by pathological examination. This research was approved by the Ethics Committee of Huai'an First People's Hospital. Written informed consents were signed by all participants before the study.

Cell Culture

The CRC cell lines Caco-2, M5, DLD1, HCT116, SW620, SW480, HT29 and normal human intestinal epithelial cells (HIECs) were all purchased from Shanghai Model Cell Bank. These cells were cultured in media Roswell Park Memorial Institute-1640 (RPMI-1640) medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C with 5% CO_2.

Plasmid and Transfection

For overexpression of SNHG1 in HCT116 cells, the SNHG1 gene was cloned into the lentiviral-vector pcDNA3.1 (System Biosciences, Palo Alto, MA, USA) and primer sequences are as follows: SNHG1: forward, 5'-GGG GTA CCG TTC TCA TTT TTC TAC TGC TCG TG-3' and reverse, 5'-CGG GAT CCA TGT AAT CAA TCA TTT TAT TAT TTT CAT C-3'. HCT116 cells were transfected with LV-Vector or LV-SNHG1.

For knockdown of SNHG1 in HT29 cells, the sequences of shRNA targeting for lncRNA-SNHG1 were designed as 5'-CAG CAG TTG AGG GTT TGC TGT GTA T-3', and the NC-shRNA was purchased from Genechem (Shanghai, China). HT29 cells were transfected with NC-shRNA or lncRNA-shRNA.

RNA Extraction and qRT-PCR

Total RNA was extracted from collected frozen tissue samples and cell lines by using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocols. Reverse Transcription Kit (TaKaRa, Dalian, China) was used to synthesize cDNAs. SNHG1 mRNA level was quantified by SYBR Green real-time PCR and normalized to GAPDH using the following primers: forwards, 5'-ACA GCA GTT GAG GGT TTG CT-3' and reverse, 5'-GTT GCA GGA AGG GGG TGA TAA-3'; and for GAPDH, forward, 5'-CGG AGT TGT TCG TAT TCG G-3' and reverse, 5'-TAC TAG CCG ATG ATG GCA TT-3'. QRT-PCR was performed by the ABI 7500 system (Applied Biosystems, Foster City, MA, USA).

MTT Assay

CRC cells in good status were seeded onto 96-well plate and incubated overnight at 37°C containing 5% CO_2. Then, 20 μL of methyl thiazolyl tetrazolium (MTT) solution was added into the 96-well plate per well and cultured for 4 h at 37°C. Then, 150 μL DMSO was added into the 96-well plate and incubated for 10 min. The absorbance was detected by the enzyme-linked immunosorbent assay (ELISA) reader at 490 nm.

Colony Formation Assay

To further investigated cell proliferation of CRC cells, cells were plated in 6-well plates at a density of 5×10^2 per well and cultured for two weeks. The colonies were fixed in ice-cold 70% methanol for 10 min and stained with 0.5% crystal violet for 10 min; then, each well was washed 3 times with phosphate-buffered saline (PBS).

Cell Cycle Analysis

Cells for cell cycle analysis were prepared with a final concentration of 2×10^5/ml, treated with RNase A, fixed with 70% ice-cold ethanol overnight, and cells were washed with PBS twice. Then, the cells were stained with 50 mg/ml PI,

100 μg/ml RNase A and 0.2% Triton X-100 for 30 min at 4ºC in the dark. The percentages of the cell population in G0/G1, S, or G2/M phase were detected with a FACSCalibur flow cytometer (BD Bioscience, Franklin Lakes, NJ, USA).

Cell Apoptosis Analysis

Cells were cultured in 6-well plates with the final concentration of 1×10^5/ml and, then, treated with 0.25% trypsin and fixed in 70% ice-cold ethanol. The cell suspension was prepared and washed twice with PBS. Afterwards, cells were double stained with 5 μl Annexin V-FITC and 1 μl propidium iodide (PI, 50 μg/ml). After incubated in the dark for 15 min, the cells were analyzed by a flow cytometer equipped with CellQuest software (BD Bioscience, Franklin Lakes, NJ, USA).

Western Blotting Analysis

To investigate the relative protein expression level, cells were washed with ice-cold PBS and lysed using lysis buffer. Then, we measured the concentration of collected protein using a protein assay kit purchased from Beyotime (Shanghai, China). The extracted protein (sum of 20 μg) was degenerated and chilled on ice. 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate protein. Then, it was shifted to polyvinylidene difluoride (PVDF) membranes purchased from Millipore (Billerica, MA, USA). 5% fat-free milk was used to block non-specific protein interactions in Tris-buffered saline and tween 20 (TBS-T) buffer which contains Tris-HCl (50 mM), NaCl (150 mM) and Tween 20 (0.05%) at 4°C for 1 h. The membranes loaded with proteins were incubated at 4°C within the fat-free milk overnight with the following primary antibody against p53 (Absci, Nanjing, China), p21 (Abnova, Taipei, Taiwan, China), BAX (Abnova, Taipei, Taiwan, China). TBS-T buffer was used to wash the unbound antibody (10 min each time for three times). The membranes were, then, incubated at room temperature with secondary antibody conjugated with horseradish peroxide (one hour). After washing these membranes three times in TBS-T buffer, we developed the membranes using ECL (Millipore, Billerica, MA, USA) following the instructions.

Xenograft Model

The research was approved by the Animal Ethics Committee of Nanjing Medical University Animal Center. HT29 cells (6×10^4/mL) transfected with NC-siRNA or lncRNA-siRNA were implanted into both axillae of NOD/SCID mice (4-5 weeks old) subcutaneously. The tumor volumes were calculated by using calipers. Mice were sacrificed, and the grafts were removed after 6 weeks.

Statistical Analysis

SPSS 20.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. Statistical data was presented with Graph PAD prism software; quantitative data was presented as mean ± SD. The independent samples *t*-test was used to perform statistical analysis. The relative expression of mRNA was measured using the method of $2^{-\Delta\Delta CT}$. Results were considered statistically significant when $p < 0.05$.

Results

Ectopic Expression of SNHG1 in CRC Cell Lines and Tissues

Using the qRT-PCR, we measured the SNHG1 expression level in both CRC tissues and adjacent normal tissues. It indicated that SNHG1 expression level was remarkably higher in CRC tissues than paraneoplastic tissues (Figure 1A), which was consistent with other previous researches, implying that the aberrant SNHG1 expression might be involved in CRC progression and development.

Then, we investigated the SNHG1 expression level in several CRC and normal cell lines with qRT-RCR. It showed that, comparing with HIECs cell line, HT29, M5, DLD1, SW620, SW480 expressed a higher level of SNHG1 while Caco-2 and HCT116 expressed a lower level of SNHG1 (Figure 1B). To explore the role of SNHG1 in LAC progression and development, we chose HT29 cell line for SNHG1 knockdown and HCT126 cell line for SNHG1 overexpression, and the transfection efficiency was both subsequently detected via RT-PCR (Figure 1C). The SNHG1 expression was effectively suppressed in HT29 cells by lncRNA-shRNA and elevated in HCT116 cells by LV-SNHG1.

Effects of SNHG1 on CRC Cell Proliferation

To explore the biological role and mechanism of SNHG1 on CRC tumorigenesis, we identified the role of SNHG1 on CRC cell viability and

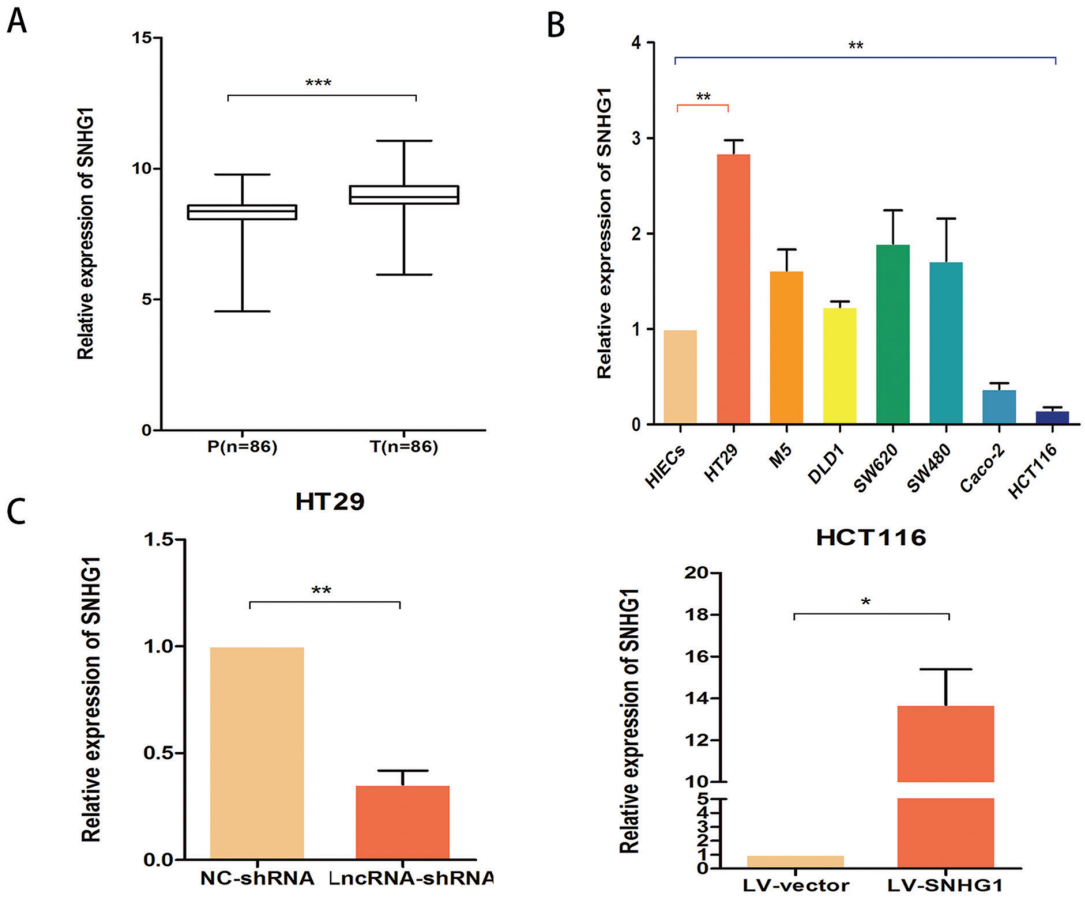

Figure 1. Expression of SNHG1 in CRC tissues and cell lines. ***A,*** Analysis of SNHG1 expression level in para-carcinoma tissues (P) and tumor tissues (T). SNHG1 was significantly elevated in CRC tumor tissues compared with the para-carcinoma tissues. ***B,*** Analysis of SNHG1 expression level in eight cell lines. ***C,*** Analysis of transfection efficiency in HT29 cells and HCT116 cells. HT29 cells was transfected with NC-shRNA or lncRNA-shRNA and HCT116 cells was transfected with LV-Vector or LV-SNHG1. Total RNA was detected by qRT-PCR and GAPDH was used as an internal control. Data are presented as the mean ± SD of three independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

proliferation. As shown with a MTT assay, cell viability was markedly impaired in HT29 cells transfected with lncRNA-shRNA compared with NC-shRNA (Figure 2A). Conversely, cell viability was enhanced in HCT116 cells transfected with LV-SNHG1 compared with LV-vector (Figure 2B).

Colony formation assay was also carried out to measure CRC cell proliferation ability. It revealed that the number of formed colonies in HCT116 cells transfected with LV-SNHG1 was larger than LV-vector (Figure 2C). Whereas, the number of formed colonies in HT29 cells transfected with lncRNA-shRNA was less than NC-shRNA (Figure 2D). Corporately, these results indicated that SNHG1 promotes CRC cells viability and proliferation.

Effects of SNHG1 on CRC Cell Apoptosis and Cell Cycle

Since knockdown of SNHG1 appeared to down-regulate CRC cell proliferation, we further examined the effects of SNHG1 on CRC cell apoptosis by using flow cytometric analysis. The results indicated that the apoptotic rate of HT29 cells transfected with lncRNA-shRNA increased remarkably compared with NC-shRNA, whereas the apoptotic rate of HCT116 cells transfected with LV-SNHG1 decreased patently compared with LV-vector (Figure 3A and B).

Next, cell cycle progression was monitored by flow cytometric assay. We found that the percentage of HT29 cells transfected with lncRNA-shRNA in G0/G1 phase increased compared with NC-shRNA, while the percentage of

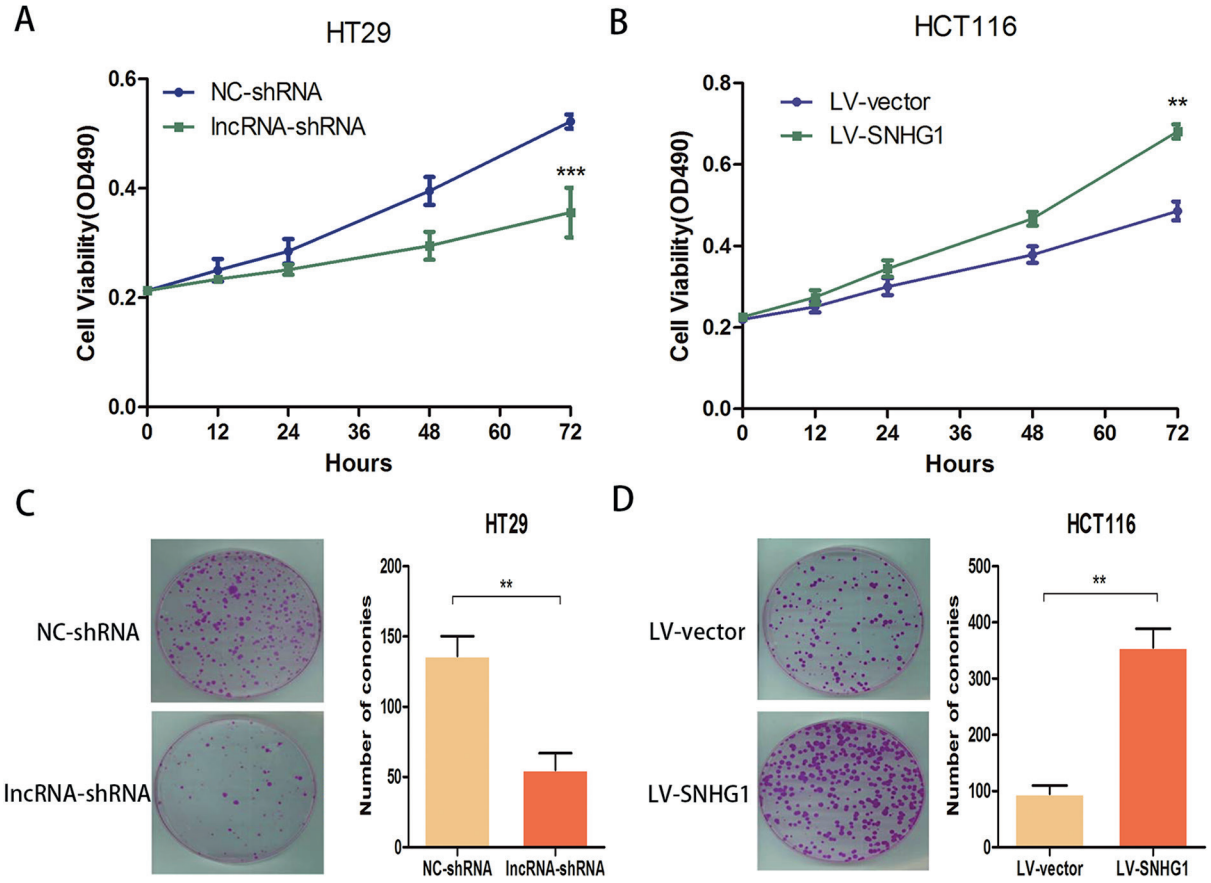

Figure 2. Effects of SNHG1 on CRC cell proliferation. ***A,*** MTT assay was performed to determine the viability of HT29 cells transfected with lncRNA-shRNA and NC-shRNA respectively. ***B,*** MTT assay was performed to determine the viability of HCT116 cells transfected with LV-SNHG1 and LV-vector respectively. ***C,*** Colony formation assay was performed to determine the proliferation of HT29 cells transfected with lncRNA-shRNA and NC-shRNA respectively. ***D,*** Colony formation assay was performed to determine the proliferation of HCT116 cells transfected with LV-SNHG1 and LV-vector respectively. Data are presented as the mean ± SD of three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$.

HCT116 cells transfected with LV-SNHG1 in G0/G1 phase decreased remarkably compared with LV-vector (Figure 3C and D). Collectively, these results demonstrated that SNHG1 knockdown promoted CRC cell apoptosis and induced G0/G1 phase arrest.

SNHG1 Inhibited p53 Expression

It has been well recognized that p53 can be modulated to regulate cancer cell growth by lncRNAs[8], and it has been reported that SNHG1 could inactivate p53 protein in HCC[12]. To better understand the underlying mechanisms of SNHG1-mediated CRC cell apoptosis and cell proliferation, we employed Western blotting to assay the expression level of p53. The results demonstrated that p53 and its target genes, p21 and BAX, were significantly upregulated in HT29 cells transfected with lncRNA-shRNA compared with the control cells (Figure 4A and B). Conversely, p53 and its target genes were remarkably downregulated in HCT116 cells transfected with LV-SNHG1 compared with the control cells (Figure 4C and D). These results confirmed that SNHG1 served as an oncogene via regulating p53 activation in CRC.

SNHG1 Knockdown Suppressed CRC Growth in vivo

HT29 cells transfected with lncRNA-shRNA or NC-shRNA were injected into mice subcutaneously. The expression of SNHG1 in tumor tissues was detected by qRT-PCR, and it showed that SNHG1 expression in the lncRNA-shRNA group was decreased compared with the expression in the LV-Vector group (Figure 5A).

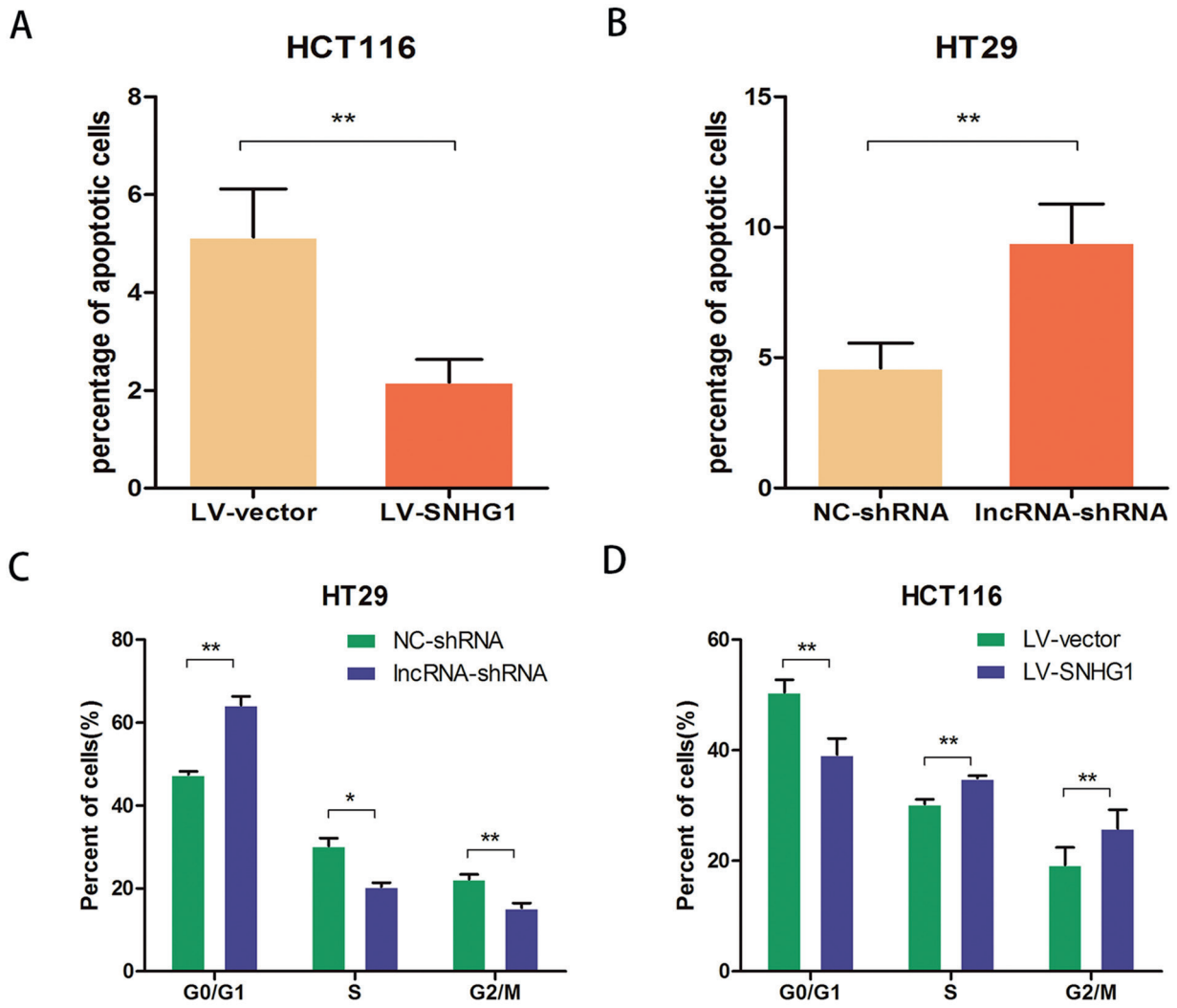

Figure 3. Effects of SNHG1 on CRC cell apoptosis and cell cycle. ***A,*** Flow cytometric analysis was performed to detect the apoptotic rates of HCT116 cells transfected with LV-SNHG1 and LV-vector. ***B,*** Flow cytometric analysis was performed to detect the apoptotic rates of HT29 cells transfected with lncRNA-shRNA and NC-shRNA. ***C,*** Flow cytometric analysis was performed to detect cell cycle progression of HT29 cells transfected with lncRNA-shRNA and NC-shRNA. ***D,*** Flow cytometric analysis was performed to detect cell cycle progression of HCT116 cells transfected with LV-SNHG1 and LV-vector. Data are presented as the mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$.

Tumor volume was significantly smaller in the lncRNA-shRNA group compared to the volume in the NC-shRNA group (Figure 5B). Tumor weight became less in the lncRNA-shRNA group compared to the weight in the NC-shRNA group (Figure 5C). These data suggested that SNHG1 knockdown suppressed CRC cell growth *in vivo.*

Discussion

High-throughput technology has demonstrated that about 98% of the human genome is non-coding genes, and lncRNAs make up more than 80% of them[13]. Increasing studies indicate that lncRNAs may be highly involved in the progression and development of various types of tumors. For instance, Liu et al[7] found that lncRNA-LET exhibited a tumor-suppressive effect on lung adenocarcinoma (LAC) progression by inhibiting EMT and Wnt/β-catenin pathway. Wang et al[14] discovered that lncRNA-LET inhibited the tumorigenesis of esophageal squamous cell carcinoma (ESCC) cells, and modulated p53 in human ESCC. Ming Sun et al[8] showed that MEG3 regulated

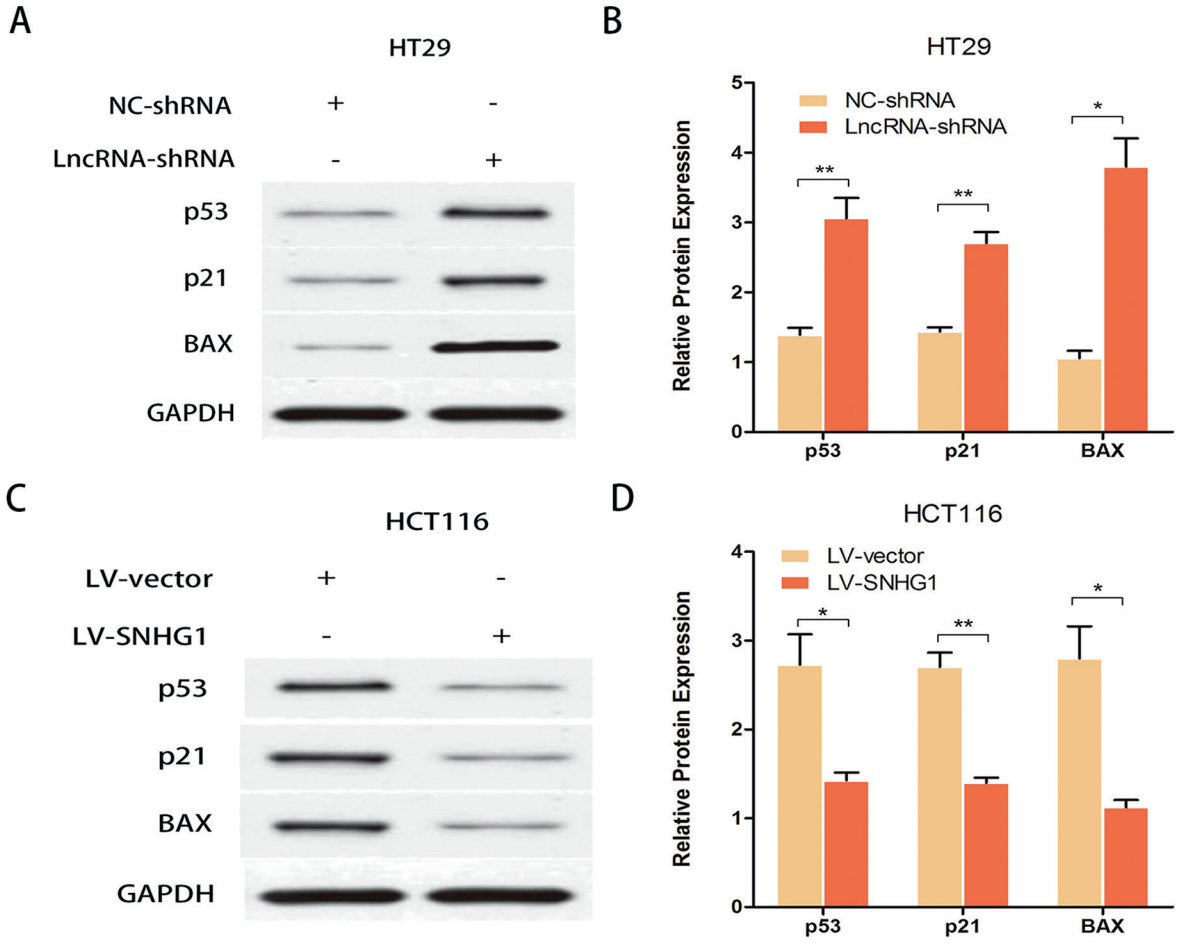

Figure 4. SNHG1 inhibited p53 expression. ***A,*** and ***B,*** p53, p21 and BAX were significantly upregulated in HT29 cells transfected with lncRNA-shRNA compared with NC-shRNA. ***C,*** and ***D,*** p53, p21 and BAX were significantly downregulated in HCT116 cells transfected with LV-SNHG1 compared with LV-vector. Data are presented as the mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$.

gastric cancer cell proliferation and apoptosis; thus, it can be identified as a poor prognostic biomarker in gastric cancer.

LncRNA-SNHG1 is a newly discovered gene that participated in the process of tumor development. You et al[10] demonstrated that SNHG1 is dysregulated in NSCLC cell lines and may provide promising therapeutic perspective for NSCLC intervention. Zhang et al[12] revealed that lncRNA SNHG1 may be involved in the malignant processes of HCC through targeting miR-195. In our investigation, we explored the role of SNHG1 in CRC. We revealed that SNHG1 expression level was markedly elevated in CRC tissues when compared to adjacent tissues, implying that SNHG1 might participate in the progression and development of CRC. Then, we observed that SNHG1 knockdown significantly attenuated HT29 cell proliferation, triggered cell cycle arrest and enhanced cell apoptosis while SNHG1 overexpression significantly functioned on the contrary. Furthermore, we revealed that SNHG1 suppressed cell growth and proliferation *in vivo*. Collectively, it documented that SNHG1 acts as a tumor-promoting gene in CRC.

P53, as a well-known tumor suppressor, is involved in tumorigenesis and development of various cancers[15,16]. It can suppress tumor growth and development by attenuating cell proliferation or via activating cell death progresses[17-19]. In our work, we found that SNHG1 knockdown significantly increases the protein level of p53 and its target genes while SNHG1 overexpression acted the opposite.

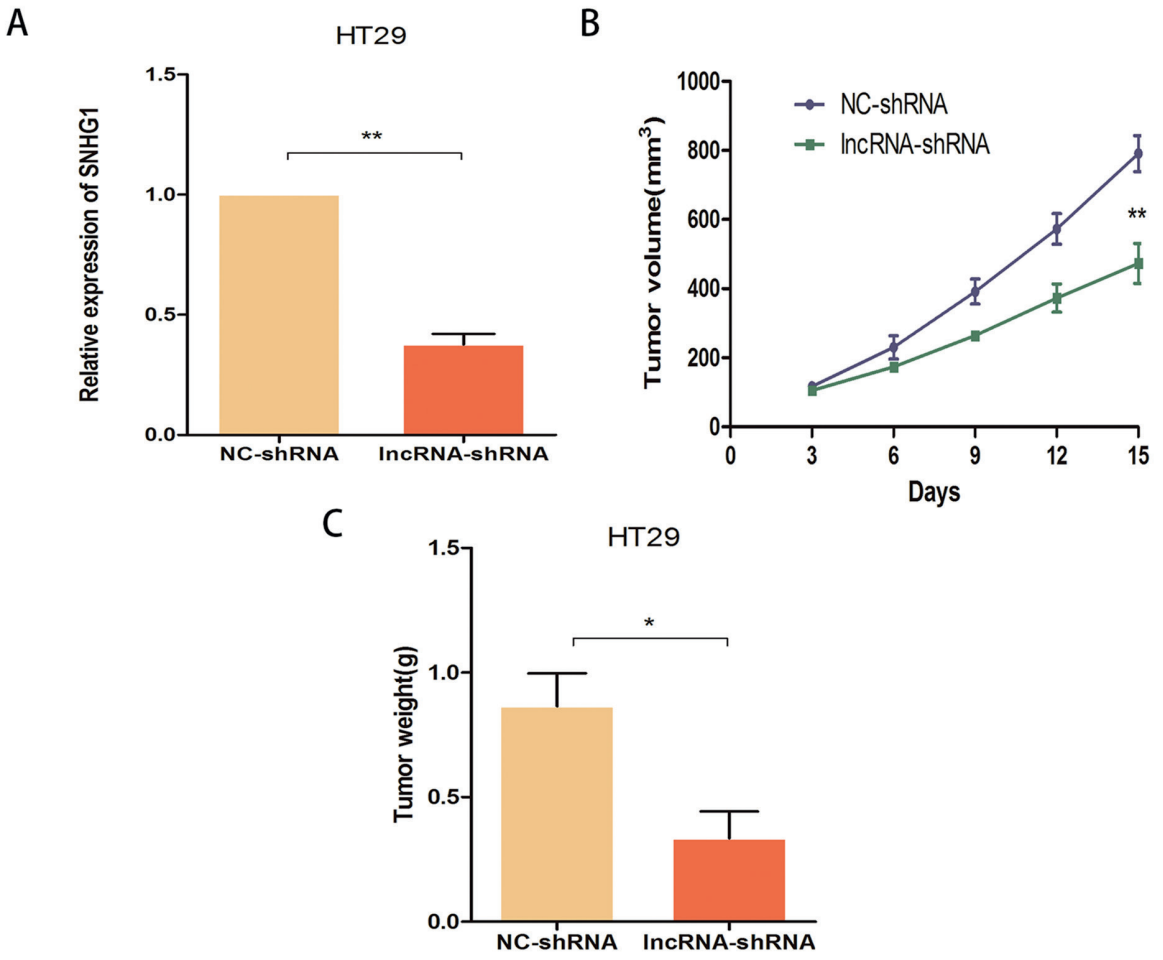

Figure 5. SNHG1 knockdown inhibited the growth of CRC *in vivo*. ***A,*** Analysis of SNHG1 expression level in tumor tissues was detected by using RT-PCR, GAPDH was used as an internal control. ***B,*** Tumor volume was determined by using calipers. ***C,*** Tumor weights are represented as means of tumor weights ± SD. *$p < 0.05$, **$p < 0.01$.

Conclusions

Our study demonstrated that SNHG1 had a tumor-promoting effect on CRC progression and proliferation *in vitro* and *in vivo*. Besides, SNHG1 could suppress p53 pathway in part. Our findings implied that SNHG1 could serve as an innovative and potential therapeutic target for CRC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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