European Review for Medical and Pharmacological Sciences 2020; 24: 3624-3632

Linc00702 inhibits cell growth and metastasis through regulating PTEN in colorectal cancer

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Abstract. – OBJECTIVE: To elucidate the expression and influence of Linc00702 on the development and progression of colorectal cancer (CRC).

PATIENTS AND METHODS: The expression of Linc00702 was evaluated using quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) in 91 paired CRC tissue samples and adjacent normal tissue samples, as well as in CRC cell lines. Cell proliferation in Caco2 and SW620 cells was detected using colony formation assay and 5-Ethynyl-2'-deoxyuridine (EdU) assay. Wound-healing assay and transwell analysis were utilized to assess the abilities of cell migration and invasion. Western blot analysis was employed to further explore the underlying mechanism of Linc00702 in CRC.

RESULTS: Linc00702 was lowly expressed in CRC tissues and cells. Over-expression of Linc00702 reduced cell proliferation, migration, and invasion of Caco2 cells, while knockdown of Linc00702 promoted cell growth and metastasis of SW620 cells comparing to control group, relatively. PTEN was verified as the target for Linc00702 in CRC, and Linc00702 promoted PTEN expression to inhibit the PI3K/AKT pathway.

CONCLUSIONS: Linc00702 was downregulated in CRC and inhibited cell proliferation, migration, and invasion by repressing the PI3K/AKT pathway via promoting PTEN expression. This might provide a new target for the biological treatment for CRC.

Key Words:
Linc00702, CRC, Growth, Metastasis, PTEN.

Introduction

Colorectal cancer (CRC), one of the most common malignant tumors of the digestive system, ranks third in the incidence and mortality of cancer in the United States, according to an assessment by the American Cancer Society in 2019[1,2]. Its prognosis remains poor due to its high rates of postoperative invasiveness and metastasis. The occurrence of CRC is controlled by a variety of genes and factors, and is a multistage complex process[3,4]. In recent years, several novel diagnosis and treatment markers for CRC have received great attention, including non-coding RNAs.

Long non-coding RNA (lncRNA) is a kind of non-coding RNA longer than 200 nucleotides with transcriptional regulation function[5,6]. Disorders of lncRNAs occur in various types of cancer, such as breast cancer, liver cancer, lung cancer, bladder cancer, and CRC. Consistently, in aggressive breast cancer, lncRNA LIMT is downregulated, which inhibits the cancer progression by targeting EGF[7]. In human liver cancer, lncRNA lncTCF7 activates the Wnt signaling to promote the self-renewal of stem cells[8]. In bladder cancer, lncRNA HCG22 reduces cell growth and metastasis *via* regulating PTBP1[9]. In addition, lncRNA FAL1 could promote the proliferation and metastasis *via* epithelial-mesenchymal transition (EMT) by regulating the PTEN (gene of phosphate and tension homology deleted on chromosome ten) signaling pathway[10]. In CRC, lncRNA CCAL regulates tumor progression by reduction of activator protein 2α to activate the Wnt/β-catenin signaling[11].

Linc00702, located on 10p15.1, has been reported to participate in the progression of malignant meningioma, non-small cell lung cancer (NSCLC), and endometrial cancer[12-14]. However, its expression and function in CRC have not been

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mentioned before. Here, we first measured the relative expression of Linc00702 in 91 CRC tissues comparing to adjacent normal colorectal tissues. Also, expression of Linc00702 in five CRC-derived cell lines was detected using quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) comparing to the normal colonic epithelial cells NCM460. Using Caco2 and SW620 cells, we studied the influence of Linc00702 on cell proliferation, migration, and invasion by functional experiments *in vitro*. Furthermore, PTEN was identified as a target for Linc00702 in CRC. Downstream molecular factors of PTEN were measured by Western blot. In this study, we first demonstrated Linc00702 expression and function in CRC, which might provide a novel target for the biological diagnosis and treatment for CRC.

Patients and Methods

CRC Tissue and Paired Adjacent Normal Tissue Species

From January 2015 to June 2018, 91 CRC patients undergoing surgery in our hospital who were confirmed by postoperative pathology were enrolled. None of them received preoperative radiotherapy or chemotherapy. Tumor tissues, and the adjacent normal tissue at 10 cm away from the tumor edge were collected. Informed contents were written by the patients or their family members and the investigation was approved by the Ethics Committee of our hospital..

Cell Lines

SW620, SW480, HT29, HCT116, and Caco2 cells were purchased from the Basic Cell Center of Chinese Academy of Medical Sciences (Shanghai, China). Normal colonic epithelial cell NCM460 was purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). All the six cell lines were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640; Gibco, Grand Island, NY, USA) medium mixing with 10% fetal bovine serum (FBS; Gibco, New York, NY, USA) in a 5% CO_2, 37°C incubator.

Cell Transfection

Lentivirus and siRNA for Linc00702 and each control (LV-Linc00702, LV-Empty, siRNA-Linc00702, siRNA-NC) were synthesized by Ribobio Co. Ltd. (Guangzhou, China). Transfection of siRNA in SW620 cells was performed using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) transfection kit and transfection of LV-Linc00702 in Caco2 cells was conducted using polybrene (Ribobio, Guangzhou, China). Cells were cultured to a density of 40-50% and the Lentivirus or siRNA were added into the medium according to the manufacturers' instructions. The efficiency of transfection was confirmed using qRT-PCR.

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

The total RNA of CRC cells and tissues were extracted from each group with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The cDNA of each tissue and cell sample was synthesized by reverse transcription using PrimeScripts RT reagent kit (TaKaRa Bio, Inc., Otsu, Shiga, Japan). PCR detection was performed by ABI 7500 PCR instrument (Applied Biosystems, Foster City, CA, USA) with reaction conditions: 50°C for 2 min and 95°C pre-denaturation for 10 min, followed by 40 cycles at 95°C for 15 s, and 65°C for 1 min. All experiments were repeated 3 times, and the expression level was calculated using the $2^{-\Delta\Delta Ct}$ method. Primers for Linc00702 and internal control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were as follows: Linc00702, Forward primer 5'-CCATGGAATGGGCAGGGATT-3' Reverse primer 5'-TGGCCTAGCCTCACTACTCA-3'; GAPDH, Forward primer 5'-GGGAGCCAAAAGGGTCATCA-3' Reverse primer 5'-GCCAAATTCGTTGTCATACTTCT-3'.

Colony Formation Assay

Cells were inoculated in a 6-well plate at a density of 3000 cells per well, and cultured for 2 to 3 weeks. The culture was terminated when the colonies were visible. Cells were washed twice with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 15 min, and stained with crystal violet for 30 min. Colonies containing more than 50 cells were count and analyzed under a microscope (Olympus Corp., Tokyo, Japan).

5-Ethynyl-2'-Deoxyuridine (EdU) Assay

Cell growth was measured by the EdU Cell Proliferation Assay Kit (Ribobio, Guangzhou, China) according to the manufacturers' instructions. Briefly, after 48 h of transfection, cells were incubated with 50 μmol/L of EdU reagent for 2 h, followed by fixation and permeabilization. Thereafter, the cells were observed by laser confocal microscopy to determine the proportion of EdU-positive cells (%).

Wound-Healing Assay

The transfected Caco2 and SW620 cells were separately cultured in a 6-well plate. After cell growth into a monolayer, a 200 μL pipette tip was used to scrape a vertical scratch of approximately 200 μm in diameter. Caco2 and SW620 cells were washed three times with PBS and incubated at 37°C. After 36 h, the average migration distance of the cells was observed under an inverted microscope. Each experiment was performed in parallel three times.

Transwell Assay

The migration and invasion assays were done in strict accordance with the manufacturers' instructions. For migration, transfected Caco2 and SW620 cells were seeded in the top chamber of the 8-μm insert (Corning, Corning, NY, USA) with serum-free medium. The bottom of the insert was immersed in RPMI-1640 containing 10% FBS. After 24 h culture, the cells were fixed with methanol for 10 min, followed by staining with 0.5% crystal violet for 15 min. Caco2 and SW620 cells that failed to migrate and invade were wiped out. Penetrating cells were observed and analyzed with a microscope in five random visions. For cell invasion assay, the top chamber of the insert was pre-covered with Matrigel (BD Biosciences, San Jose, CA, USA).

Western Blot

The total protein was extracted using radioimmunoprecipitation assay (RIPA) reagent (Beyotime, Shanghai, China) and analyzed using bicinchoninic acid (BCA) Kit (Beyotime, Shanghai, China). A total of 40 μg of protein in each sample was added to the well of 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) for gel electrophoresis. After electrophoresis, the protein was transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). 0.5% bovine serum albumin was applied to block non-specific antigens on the membrane for 2 h. Then, the membrane was incubated with PTEN [1:2 000, Cell Signaling Technology (CST), Danvers, MA, USA), PI3K (1:1 000, CST, Danvers, MA, USA), p-PI3K (1:500, CST, Danvers, MA, USA), AKT (1:1 000, CST, Danvers, MA, USA), p-AKT (1:500, CST, Danvers, MA, USA), GAPDH (1:2 000, CST, Danvers, MA, USA) overnight at 4°C. The membrane was washed 3 times and incubated with secondary antibody (1:2 500, CST, Danvers, MA, USA) for 2 h at room temperature. Chemiluminescence detection kit (ECL; Millipore, Billerica, MA, USA) was applied to test the relative expression of proteins.

Statistical Analysis

Data were analyzed using Statistical Product and Service Solutions (SPSS) 22.0 statistical analysis (IBM Corp., Armonk, NY, USA) and displayed using GraphPad 6.0 Software (La Jolla, CA, USA). Measurement data were described as mean ± standard deviation (SD). Comparison between multiple groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). $p<0.05$ was considered statistically significant.

Results

Linc00702 Was Lowly Expressed in Colorectal Cancer (CRC) Tissues and Cells

To explore the expression level of Linc00702 in CRC, we collected 91 CRC tissue and paired adjacent normal tissue samples. Linc00702 expression was found to be significantly lower in CRC tissues comparing to the adjacent normal tissues (Figure 1A). Also, Linc00702 was downregulated in five CRC-derived cell lines comparing to the NCM460 cells (Figure 1B). These data indicated that Linc00702 might function as a tumor suppressor in CRC.

Next, we over-expressed Linc00702 level using LV-Linc00702 in Caco2 cells and silenced Linc00702 level using siRNA-Linc00702 in SW620 cells. After transfection of LV-Linc00702, Caco2 cells expressed significantly higher Linc00702 level than LV-Empty group (Figure 1C). SW620 cells transfected with siRNA-Linc00702 showed a markedly reduced Linc00702 expression (Figure 1D).

Ectopic Linc00702 expression Influenced proliferation of CRC cells

To study the influence of Linc00702 on cell proliferation, we employed colony formation assay and EdU assay. Significantly, Caco2 cells overexpressing Linc00702 formed more colonies than LV-Empty group (Figure 2A). In contrast, SW620 cells showed a smaller number of colonies after knockdown of Linc00702 (Figure 2B). Similarly, EdU assay demonstrated that proliferation ability in Caco2 cells was repressed by overexpression of Linc00702, and the opposite trend was observed in SW620 cells with Linc00702

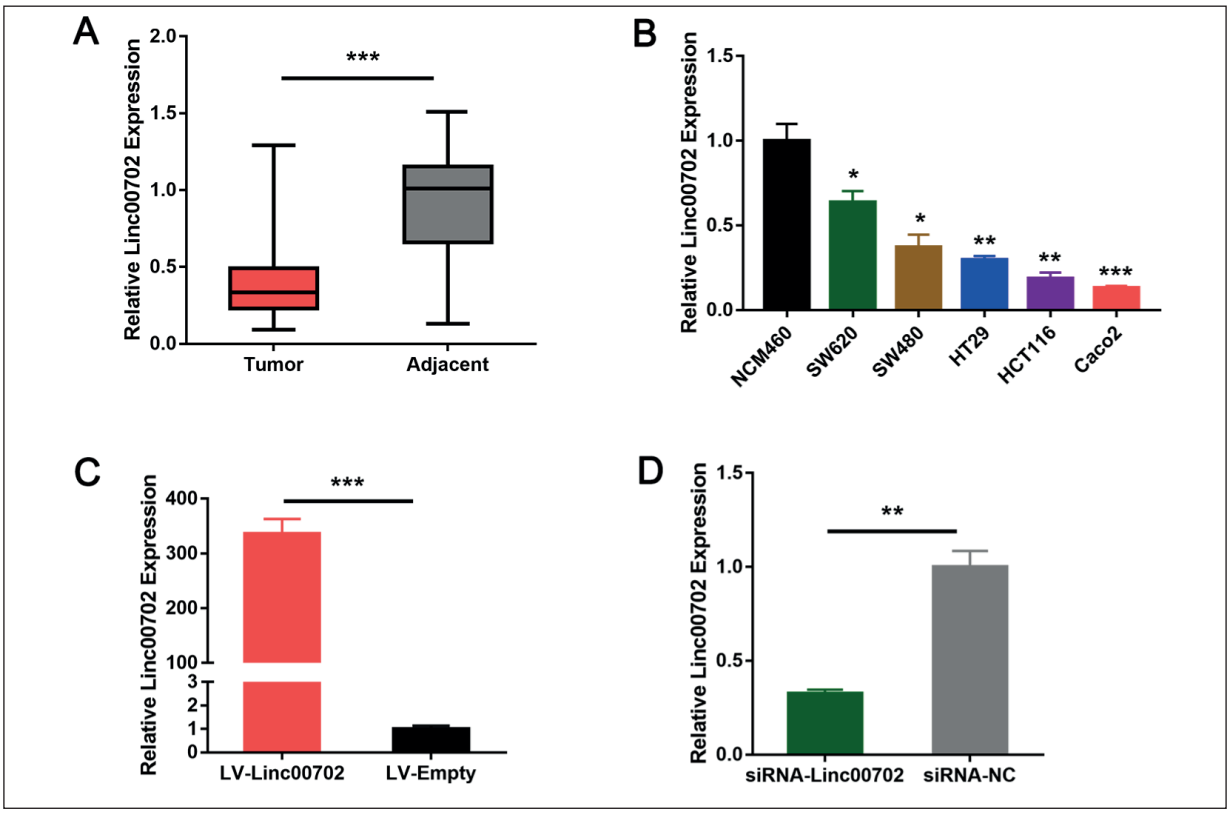

Figure 1. Linc00702 was lowly expressed in CRC tissues and cells. **A,** QRT-PCR showed the Linc00702 expression level in total of 91 CRC tissues and paired non-tumor tissues. **B,** Linc00702 expression level in CRC cell lines (SW620, SW480, HT29, HCT116, and Caco2) and normal colonic epithelial cells NCM460. **C,** LV-Linc00702 or LV-Empty was transfected into Caco2 cells. **D,** SiRNA targeting Linc00702 (siRNA-Linc00702) or negative controls (siRNA-NC) was transfected into SW620 cells. ***p<0.001, **p<0.01 *p<0.05 compared to control group.

knockdown (Figure 2C, 2D). These results suggested that Linc00702 could inhibit cell proliferation of CRC cells.

Linc00702 Affected Cell Migration and Invasion of CRC Cells

We determined the effect of Linc00702 on cell metastasis of CRC using wound-healing assay and transwell assay. Clearly shown in Figure 3, the wound-healing rate of Caco2 cells was significantly reduced by LV-Linc00702 transfection (Figure 3A, 3B), which was enhanced in SW620 cells by knockdown of Linc00702 (Figure 3C, 3D). Transwell assay showed that the ability of cell migration was inhibited by Linc00702 over-expression in Caco2 cells, but improved by Linc00702 down-regulation in SW620 cells (Figure 4A, 4B), which corroborated with wound-healing assay results. Similarly, cell invasion was inhibited in Caco2 cells over-expressing Linc00702, which was stimulated by knockdown of Linc00702 in SW620 cells (Figure 4C, 4D). These results suggested that Linc00702 could reduce cell migration and invasion of CRC cells.

Linc00702 Improved the Expression of PTEN to Regulate the PI3K/AKT Signaling Pathway

Several studies have identified that PTEN was involved in the regulation of CRC progression. Yu et al[14] found that Linc00702 could regulate PTEN in non-small cell lung cancer. Herein, we detected the expression of PTEN in the experimental cells. The expression of PTEN was significantly upregulated in Caco2 cells overexpressing Linc00702, and decreased in SW620 cells transfected with siRNA-Linc00702 (Figure 5). This indicated that Linc00702 could positively regulate PTEN expression in CRC. The activity of PI3K/AKT signaling was determined using Western blot. The phosphorylation

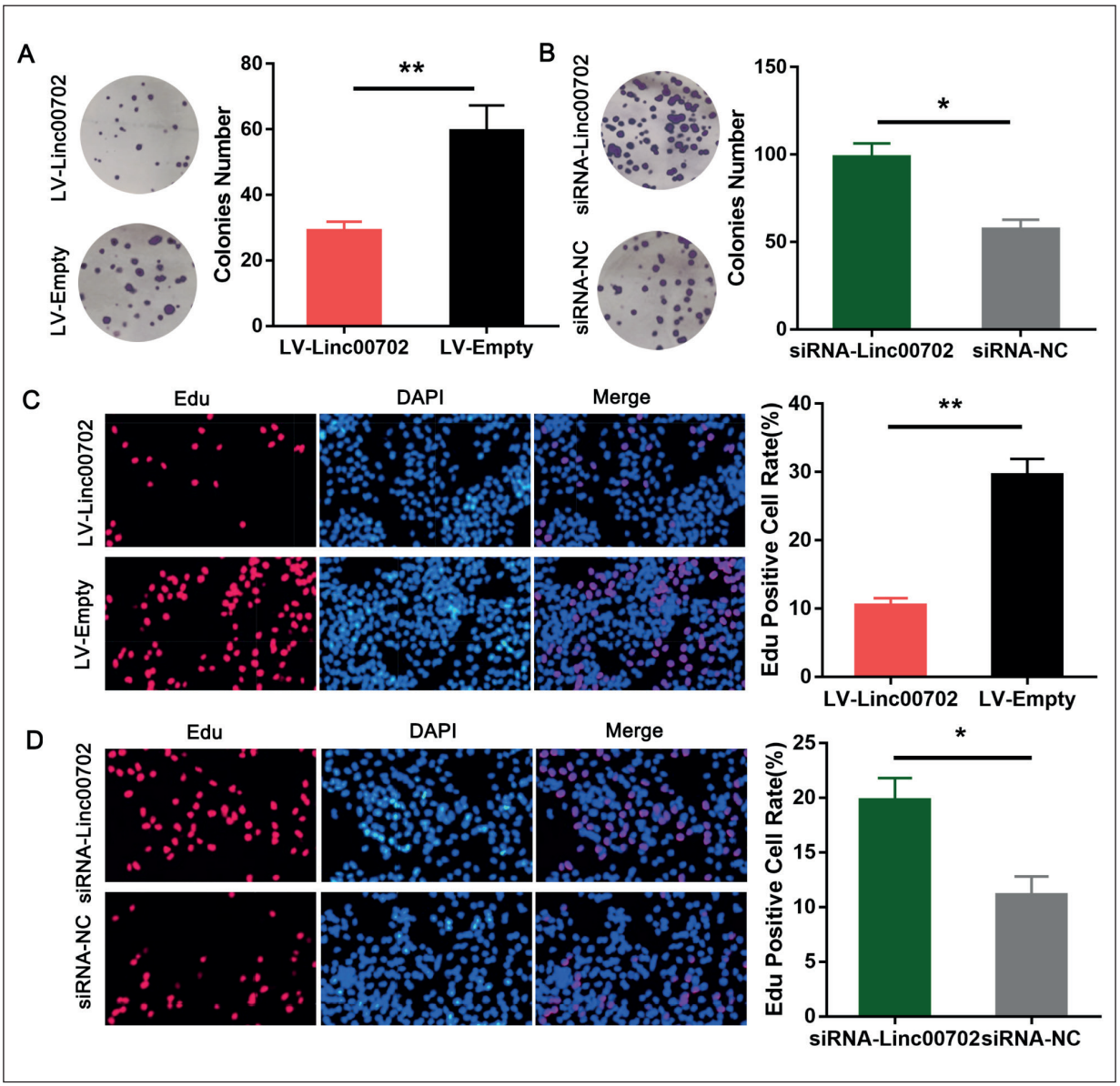

Figure 2. Linc00702 affected the proliferation of CRC cells *in vitro*. **A, B,** Colony formation assay showed the colony formation ability of Caco2 cells transfected with LV-Linc00702 or LV-Empty (**A**), and SW620 cells transfected with siRNA-Linc00702 or siRNA-NC (**B**). **C, D,** EdU assay showed the proliferation ability of Caco2 cells transfected with LV-Linc00702 or LV-Empty (**B**), and SW620 cells transfected with siRNA-Linc00702 or siRNA-NC (original magnification ×200). $^{**}p<0.01$, $^{*}p<0.05$ compared to control group.

of PI3K and AKT was significantly inhibited in Caco2 cells transfected with LV-Linc00702. However, in SW620 cells transfected with siRNA-Linc00702, the expressions of p-PI3K and p-AKT were remarkably improved comparing to control group (Figure 5). These suggested that Linc00702 could promote the expression of PTEN to regulate the PI3K/AKT, thus affecting the progression of CRC.

Discussion

LncRNA has been identified to play an important role in the growth, differentiation, proliferation, development, apoptosis, and metabolism of cancer cells. It could act as a suppressor or oncogene in several tumors[15]. Abnormally expressed lncRNA in a variety of malignant tumors plays an important role in tumor development and pro-

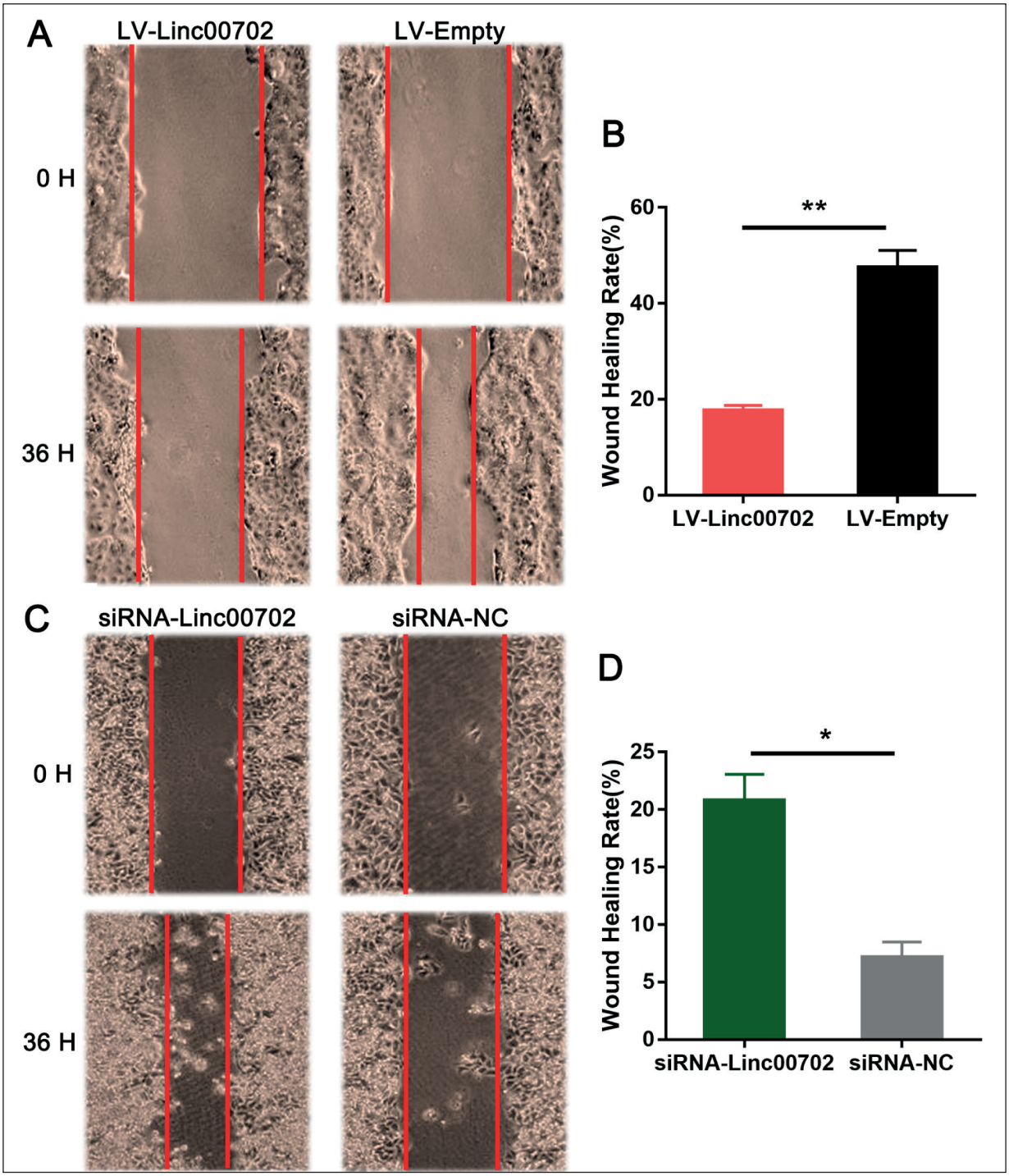

Figure 3. Linc00702 effected the wound-healing of CRC cells. Wound-healing assay indicated the wound-healing rate in established Caco2 cells (**A, B**) and SW620 cells (**C, D**; original magnification ×100). $^{**}p<0.01$, $^{**}p<0.05$ compared to control group.

gression, including CRC[16]. LncRNA UPAT could promote tumorigenesis of CRC *via* repressing the UHRF1 degradation[17]. LncRNA CCAT1 and CCAT2, which located on 8q.24.21, could function as important prognostic biomarkers for CRC patients[18]. Also, lncRNA OCC-1 acts as a CRC suppressor and reduces cell proliferation *via* destabilizing HuR protein[19]. LncRNA UICLM promotes liver metastasis of CRC by sponging miRNA-215, and then regulates expression of ZEB2[20].

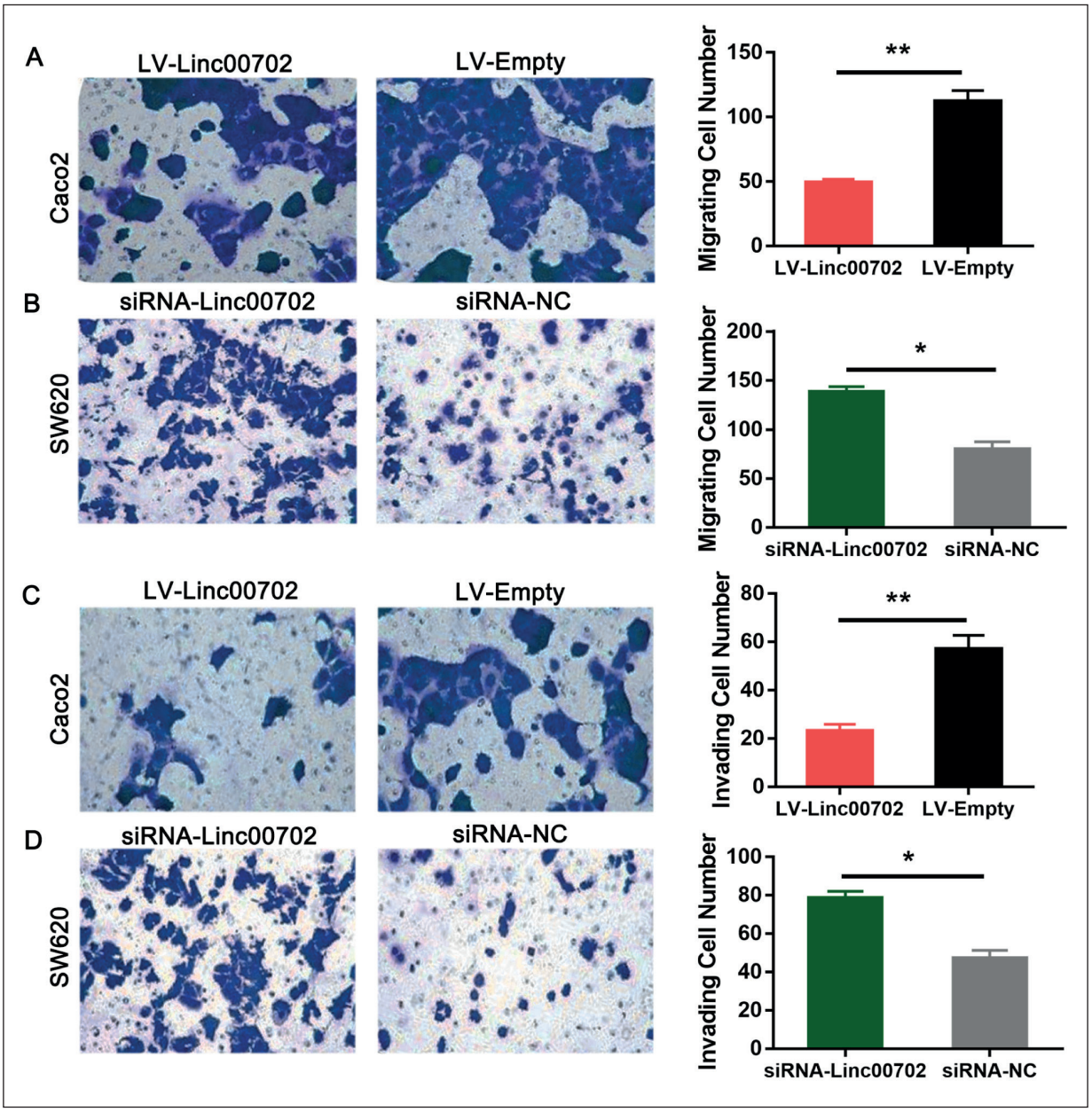

Figure 4. Linc00702 effected the migration and invasion of CRC cells. **A, B,** Transwell migration assay indicated the invaded cell number in established Caco2 cells and SW620 cells. **C, D,** Transwell invasion assay showed the migrated cell number in established Caco2 cells and SW620 cells (original magnification ×100). $^{**}p<0.01$, $^{*}p<0.05$ compared to control group.

Here, we detected Linc00702 expression in 91 CRC tissues and cells for the first time. We found that Linc00702 was lowly expressed in CRC tissues and cells, which was similar to its expression pattern in non-small cell lung cancer[14]. Further, we conducted functional experiments by altering Linc00702 expression in Caco2 or SW620 cells. Colony formation and EdU assays verified that over-expression of Linc00702 inhibited cell growth of Caco2 cells, and knockdown of Linc00702 promoted cell proliferation of SW620 cells. Next, we found Linc00702 could inhibit cell migration and invasion of CRC cells as well. These indicated that Linc00702 acted as a tumor suppressor in CRC, which could provide a target for the treatment of CRC.

Besides, we found PTEN was a potential target for Linc00702 according to the previous

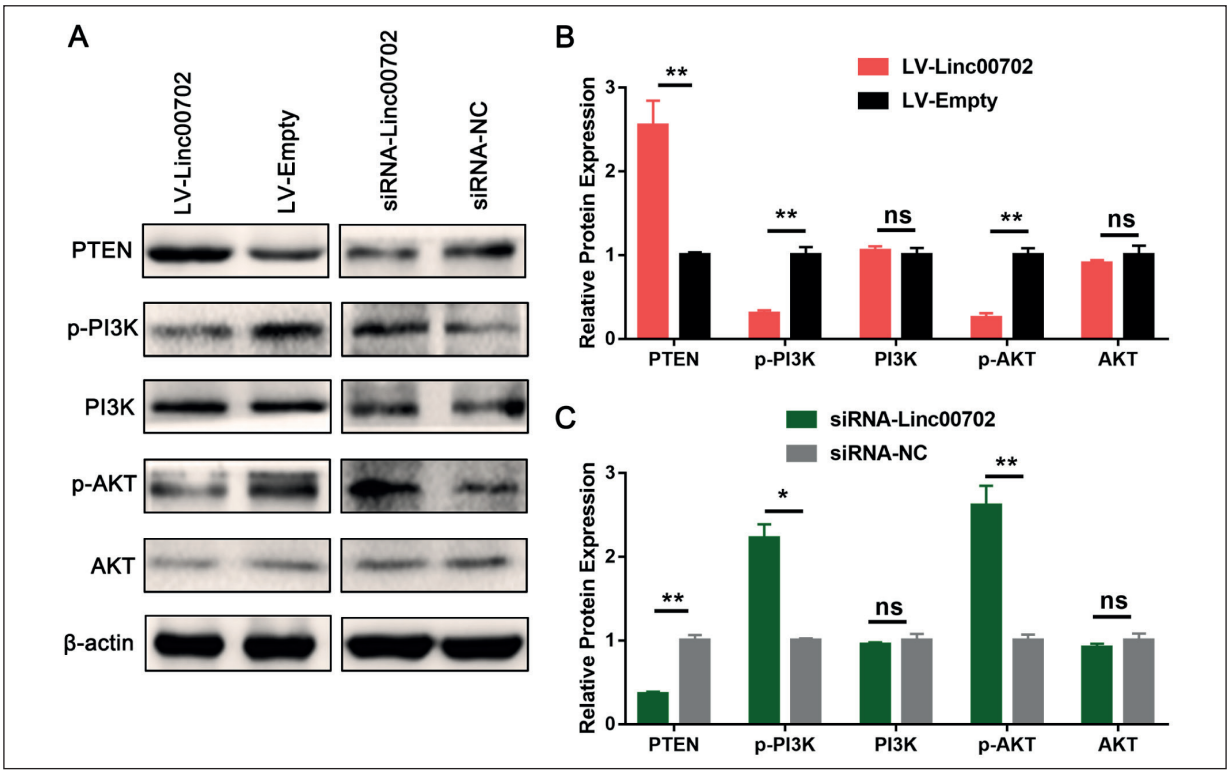

Figure 5. Linc00702 promoted expression of PTEN. Western blot assay indicated the PTEN, PTEN, PI3K, p-PI3K, AKT, p-AKT protein expression in established Caco2 cells (**A, B**) and SW620 cells (**A, C**). **p<0.01, *p<0.05, ns non-sense compared to control group.

study. Overexpression of Linc00702 promoted expression of PTEN in Caco2 cells, while knockdown of Linc00702 inhibited PTEN expression. PTEN is a classical tumor-suppressor gene that is expressed in a variety of cancers and encodes a phosphatase by negatively regulating the PI3K/AKT/mTOR signaling pathway[21-23]. In CRC, it could act as a target for miRNAs and lncRNAs, such as miRNA-543, Linc02023[24-26]. Next, we measured the phosphorylation of PI3K and AKT to confirm the effect of Linc00702. Clearly, over-expression of Linc00702 inhibited the phosphorylation of PI3K and AKT, while knockdown of Linc00702 promoted the phosphorylation of PI3K and AKT. These confirmed that Linc00702 could promote PTEN expression to regulate the activation of the PI3K/AKT signaling pathway. Herein, Linc00702 could inhibit the proliferation and metastasis of CRC. Despite the lack of evidence for further *in vivo* experiments, our study could still explain the expression and role of Linc00702 in CRC from a certain perspective.

Conclusions

Taken all together, our study elucidated the low expression of Linc00702 in CRC tissues and cells for the first time. Linc00702 could reduce the proliferation, migration, and invasion of CRC cells *via* repressing the PI3K/AKT axis by positively regulating PTEN. This might provide a novel target for the study of CRC tumorigenesis and progression and biological treatment for CRC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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