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LINC01198 promotes colorectal cancer cell proliferation and inhibits apoptosis *via* Notch signaling pathway

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Abstract. – OBJECTIVE: To detect the expression level of long intergenic non-protein coding RNA 1198 (LINC01198) in colorectal cancer (CRC) tissues and cells, to investigate the effect of LINC01198 on the biological function of CRC cells through *in vivo* and *in vitro* experiments, and to explore its molecular mechanism.

PATIENTS AND METHODS: Tissue samples were collected from 32 patients with CRC. Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was utilized to detect the relative expression level of LINC01198 in CRC tissues and cells. In vitro experiments [Cell Counting Kit-8 (CCK-8) and flow cytometry] were conducted to explore the effect of interfering with the expression of LINC01198 on the proliferation, cycle and apoptosis of CRC cells. Tumorigenesis assay was undertaken in nude mice to investigate the influence of LINC01198 on the tumorigenic ability of CRC cells in vivo. Besides, Western blotting was performed to determine the changes in the downstream signaling pathway of LINC01198.

RESULTS: Among the 32 cases of tissue samples of CRC patients, 28 cases had an upregulated expression of LINC01198 compared with paracancerous tissues. The results of qRT-PCR indicated that LINC01198 expression was upregulated in CRC cells, and the interference efficiency of si-LINC01198 was measured via qRT-PCR. The results of in vitro experiments demonstrated that after interfering with the expression of LINC01198 in CRC cells, cell proliferation capacity was inhibited, cell cycle was arrested at G1/G0 phase, and the apoptosis rate was increased. The results of nude mice tumorigenesis experiments revealed that after interfering with the expression of LINC01198, the tumorigenic ability of CRC cells in vivo declined. Additionally, Western blotting assay results confirmed that after interfering with the expression of LINC01198, the expression of molecular markers in the Notch signaling pathway was inhibited.

CONCLUSIONS: The expression of LINC01198 is upregulated in the case of CRC, which promotes proliferation and inhibits apoptosis of CRC cells by regulating the Notch signaling pathway. Our findings provide a novel biomarker for the diagnosis and treatment of HCC patients and treatment strategies.

Key Words:
LINC01198, Proliferation, Apoptosis, Notch signaling pathway.

Introduction

Colorectal cancer (CRC) is one of the most common gastrointestinal malignancies in the world. With the improvement of living standards and changes in dietary structure, the incidence and mortality rates of CRC in China have gradually increased, ranking fourth in all malignant tumors[1-3]. As the medical technology advances constantly, most CRC patients can be treated with surgery in time, but the average survival time is still less than 30 months[4]. Therefore, it is of great clinical significance to deeply investigate the pathogenesis of CRC and seek potential therapeutic targets.

Long non-coding ribonucleic acids (lncRNAs), a kind of non-coding RNAs with transcripts more than 200 bp in length, are widely present in the nucleus and cytoplasm, and also exist in organelles, and they are not involved or rarely participate in the encoding of proteins due to the lack of open reading frame but regulate gene expression at multiple levels in the form of RNAs[5,6]. Moreover, thousands of lncRNAs that are abnormally expressed or mutated in various tumor, such as CRC, have been discovered through a new generation sequencing technology[7].

LINC01198 is located on chromosome 13q14.13, with a total length of 955 bp. There are relatively few reports about linc01198 in tumors. Chen et al[8] reported that the expression of LINC01198 is

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upregulated in gliomas. The high expression of linc01198 promoted the proliferation of glioma cells and increased their resistance to temozolomide. Through bioinformatics analysis, Sun et al[9] reported that the downregulated expression of LINC01198 in bladder cancer prompts a better prognosis. Xie et al[10] reported that LINC01198 facilitates gliomagenesis by activating the PI3-K/AKT pathway to regulate PI3-KCA and PTEN. However, LINC01198 expression and its clinical significance in CRC have not been reported. In the present study, therefore, the expression level of LINC01198 in CRC tissues and cells was detected through *in vitro* experiments, and the effect of LINC01198 on the biological function of CRC cells was further investigated through *in vivo* and *in vitro* experiments.

Patients and Methods

Tissue Samples

A total of 32 CRC patients who underwent surgical treatment from January 2016 to June 2018 were selected, including 13 females and 19 males, with an average age of (60.3±8.1) years old. All patients had not undergone radiotherapy and chemotherapy before surgery, and had no family history of intestinal polyps or chronic intestinal diseases. All patients enrolled in this study signed the informed consent. During operation, CRC tissues and para-cancer tissues (colorectal tissues more than 5 cm from the tumor margin) were collected, and immediately cryopreserved at -80°C. The samples were diagnosed as intestinal adenocarcinoma by postoperative pathological examinations. The selection of patients was based on the guideline proposed by the Union for International Cancer Control (UICC). This investigation was approved by the Ethics Committee of Zhejiang Provincial People's Hospital.

Cell Culture

CRC cell lines (HT-29, SW480, SW620, LOVO) and FHC cells were purchased from Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd. (Shanghai, China). Cells were cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% penicillin/streptomycin (Beyotime, Shanghai, China) or Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco; Rockville, MD, USA) in an incubator at 37°C with 5% CO_2. They were passaged once every 2 to 3 days, and cells in the logarithmic growth phase were taken for subsequent experiments.

Reverse Transcription and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and then utilized to synthesize complementary deoxyribonucleic acid (cDNA) *via* reverse transcription according to the instructions (TaKaRa, Tokyo, Japan). Later, qRT-PCR system (TOYOBO, Osaka, Japan) composed of internal reference and target genes was prepared for all cDNA samples, with cDNA as a template and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal reference, and triplicate wells were set for each reaction tube. Then, qRT-PCR was conducted using a PCR amplifier with the prepared reaction solution under the following conditions: pre-denaturation at 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min, followed by melting curve analysis. The relative expression level of LINC01198 was evaluated by $2^{-\Delta\Delta Ct}$ method. The primer sequences for qRT-PCR are shown as follows: LINC01198-F: CCTTTCTCGGGGAAGATGAC, LINC01198-R: GCAACTGCTGGACGATAACAA, GAPDH-F: 5′-CACCATCTTCCAGGACGAG-3′, GAPDH-R: 5′-CCTTCTCCATGGTGGTGAAGA-3′.

Cell Transfection

Cells were inoculated into a 6-well plate. When the cells were observed to cover 60-70% of the plate, si-LINC01198 and si-NC were transiently transfected into CRC cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) reagent. About 48 h later, the transfection efficiency was verified *via* qRT-PCR.

Cell Counting Kit-8 (CCK-8) Assay

Cells in the logarithmic growth phase were inoculated into a 96-well plate (5×10^3 cells/well) with 100 µL of complete medium in each well. The cells were then added with 10 µL of CCK-8 reagent (Beyotime, Shanghai, China) when they were transfected for 0 h, 24 h, 48 h, 72 h and 96 h. After incubation for 2 h, the absorbance was measured at 450 nm on a microplate reader.

Detection of Cell Apoptosis Via Flow Cytometry

At 48 h after transfection, the cells were digested and centrifuged at 8,000 rpm in a 15 mL

centrifuge tube for 3 min. Then, 1 mL of phosphate-buffered solution (PBS) was used to prepare cell suspension, and the cells were resuspended with 200 μL of binding buffer. Subsequently, each 5 μL of Annexin V-FITC and propidium iodide (PI) staining solution was added into the tubes. The samples were determined using FACSAria II Flow Cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Then, the cell apoptosis rate was recorded.

Determination of Cell Cycle Distribution Via Flow Cytometry

At 48 h after transfection, cells were digested with trypsin to prepare a cell suspension, and the cells were harvested and centrifuged at 4°C for 5 min. Next, the cells were resuspended with 0.5 mL of PBS, fully mixed with pre-cooled 70% ethanol, and fixed at 4°C overnight. Finally, the fixed cells were then stained with PI in a dark water bath at 37°C for 30 min. After the cells were filtered through 0.75 μm filter membrane, the proportion of cell cycle was determined using BD FACSCalibur flow cytometer.

Nude Mice Tumorigenesis

A total of 12 female nude mice aged 5 weeks old were selected and fed in SPF-grade barrier system. This study was approved by the Animal Ethics Committee of Zhejiang Provincial People's Hospital Animal Center. The HT-29 cells (1×10^6) in si-NC group and si-LINC01198 group were injected into the axilla of nude mice. After 5 weeks of feeding, the volume and weight of tumor xenografts in nude mice were tested. Tumor xenograft volume was calculated as follows: volume=1/2 (shortest diameter)^2×(longest diameter).

Western Blotting

Cells were collected from experimental group and control group, washed twice with pre-cooled PBS, and lysed with cell lysate containing protease inhibitors, and then, lysates were collected into a micro-centrifuge tube using a curette and shaken 3 times (5 s/time) in the ultrasonic apparatus. After that, the samples were centrifuged at 13,000 rpm for 20 min at 4°C. After that, the total protein was obtained, and the concentration was measured by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). Subsequently, the protein samples were loaded with 5× loading buffer, denaturated in metal bath at 100°C for 5 min, separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; constant pressure: 100 V, 120 min), and electrotransferred (constant current: 200 mA, 120 min, ice-water bath) onto a membrane. The membrane was sealed with skim milk powder for 2 h. Next, the membrane was incubated with primary antibody at 4°C overnight. The next day, after washing in Tris-buffered saline with Tween-20 (TBST) for three times (5 min/time), the membrane was incubated with secondary antibody for 1 h. Then, the membrane was washed with TBST twice (20 min/time). Finally, images were developed with enhanced chemiluminescence (ECL) reagent and photographed. GAPDH was used as an internal reference.

Immunohistochemistry

The tumor xenografts were removed, fixed in 4% formalin, and then, embedded in paraffin. After blocking endogenous peroxides and proteins, the sections were incubated with primary antibody at 4°C overnight. After washing with PBS, the sections were incubated with horse radish peroxidase (HRP)-polymer-conjugated secondary antibody at 37°C for 1 h. Then, the sections were stained with 3,3'-diaminobenzidine solution for 3 min, and the nuclei were counterstained with hematoxylin.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 software (IBM Corp., Armonk, NY, USA) was applied for statistical analysis. Measurement data were expressed as mean ± standard deviation ($\bar{\chi}\pm s$). The *t*-test was used for analyzing measurement data. The differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ suggested that the difference was statistically significant.

Results

Expression of LINC01198 in CRC Tissues and Cells

Among the 32 cases of tissue samples of CRC patients, 28 cases had an upregulated expression of LINC01198 compared with para-cancer tissues (Figure 1A). The results of qRT-PCR indicated that LINC01198 expression was upregulated in CRC cells (Figure 1B). The sequence of si-LINC01198 and si-NC were transiently transfected into CRC cells. About 48 h later, RNA was collected from

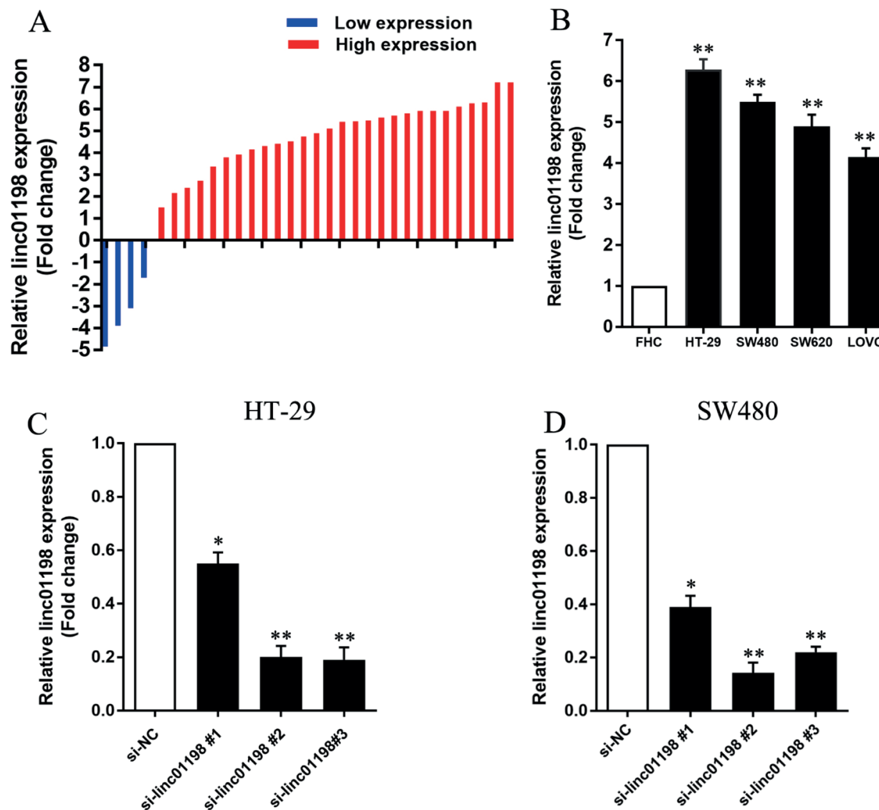

Figure 1. Expression of LINC01198 in CRC tissues and cells. **A,** Relative expression level of LINC01198 in the 32 cases of tissue samples of CRC patients was detected by qRT-PCR, and the results indicated that LINC01198 expression was up-regulated in 28 cases of CRC tissues (GAPDH was used as an internal reference). **B,** The results of qRT-PCR showed that LINC01198 was up-regulated in CRC cells (GAPDH was used as an internal reference). **C-D,** Si-LINC01198 and si-NC were transiently transfected into CRC cells. The transfection efficiency was verified *via* qRT-PCR.

cells, and the transfection efficiency was verified *via* qRT-PCR (Figure 1C, 1D).

Biological Function of LINC01198 in CRC Cells

After interfering with the expression of LINC01198 in CRC cells, CCK-8 assay results exhibited that the cell proliferation capacity was inhibited in the experimental group compared with that in the si-NC group (Figure 2A, 2B). The results of flow cytometry demonstrated that after interfering with the expression of LINC01198 in CRC cells, cell cycle was arrested at G1/G0 phase (Figure 2C, 2D). Besides, through the same treatment, flow cytometry results suggested that the apoptosis rate increased in experimental group compared with that in control group (Figure 2E, 2F).

Effect of LINC01198 on the Tumorigenic Ability of CRC Cells In Vivo

The results of nude mice tumorigenesis experiments (Figure 3A) revealed that after 5 weeks, the weight of tumor xenografts in nude mice decreased in si-LINC01198 group compared with that in si-NC group (Figure 3B), and the difference was statistically significant. Moreover, the increase in the tumor volume in si-LINC01198 group was also smaller than that in si-NC group as time passed, suggesting a statistically significant difference (Figure 3C). Additionally, Ki67 expression in the tumor xenografts was measured by immunohistochemistry (Figure 3D). The above findings indicated that after interfering with the expression of LINC01198, the tumorigenic ability of CRC cells *in vivo* declined.

LINC01198 Regulated Notch Signaling Pathway

To explore the molecular mechanism of LINC01198 in exerting oncogene-like functions in CRC cells, the cells were collected from si-LINC01198 and si-NC groups to extract the proteins. Western blotting results prompted that after interfering with the expression of LINC01198, the

expression of molecular markers (Nothc-1, P300, Hes1) in the Notch signaling pathway was inhibited (Figure 4A, 4B).

Discussion

Previous research[11] confirmed that most human genomes are transcribed into non-coding RNAs. LncRNAs refer to non-coding RNAs with more than 200 bases in length. LncRNAs play an important role in various pathophysiological processes[12], especially in the occurrence and progression of tumors, such as CRC. During the development of normal epithelial cells to malignant tumors, a variety of normal growth mechanisms are destructed, including cell proliferation, differentiation and apoptosis, and abnormal proliferation is regarded to be a typical feature of tumor cells[13,14]. Therefore, inhibiting the proliferation or

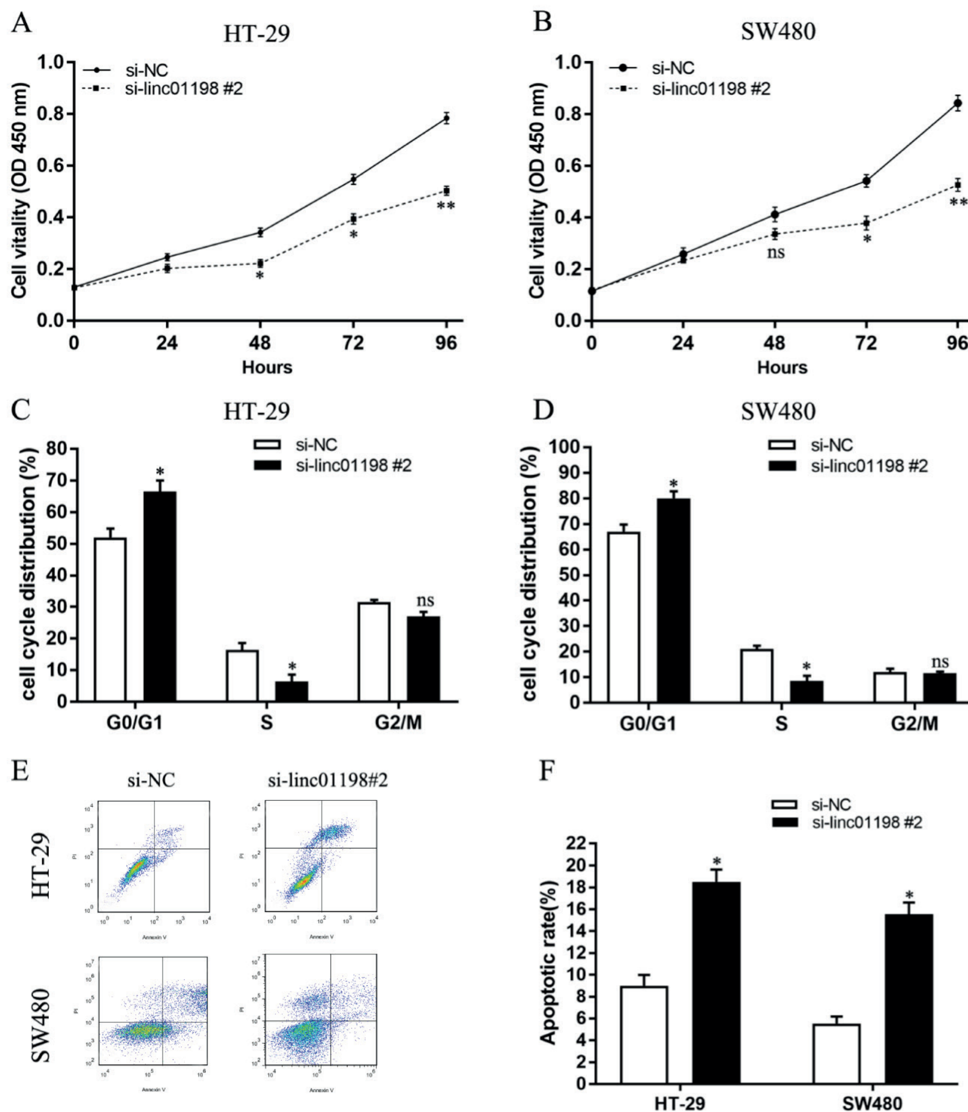

Figure 2. Biological function of LINC01198 in CRC cells. **A-B,** CCK-8 assay results exhibited that the cell proliferation capacity was inhibited after interfering with the expression of LINC01198 in CRC cells. **C-D,** The results of flow cytometry demonstrated that cell cycle was arrested at G1/G0 phase in experimental group compared that in control group. **E-F,** The results of flow cytometry suggested that the apoptosis rate was increased in si-LINC01198 group compared with that in si-NC group.

promoting the apoptosis of cancer cells has become the key to anti-tumor and also anti-CRC research.

The effects of lncRNAs on the proliferation and apoptosis of CRC cells are gradually being emphasized. LINC00461[15] and lncRNA-LUNAR1[16] have been demonstrated to exert crucial roles in the pathological proliferation of CRC cells. The present study showed that LINC01198 was highly expressed in CRC tissues, and downregulating LINC01198 inhibited the proliferation of CRC cells HT-29 and SW480. The results of nude mice tumorigenesis experiments revealed that downregulating LINC01198 repressed the tumorigenic ability of CRC cells *in vivo*. These findings indicated that highly expressed LINC01198 may promote the proliferation and inhibit the apoptosis of CRC cells. Thus, LINC01198 can serve as a potential prognostic biomarker and therapeutic target for CRC.

The Notch signaling pathway is a crucial player in the development of normal tissues and cells[17]. Currently, four receptors of the Notch signaling pathway have been discovered in mammals[18,19]. After ligand binding and a series of modifications, Notch receptors interact with CSL (CBF-1, Suppressor of hairless, Lag) transcription factor, thereby regulating Notch-mediated transcription and initiating negative feedback inhibition of the Notch signaling pathway[20,21]. Notch gene is abnormally expressed in CRC tissues, proving that the Notch signaling pathway is closely associated with the occurrence and progression of CRC. LncRNAs, important regulatory factors, can participate in the regulation of the Notch signaling pathway, such as FAM83H-AS1 and FOXD2- AS1[22,23]. The results of *in vitro* assays in the present study revealed that after interfering with the expression of LINC01198, the expression of molecular markers in the Notch signaling pathway was inhibited.

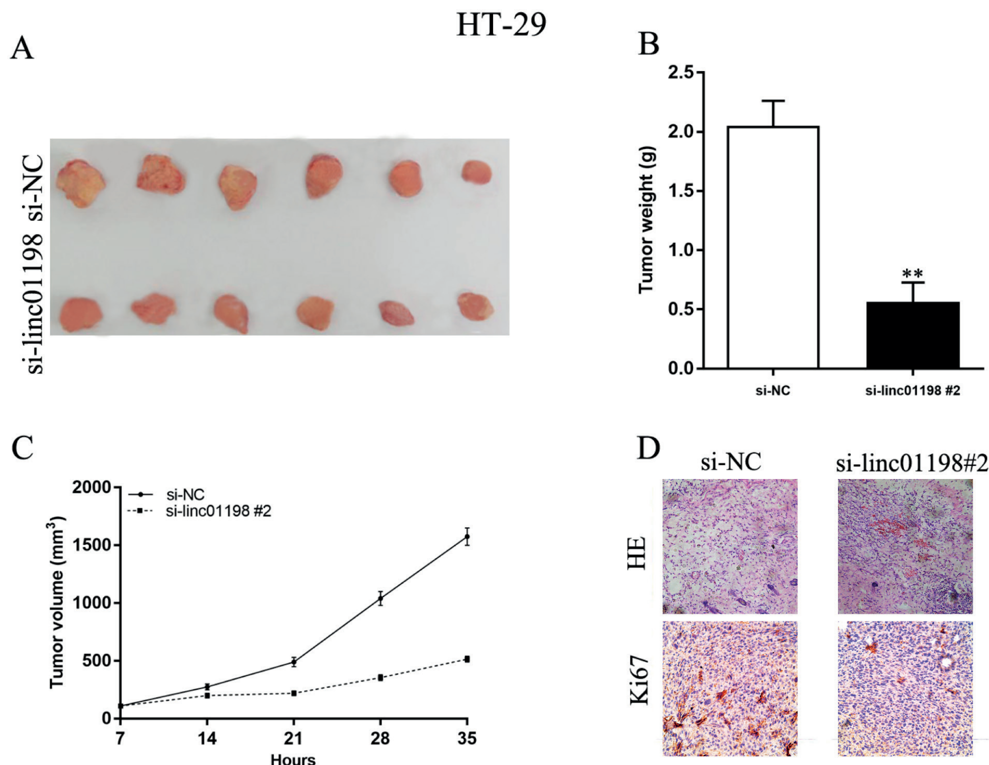

Figure 3. Effect of LINC01198 on the tumorigenic ability of CRC cells *in vivo.* **A,** A model of subcutaneous tumor xenograft in nude mice was established. The results exhibited that after interfering with the expression of LINC01198, the tumorigenic ability of CRC cells *in vivo* declined. **B,** The results showed that the weight of the tumor xenograft was decreased in experimental group compared with that in control group. **C,** The volume of tumor xenograft was measured and the growth curve was drawn. The results illuminated that the volume declined in experimental group compared with that in control group. **D,** Immunohistochemistry results prompted that the expression level of Ki67 decreased in tumor xenograft in experimental group, suggesting that the tumorigenic ability was inhibited (magnification: 200×).

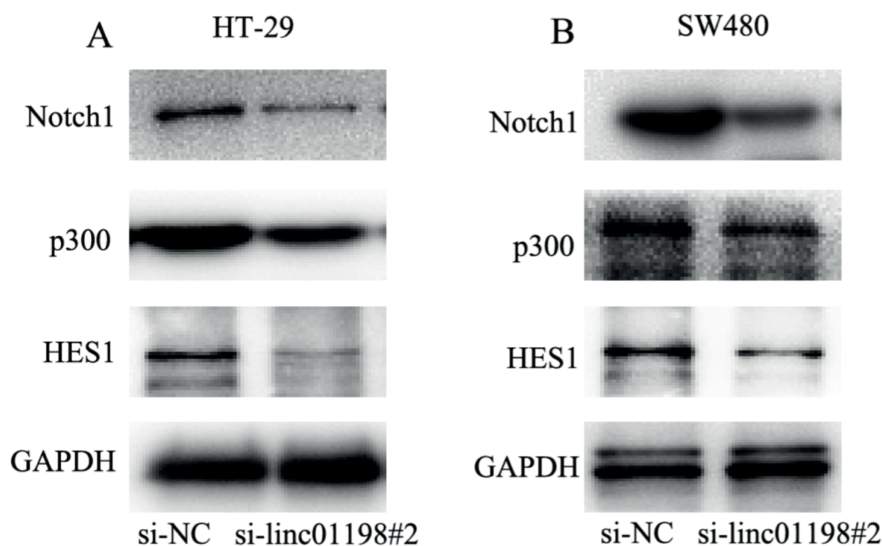

Figure 4. LINC01198 regulated Notch signaling pathway. **A-B,** The expression of LINC01198 was interfered in CRC cells. Western blotting assay was utilized to detect the expression of molecular markers in the Notch signaling pathway.

Conclusions

The results in the present study demonstrated that LINC01198 is highly expressed in CRC tissues, and downregulating LINC01198 can notably inhibit the proliferation and promote the apoptosis of CRC cells, and the Notch signaling pathway can be regulated by LINC01198, which was further investigated through *in vitro* experiments. Thus, LINC01198 may be a potential target for the treatment of CRC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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