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Promoting effect of PAX5-activated lncRNA UASR1 on growth of colorectal cancer by regulating the mTOR pathway

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Abstract. – OBJECTIVE: To study the expression of long non-coding ribonucleic acid (lncRNA) UNC5B antisense RNA 1 (UASR1) in colorectal cancer (CRC) and its biological functions, and to discuss the regulatory effect of the transcription factor on lncRNA UASR1.

PATIENTS AND METHODS: The expressions of lncRNA UASR1 in the CRC tissues and cells were detected *via* quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) assay. After the expression of lncRNA UASR1 was interfered, the change in the CRC cell proliferation ability was investigated through cell counting kit-8 (CCK-8) assay and colony formation assay, respectively. Changes in cell cycle distribution and apoptosis rate in CRC cells after transfection of small-interfering UASR1 (si-UASR1) were detected using flow cytometry. Potential transcription factors binding UASR1 promoter region were analyzed through bioinformatics. The change in the UASR1 expression was measured through the qRT-PCR assay after the paired box 5 (PAX5) expression was interfered. Following the interference in the expressions of PAX5 and UASR1, expression changes in the molecular markers of the mammalian target of rapamycin (mTOR) signaling pathway were detected *via* Western blotting assay.

RESULTS: The qRT-PCR results indicated that the UASR1 expression was up-regulated in 39/45 CRC tissues, and it identically presented the up-regulated expression level in the CRC cells. After the UASR1 expression was interfered, the CRC cell proliferation ability was degraded according to the CCK-8 assay and colony formation assay. Based on the flow cytometry results, compared with the small-interfering-negative control (si-NC) group, the cell cycle was arrested in the G1/G0 phase in the si-UASR1 group, and apoptosis rate increased. Bioinformatics and qRT-PCR results showed that the transcription factor PAX5 regulated the UASR1 expression. The Western blotting assay indicated that the expressions of the molecular markers of the mTOR signaling pathway were changed after the expressions of PAX5 and UASR1 were interfered.

CONCLUSIONS: The transcription factor PAX5 promotes the expression of lncRNA UASR1 in CRC. The highly expressed UASR1 facilitates the malignant proliferation of CRC *via* the mTOR signaling pathway.

Key Words:
Colorectal cancer, LncRNA UASR1, PAX5, mTOR.

Introduction

Morbidity and mortality rates of colorectal cancer (CRC), which is one of the three major malignant tumors in the world, are still presenting a rising tendency globally[1,2]. The genesis and development of CRC are closely related to dietary factors, hereditary factors, polyps, chronic inflammatory stimuli, etc. Malignant proliferation, invasion, and metastasis of the cancer cells are the main causes for the high mortality rate of CRC, but the detailed molecular mechanism remain unclear[3,4].

With the development of the whole genome and transcriptome sequencing technology, long-coding ribonucleic acids (lncRNAs), which are initially considered as "noise" in the transcription process, have been well explored nowadays[5]. As a kind of non-coding RNA molecules with a length

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greater than 200 nt, lncRNAs regulate cell proliferation, differentiation, and apoptosis mainly at the epigenetic, transcriptional, and posttranscriptional levels. They are extensively involved in chromatin modification, gene imprinting, protein folding, and alternative RNA splicing, thus influencing metabolism and disease progression[6-8].

The genesis and development processes of CRC are usually accompanied by the abnormally expressed lncRNAs, which are functionally similar to the proto-oncogenes and cancer-suppressor genes in the tumorigenesis process. As previously reported, highly expressed linc00668 regulates the expression of USP47 by sponging miR-188-5p, so as to promote the genesis and development of CRC[9]. Yang et al[10] reported that lncRNA KIAA0125, a tumor-inhibitor, suppresses CRC cell proliferation and metastasis by regulating the Wnt/β-catenin signaling pathway. Through an *in-vitro* experiment carried out by this research group, it was found that lncRNA UNC5B antisense RNA 1 (UASR1) is up-regulated in the CRC tissues and cells, and the highly expressed UASR1 promotes the CRC proliferation and inhibits the apoptosis. This research provided a direction and early-stage foundation for exploring the malignant phenotype formation of CRC.

Patients and Methods

Cell Culture and Transfection

Normal human colonic epithelial cell NCM460 and CRC cells HT29, LOVO, SW480, SW620, and HCT116 were all preserved in the lab. The cells were inoculated in the Roswell Park Memorial Institute-1640 (RPMI-1640) or Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin, in an incubator containing 5% CO_2 at 37°C. Cell transfection was conducted using Lipofectamine 2000. The interfering sequences were: si-PAX5: 5′-GCAGUCUGUUCUGAACAUA-3′; si-UASR1 #1 5′-CCATTCCATTCATTTCTCTTTCCTA-3′; si-UASR1 #2 5′-GGCGTAGGC GATTGGGGATCG-3′; si-UASR1#3: 5′-ATTTAAGGAGCGGATTTAGC-3′.

Tissue Specimens

A total of 39 cases of operative specimens and paired para-carcinoma tissues of CRC patients in Changhai hospital from January 2017 to June 2018 were collected. The patients were definitely diagnosed with CRC in the Pathology Department of the hospital. After the operative specimens were marked, they were preserved in a –80°C ultralow-temperature freezer. This investigation was carried out with the informed consent from the patients and the approval from the Medical Ethics Committee of Changhai hospital.

RNA Extraction and Reverse Transcription

Tissues (30 mg) were grinded and smashed repeatedly using liquid nitrogen. Cells were rinsed in precooled phosphate-buffered saline (PBS) and then centrifuged to discard the supernatant. TRIzol lysate (Invitrogen, Carlsbad, CA, USA) was added and RNAs in the tissues or cells were extracted following the instructions, which were then preserved at –80°C.

Reverse transcription was performed using lnRcute lncRNA First-Strand complementary deoxyribose nucleic acid (cDNA) Synthesis Kit (Guangzhou TIANGEN Biotech Co., Ltd., Guangzhou, China), and the reaction system was prepared according to the instructions. The operation procedure of the reverse transcription was: 42°C for 15 min and 95°C for 3 min. Afterwards, the samples were preserved in the refrigerator at –20°C.

QRT-PCR

The lnRcute lncRNA qPCR Detection Kit (TIANGEN Biotech Co., Ltd., Beijing, China) was utilized to perform quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) assay. The primer sequences were: glyceraldehyde 3-phosphate dehydrogenase (GAPDH) sense, 5′-GCACCGTCAAGGCTGAGAAC-3′ and antisense, 5′-TGGTGAAGACGCCAGTGGA-3′. LncRNA UASR1 sense, 5′-TGTCGCTCAGCCAACATGC-3′ and antisense, 5′-AAAGTGCAAGTTGCATCGCTC-3′. PAX5 sense, 5′-CTCGCTTCGGCAGCACA-3′ and antisense, 5′-AACGCTTCACGAATTTGCGT-3′. The PCR reaction system was configured according to the instructions. PCR operation procedure: 95°C for 3 min, 95°C for 5 s, and 60°C for 15 s, for a total of 38 cycles. The relative expression level was calculated according to the $2^{-\Delta\Delta ct}$ value.

Cell Counting Kit-8 (CCK-8)

CRC cells in the logarithmic growth phase were transfected with small-interfering-negative control (si-NC) or small-interfering-UASR1 (si-UASR1), respectively. 10 μL of CCK-8 solution (Dojindo Laboratories, Kumamoto, Japan) was

added into each well in each group at 0 h, 24 h, 48 h, 72 h, and 96 h, respectively, after the transfection. The cells were incubated under conditions with saturated humidity and 5% CO_2 at 37°C for 3 h. Absorbance (A) value at the wavelength of 450 nm in each well was detected using Multiskan MK3 microplate reader. A_{450} value represented the cell proliferation ability.

Colony Formation Assay

The cells were inoculated in a 6-well plate, followed by continuous culture for 2-3 weeks. The culture was terminated when visible colonies formed. Cells were rinsed twice using PBS and fixed in 4% paraformaldehyde for 15 min, followed by staining with crystal violet for 30 min. Afterwards, the staining solution was slowly washed off with running water, and colonies were photographed, counted, and analyzed.

Cell Apoptosis Assay

The cells were suspended in 200 μL of PBS and incubated with fluorescein isothiocyanate (FITC)-labeled Annexin V and propidium iodide (PI; BD Biosciences, Franklin Lakes, NJ, USA) for 20 min at room temperature. Apoptosis rate was analyzed *via* a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Cell Cycle Detection

About 1×10^6 cells were harvested after the trypsin digestion and then fixed in precooled 70% ethanol (4°C) at –20°C overnight. After the cells were rinsed twice using PBS, they were re-suspended in PI (50 μg/mL) and RNAseA (0.1 mg/mL, Sigma-Aldrich, St. Louis, MO, USA), followed by staining for 30 min at room temperature and the analysis *via* the flow cytometer.

Western Blotting

Immediately after the cells were treated using protein lysate, the total protein concentration was determined using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). The target proteins (35 mg) were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). Membranes were sealed in 5% milk for 2 h and incubated with primary antibodies (anti-GAPDH: 1: 1000. Anti-PAX5: 1:1000. anti-p-mTOR:1:800, anti-mTOR:1:800) at 4°C overnight. Subsequently, they were incubated using horseradish peroxidase (HRP)-conjugated second antibodies for 2 h. Chemiluminescence was performed *via* enhanced chemiluminescence (ECL) apparatus (Bio-Rad, Hercules, CA, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 software (SPSS Inc., IBM, Armonk, NY, USA) was used for the statistical analysis. Measurement data were expressed by ($\bar{x} \pm s$), and enumeration data by percentage (%). Student's *t*-test was used for the comparison of measurement data, and χ^2-test for the comparison of enumeration data. $p<0.05$ indicated that the difference was statistically significant.

Results

Expression of LncRNA UASR1 Was Up-Regulated in CRC

First, the expression levels of USAR1 in 45 CRC cases of tissues and corresponding para-carcinoma tissues were detected *via* qRT-PCR assay. The results showed that compared with that in the para-carcinoma tissues, the expression of USAR1 was up-regulated in 39 cases of CRC tissues (Figure 1A). Next, the relative expression of USAR1 in the CRC cells was detected. Compared with the colonic epithelial cell NCM460, the CRC cells exhibited up-regulated expression of USAR1 (Figure 1B). To investigate the biological functions of USAR1, its specific interfering sequences were designed and transiently transfected into the CRC cells, and 48 h later, the interference efficiency was measured (Figure 1C and D).

LncRNA UASR1 Promoted the CRC Cell Proliferation

CCK-8 assay showed that after transfection of si-USAR1, the CRC cell proliferation ability was degraded (Figure 2A and 2B). The colony formation assay result was identical with the CCK-8 assay result (Figure 2C and 2D). Afterwards, the effect of UASR1 on the CRC cell cycle was investigated, and the result manifested that in comparison with that in the si-NC group, the cell cycle was arrested in the G1/G0 phase in si-UASR1 group (Figure 2E and 2F).

Paired Box 5 (PAX5) Promoted Transcription of UASR1 and Inhibited CRC Cell Apoptosis

According to the flow cytometry results, after the interference in UASR1 expression, the CRC cell

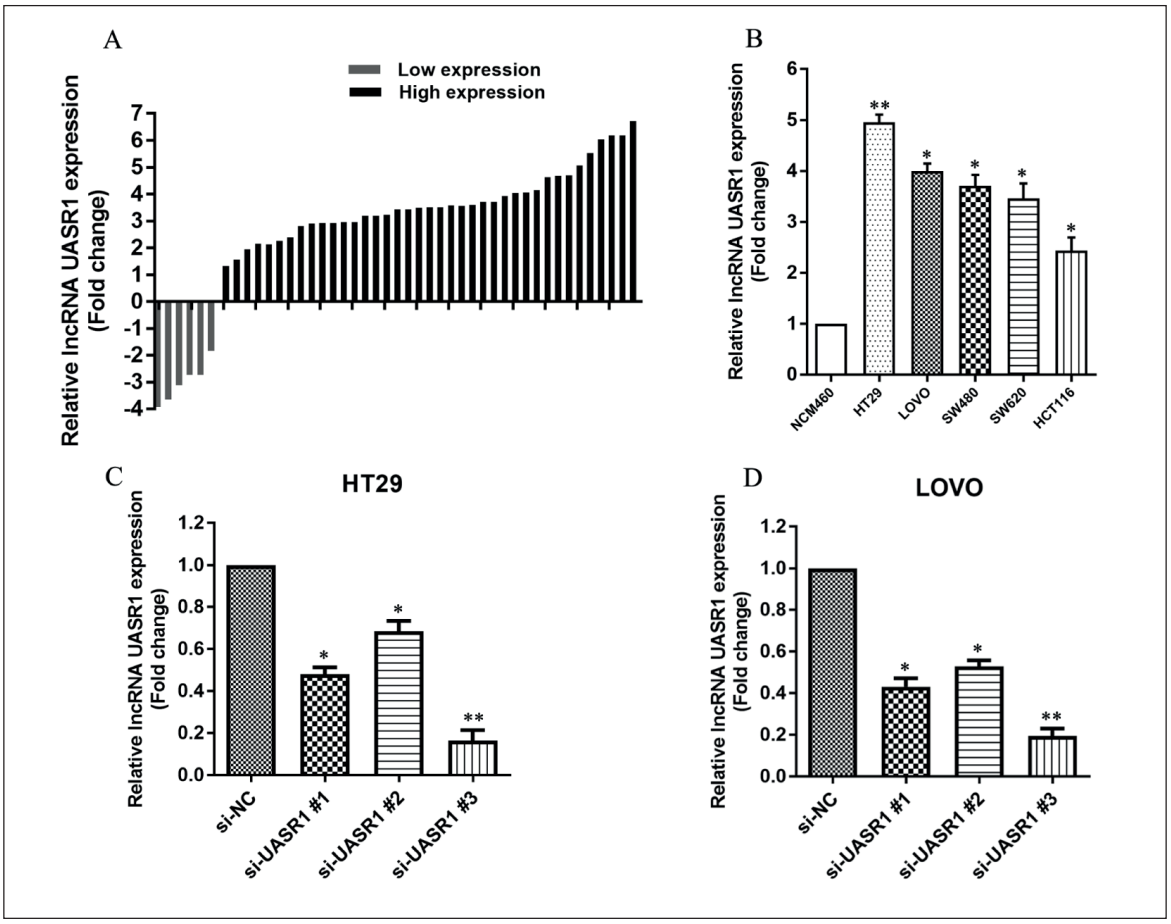

Figure 1. Expression of lncRNA UASR1 in CRC is up-regulated. **A,** Expressions of UASR1 in 47 cases of CRC tissues are detected through qRT-PCR assay. The results indicate that UASR1 presents the up-regulated expressions in 39 cases of CRC tissues, and GAPDH is taken as the internal reference. **B,** Relative expressions of UASR1 in CRC cells are detected via qRT-PCR assay, and the results indicate that the UASR1 expression is up-regulated. **C,** and **D,** Si-UASR1 is transiently transfected into CRC cells, and the transfection efficiency is measured using qRT-PCR assay 48 h later. (**p<0.01, *p<0.05).

apoptosis rate markedly increased (Figure 3A and 3B). To study the possible mechanism of UASR1 in regulating phenotype changes of CRC, the bioinformatics method (http://genome.ucsc.edu/) was applied to obtain the UASR1 promoter sequence. Meanwhile, the PROMO database (http://alggen.lsi.upc.es/ cgi-bin/promo_v3/promo/promoinit.cgi?irDB=TF_8.3) was utilized to predict the transcription factors binding UASR1 promoter region (Figure 3C and 3D). Furthermore, qRT-PCR assay verified that the transcription factor PAX5 promoted the UASR1 expression (Figure 3E).

LncRNA UASR1 Regulated the Mammalian Target of Rapamycin (mTOR) Signaling Pathway

The downstream molecular signaling pathways involved in UASR1 regulation were explored. After the UASR1 expression was knocked down, the expression changes in the molecular markers of the mTOR signaling pathway were determined *via* Western blotting assay (Figure 4A). After the PAX5 expression was interfered, the Western blotting assay result was consistent with that in the si-UASR1 group (Figure 4B).

Discussion

As a common malignant tumor, CRC takes the 3rd place among the death causes of global malignant tumors[11]. The development of CRC is a multistep and multifactor process, and the complicated pathogenesis of CRC remains to be clearly clarified[12].

The genetic expression is regulated by all kinds of molecular substances at various levels.

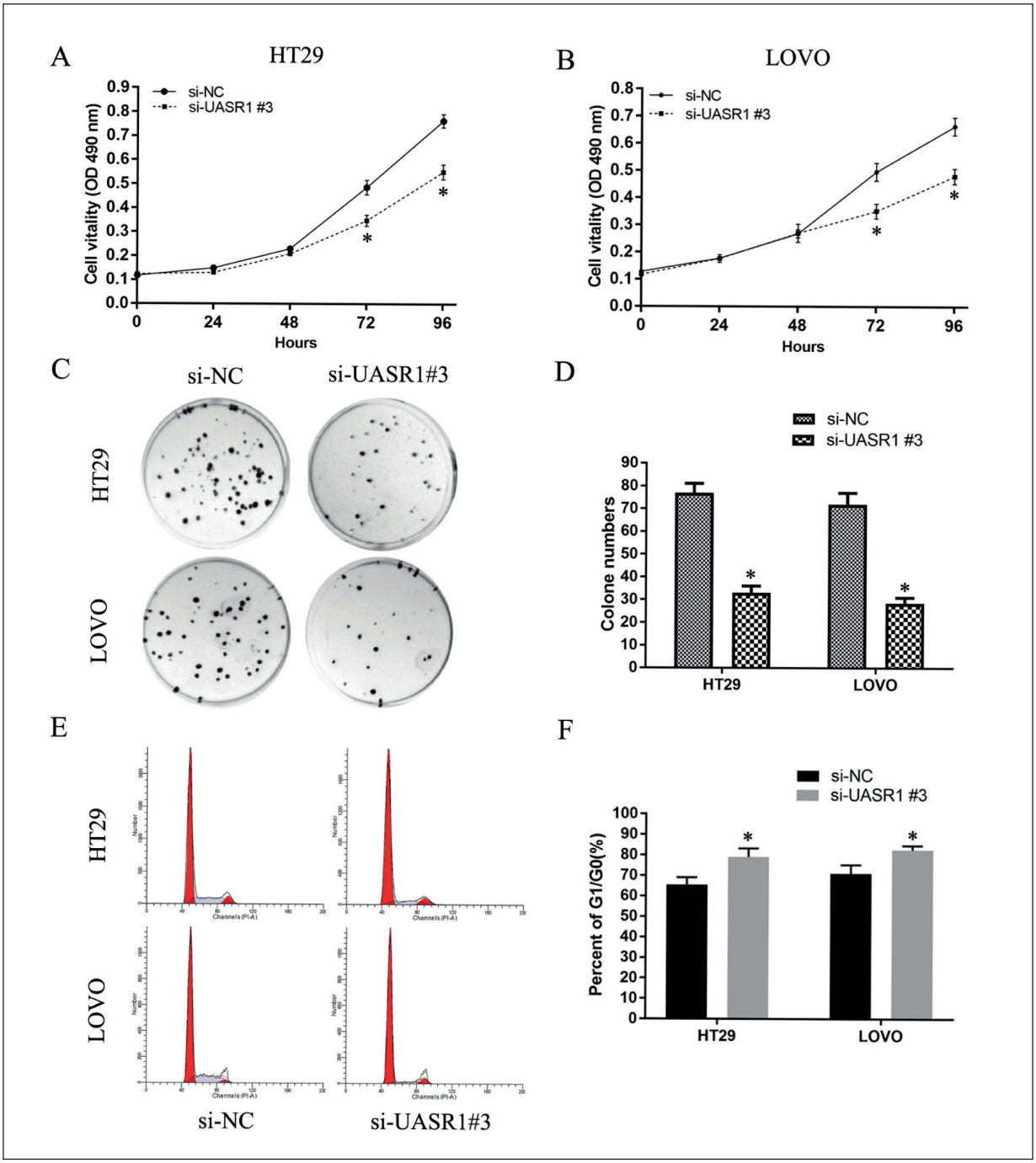

Figure 2. LncRNA UASR1 promotes CRC cell proliferation. **A,** and **B,** According to the CCK-8 assay results, compared with that in the si-NC group, the cell proliferation ability is degraded in the si-UASR1 group. **C,** and **D,** Colony formation assay results show that the interference in the UASR1 expression suppresses the CRC cell proliferation (40×). **E,** and **F,** Si-NC and si-UASR1 are transfected into the CRC cells, and the flow cytometry results indicate that the cell cycle is arrested in the G1/G0 phase in si-UASR1 group. (**p<0.01, *p<0.05).

With the length greater than 200 nt and the structure similar to that of coding RNAs, lncRNAs can bind to the homologous DNA sequences (genes transcribing lncRNAs and possessing similar sequences) and homologous RNA sequences. Moreover, it can bind to proteins as it can be folded into a complicated secondary structure, indicating that it plays a significant role in the

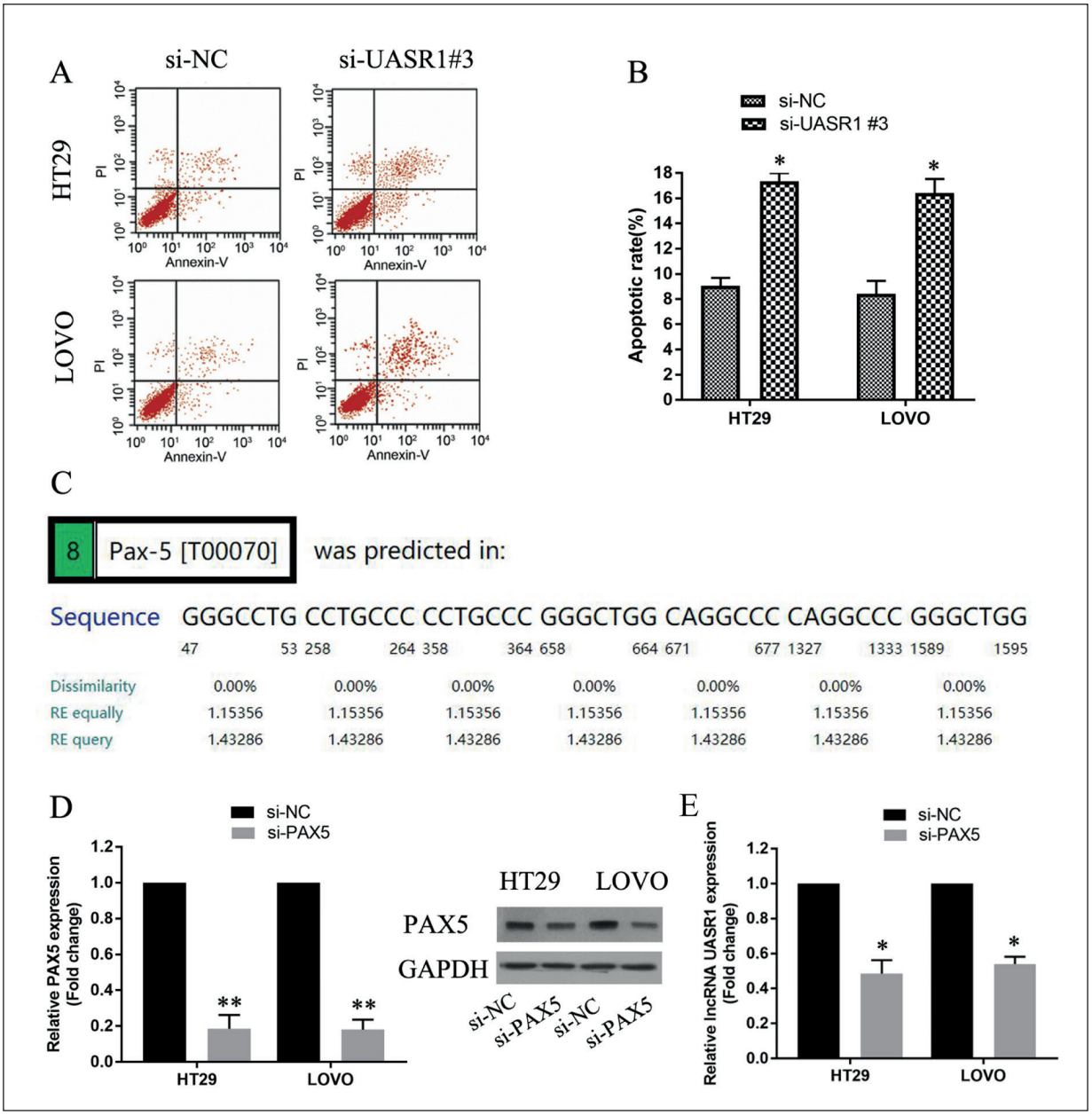

Figure 3. PAX5 promotes transcription of UASR1. **A,** and **B,** Based on the flow cytometry results, the cell apoptosis rate is increased after the UASR1 expression is knocked down. **C,** Bioinformatics method is utilized to predict the binding sites of the transcription factor PAX5, which exist in the lncRNA UASR1 promoter region. **D,** Interference efficiency of si-PAX5 determined by qRT-PCR assay and Western blotting assay. **E,** According to the qRT-PCR assay, the UASR1 expression declines after the PAX5 expression is interfered. (**p<0.01, *p<0.05).

genetic expression process[13,14]. A large quantity of lncDNAs has been verified related to the genesis and development of CRC, such as HOXB-AS3, lncRNA-422, CLMAT3, and LOC554202[15,16]. LncRNA UASR1 is located on the chromosome 10q22.1 region with an overall length of 652 bp. As reported, the UASR1 presents the up-regulated expression in breast cancer and promotes its genesis and development[17]. However, the related situation of UASR1 in CRC has not been reported yet. The research group is the first to find that the UASR1 expression was up-regulated in the CRC tissues and cells, and it promoted the CRC proliferation and inhibited the apoptosis.

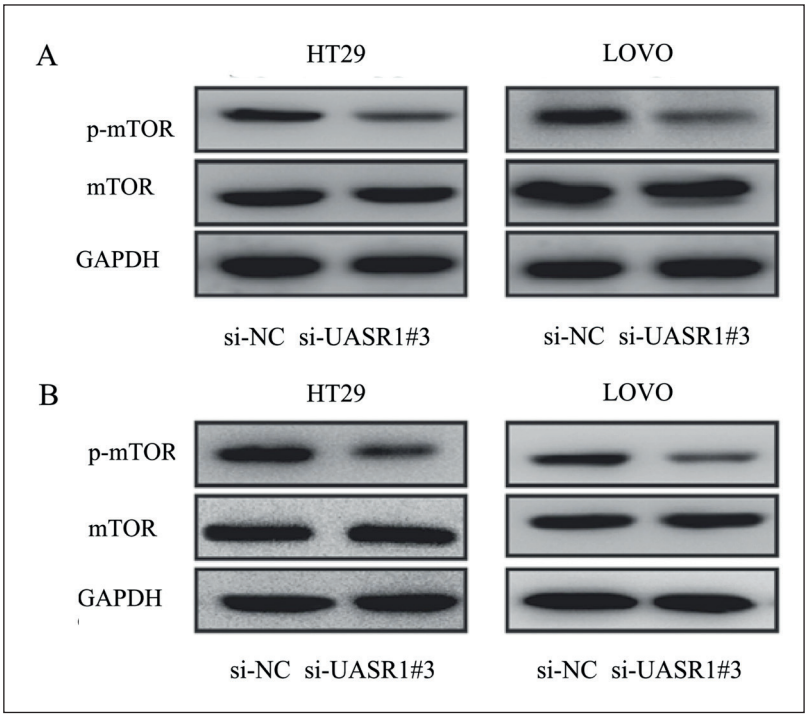

Figure 4. LncRNA UASR1 regulates mTOR signaling pathway. **A,** After the interference in UASR1 expression, the expression changes in the molecular markers of the mTOR signaling pathway are detected via Western blotting assay. **B,** After the PAX5 expression is interfered, Western blotting assay is performed to detect the expression changes in the molecular markers of the mTOR signaling pathway.

The genetic expression is regulated at various levels (epigenetics, transcriptional level, translational level, and posttranscriptional modification, etc.)[18,19]. To explore the reason for the high expression of UASR1 in CRC, transcriptional regulation mechanism of UASR1 in CRC was highlighted in this research. It was discovered through the bioinformatics method that PAX5 may be a target binding UASR1 promoter region. Moreover, PAX5 exerts important effects on the tumor cell proliferation, apoptosis, and phenotype transformation[20]. It has been reported that PAX5 can promote the transcription of lncRNA FOXP4-AS1, thus facilitating the proliferation of prostatic cancer[21]. It was verified *via* qRT-PCR assay in this research that PAX5 could promote the UASR1 transcription.

Conclusions

We first found that the transcription factor PAX5 promotes the expression of lncRNA UASR1 in CRC. The highly expressed UASR1 facilitates the malignant proliferation of CRC *via* the mTOR signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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