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Circ-0104631 promotes cell proliferation and invasion in colorectal cancer and predicts poor prognosis

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Abstract. – OBJECTIVE: The aim of this study was to elucidate the effect of Circ-0104631 on the progression of colorectal cancer (CRC) and to demonstrate the underlying mechanism. Our research might provide new biological markers and molecular therapeutic targets for the diagnosis and therapy of CRC.

PATIENTS AND METHODS: Quantitative real-time polymerase chain reaction (qRT-PCR) was applied to detect Circ-0104631 expression in human colorectal cancer tissues and normal control tissues. To further explore the effect of Circ-0104631 on CRC *in vitro*, we first knocked down Circ-0104631 in colorectal cancer cells (SW480 and LoVo) by shRNA transfection. Subsequently, we detected its effect on cell proliferation and invasion by cell counting kit-8 (CCK-8) assay, colony formation assay and cell invasion assay, respectively. The regulation of Circ-0104631 on the expressions of phosphate and tension homology deleted on chromosome ten (PTEN)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) signaling pathway-related proteins was detected by Western blot. Besides, the regulatory mechanism of Circ-0104631 on the progression and metastasis of CRC was further verified by recovery experiments.

RESULTS: QRT-PCR results showed that Circ-0104631 was highly expressed in tissues of patients with CRC when compared with that of normal control tissues. At the same time, we also found that the expression of Circ-010463 was significantly up-regulated in CRC tissues with high topography lymph node metastasis (TNM) stage and distant metastasis. Survival curve analysis indicated that high expression of Circ-010463 predicted poor prognosis of CRC patients. *In vitro* experiment demonstrated that inhibition of Circ-0104631 in SW480 and LoVo cells could markedly decrease cell growth and metastasis abilities. Meanwhile, Western blot results indicated that the protein expression of PTEN increased significantly, while p-Akt and p-mTOR decreased remarkably after knock-down of Circ-0104631 in CRC cells. Furthermore, recovery experiments illustrated that knockdown of PTEN in SW480 and LoVo cells partially attenuated the inhibitory effect of shRNA-Circ-0104631 on cell growth and metastasis.

CONCLUSIONS: Circ-0104631 was highly expressed in CRC tissues. Furthermore, knockdown of Circ-0104631 could inhibit the growth and metastasis of CRC cells by regulating PTEN/Akt/mTOR signaling pathway.

Key Words:
Colorectal cancer (CRC), Circ-0104631, Cell proliferation, Cell invasion, PTEN/Akt/mTOR.

Introduction

Colorectal cancer (CRC) is a common malignant tumor of the digestive tract with high incidence and mortality. Meanwhile, it is also one of the three major tumors in the world[1]. The incidence of CRC is related to various factors, including high-fat diet, inadequate intake of plant fiber, smoking and obesity. The clinical manifestations of CRC include abdominal pain, bloating, constipation, blood in the stool, intestinal obstruction, weight loss, and fever. At present, the main treatment methods for clinical CRC are surgery, radiotherapy, chemotherapy, and targeted therapy[2]. Previous studies have indicated that the prognosis of CRC is closely related to early diagnosis. It is reported that the 5-year survival rate of early CRC can be as high as 90% through radical resection.

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However, the long-term survival rate and prognosis of advanced CRC are poor, which is less than 10% in patients in late stages. Therefore, recurrence and metastasis remain as the leading causes of death in CRC patients. Even if no metastasis is detected in lymph nodes other than tumor tissues, the recurrence rate can reach 25%[3].

Exploring the mechanism of recurrence and metastasis of CRC and finding biomarkers for CRC diagnosis and therapeutic targets are the main tasks and directions of current research[4]. Studies showned[5-7] that the occurrence, development and metastasis of CRC involve changes in multiple genes, such as tumor suppressor gene p53, oncogene C-myc, deleted in colorectal carcinoma (DDC), matrix metalloproteinases (MMPs), etc. Currently, multiple studies revealed that non-coding RNAs, such as micro RNA, long noncoding RNA, and circular RNA, play important roles in the development of CRC[8-10].

Cyclic ribonucleic acid (RNA) is an RNA molecule that is ubiquitous in animal and plant cells. It can regulate gene expression through a variety of pathways. As early as the 1970s, it was discovered that cyclic RNA molecules existed in RNA viruses[11]. However, the research on circRNA was not deep enough, and they had not received sufficient attention. By 2000, the number of circRNAs discovered was not enough. With the rapid development of RNA sequencing technology and bioinformatics, it has been found that transcripts of many human exons are different from previous linear splicing. However, they are reverse spliced or formed by gene rearrangement into CircRNAs. Meanwhile, these circular RNAs account for a large proportion of all spliced transcripts[12]. At present, increasing evidence has demonstrated that CircRNA is not only closely related to many human diseases but also exerts potential diagnostic and adjuvant therapeutic value[13,14].

Previous research has revealed that Circ-0104631 is highly expressed in CRC tissues. However, its specific role in CRC and the possible mechanism remains unclear[15]. In this experiment, we analyzed the expression of Circ-0104631 in CRC tissues and normal control tissues. The relationship between its expression and prognosis of CRC was explored as well. Furthermore, by knocking down the expression of Circ-0104631 in cells, Circ-0104631 function in CRC was studied, and its mechanism was preliminarily explored. Our findings might provide experimental evidence for early diagnosis and treatment of CRC.

Patients and Methods

Specimen Collection and Processing

Forty-six CRC tissues and 32 normal control tissues were obtained from patients with CRC who underwent surgery at Shanxian Central Hospital from April 2017 to July 2018. All specimens were confirmed by pathology. No radiotherapy, chemotherapy or other adjuvant treatment was performed before surgery. All collected samples were quickly frozen in liquid nitrogen and placed in a -80°C freezer for storage. Informed consent was obtained from each subject before the study. This research was approved by the Ethics Committee of Shanxian Central Hospital.

Cell culture and transfection

CRC cell lines (SW480, LoVo, HCT116, and HT29), as well as normal control cell line (NCM460), were purchased from ATCC (Manassas, VA, USA). All cells were cultured in radioimmunoprecipitation assay-1640 (RIPA-1640: Hyclone, South Logan, UT, USA) medium containing 10% inactivated newborn fetal bovine serum (FBS: Gibco, Grand Island, NY, USA), 100 U/mL penicillin and streptomycin (Gibco, Grand Island, NY, USA) in a 5% CO_2 incubator at 37°C. For Circ-0104631 or phosphatase and tensin homolog deleted on chromosome ten (PTEN) silencing, shRNAs against Circ-0104631 or PTEN were constructed into pSicoR lentiviral vectors. Subsequently, CRC cells were transfected with lentivirus and Circ-0104631 or PTEN-silenced shRNAs for 2 weeks using puromycin. Transfection efficiency was evaluated by quantitative real-time polymerase chain reaction (qRT-PCR). Moreover, the cells were harvested for subsequent experiments.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

RNA in cells or tissues was extracted according to the instructions of TRIzol REAgent (Invitrogen, Carlsbad, CA, USA). Subsequently, extracted RNA was reverse transcribed into complementary deoxyribonucleic acid (cDNA) by PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). QRT-PCR was performed by the SYBR® Green Master Mix (TaKaRa, Otsu, Shiga, Japan) according to the instructions. U6 was used as an internal reference. Relative gene expressions were calculated by the $2^{-\Delta\Delta CT}$ method. Primer sequences used in this study were as follows: Circ-0104631, F: 5'-CGGTAAACCTCACTTGGCTC-3', R:

5'-CACCGTTGTGAGGAGAACGGA-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'.

Cell Counting Kit-8 (CCK-8) Assay

Circ-0104631 or PTEN-silenced cells and normal control cells were digested and inoculated into 96-well plates at a density of 3000 cells per well. 8 replicate wells were set in each group. The viability of cells was measured by CCK-8 (Dojindo, Kumamoto, Japan) at 24 h, 48 h, 72 h, and 96 h, respectively. 2 h before CCK-8 assay, the cells were added with 10 μL of CCK-8 solution and then incubated at 37°C for 1 h. The absorbance of each well at 450 nm was measured by a microplate reader.

Colony Formation Assay

Circ-0104631 or PTEN-silenced and negative control SW480 and LoVo cells were first seeded into 12-well plates, followed by culture in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Hyclone, South Logan, UT, USA) for two weeks. After that, formed cell colonies were stained with crystal violet. Finally, the cells were photographed, and the number of formed colonies was counted for statistical analysis.

Cell Invasion Assay

Transfected SW480 and LoVo cells in serum-free medium were seeded into the upper chamber at a density of 1 x 10^5 cells per well. Matrigel BioGlue (Invitrogen, Carlsbad, CA, USA) was covered at the bottom of the upper layer. Meanwhile, medium containing 10% FBS was added to the lower chamber as a chemotactic agent. Subsequently, the cells were placed at 37°C for 48 h. After that, the cells invading the lower surface of the filter were fixed in 70% ethanol for 30 min and stained with 0.1% crystal violet for 10 min. The number of cells migrating to the lower side was counted under an inverted microscope. 5 fields were randomly selected for each sample.

Western Blot

Total protein in cells was first extracted by lysis buffer. The concentration of extracted protein was determined using the bicinchoninic acid (BCA) kit (Beyotime, Shanghai, China). 50 μg of total protein was separated by sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto membranes. After blocking with skimmed milk for 2 h, the membranes were incubated with primary antibodies (Abcam, Cambridge, MA, USA) overnight. On the next day, the membranes were incubated with corresponding secondary antibody (Abcam, Cambridge, MA, USA). Immuno-reactive bands were finally visualized by enhanced chemiluminescence (ECL: Thermo Fisher Scientific, Waltham, MA, USA) method.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Armonk, NY, USA) and GraphPad Prism 7 (Version X; La Jolla, CA, USA) were used for all statistical analysis. Measurement data were displayed as average ± standard deviation. The *t*-test was used to compare the difference between the two groups. $p<0.05$ was considered statistically significant.

Results

Up-Regulated Expression of Circ-0104631 was Associated with the Progression of CRC

First, we detected the expression level of Circ-0104631 in CRC tissues and adjacent normal tissues by qRT-PCR. As shown in Figure 1A, Circ-0104631 expression in CRC tissues was significantly higher than adjacent normal tissues. Furthermore, the expression of Circ-0104631 was also obviously elevated in most CRC tissues in 32 paired CRC tissues and adjacent normal tissues (Figure 1B). At the same time, Circ-0104631 expression was positively correlated with TNM stage of CRC (Figure 1C). Similarly, Circ-0104631 expression in metastatic CRC samples was remarkably higher when compared with non-transferred CRC samples (Figure 1D). According to the median value of Circ-0104631 expression, we divided CRC tissues into two groups, including Circ-0104631 high expression group and Circ-0104631 low expression group. Survival analysis indicated that the survival rate of patients with high Circ-0104631expression was relatively low (Figure 1E). These results suggested that Circ-0104631 was associated with the progression of CRC.

Knockdown of Circ-0104631 Inhibited the Proliferation and Invasion of CRC Cells

To further explore the function of Circ-0104631 *in vitro*, we detected the expression of Circ-0104631 in CRC cell lines as well. Results

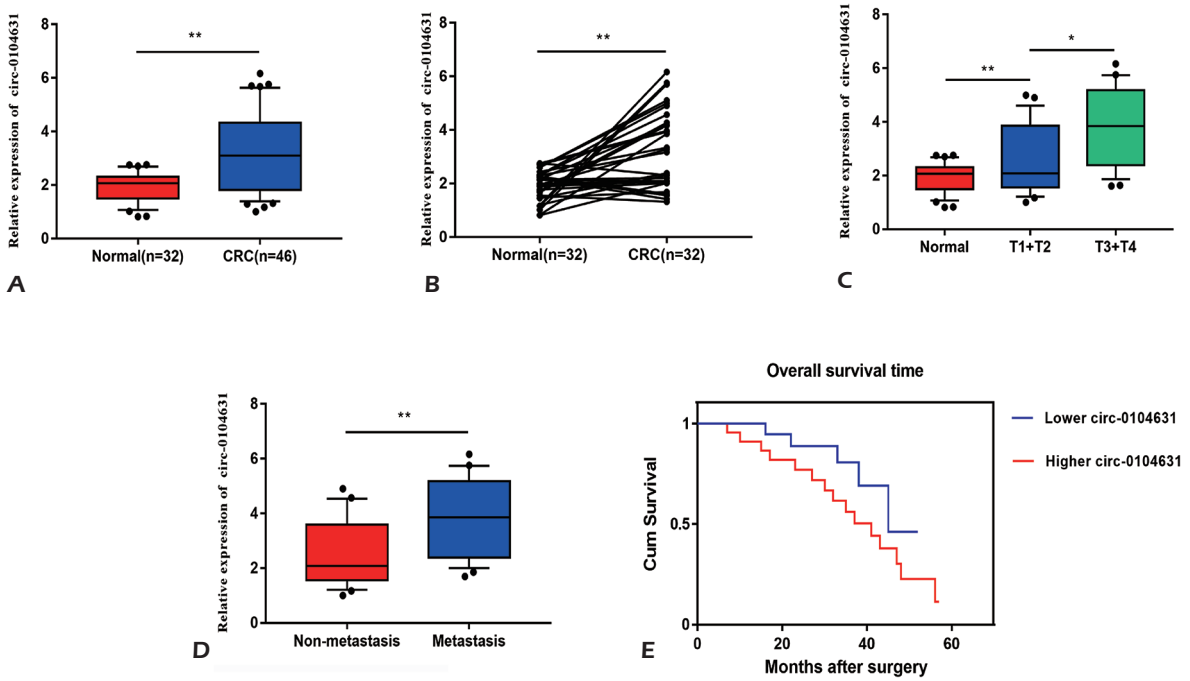

Figure 1. Up-regulated expression of Circ-0104631 was associated with progression of CRC. ***A,*** Expression of Circ-0104631 in CRC tissues and adjacent normal tissues was analyzed by qRT-PCR assay. ***B,*** Expression of Circ-0104631 in 32 paired CRC tissues and adjacent normal tissues was analyzed by qRT-PCR assay. ***C,*** Expression of Circ-0104631 in adjacent normal tissues and different stages of CRC tissues was analyzed by qRT-PCR assay. ***D,*** Expression of Circ-0104631 expression in CRC tissues with distant metastases and no distant metastasis was detected by qRT-PCR assay. ***E,*** Survival curves were plotted by analyzing the expression of Circ-0104631 in CRC tissues. *$p<0.05$, **$p<0.01$.

found that Circ-0104631 was highly expressed in SW480 and LoVo cells than normal cells (Figure 2A). Then shRNA was transfected into these two cell lines to knock down the expression of Circ-0104631 (Figure 2B). Subsequently, we explored the effects of Circ-0104631 silencing on the proliferation of SW480 and LoVo cells by CCK8 and colony formation assays. As shown in Figure 2C, 2D, the proliferation ability was markedly attenuated after knocking down Circ-0104631. Furthermore, the invasive ability of SW480 and LoVo cells was significantly attenuated after knockdown of Circ-0104631 *in vitro* (Figure 2E). These results demonstrated that Circ-0104631 might act as an oncogene in CRC progression.

Circ-0104631 Knock Down Could Inhibit PTEN/Akt/mTOR Signaling Pathway

PTEN is a tumor suppressor that is involved in a variety of activities in multiple malignant tumors, including CRC. We speculated that Circ-0104631 might inhibit PTEN expression through the PTEN/Akt/mTOR pathway, as well as promote cell proliferation and enhance cell invasion. Subsequently, we silenced Circ-0104631 in SW480 and LoVo cells. The expressions of PTEN/Akt/mTOR pathway-related proteins were detected by Western blot. As shown in Figure 3A, 3B, Circ-0104631 silencing markedly increased the protein expression of PTEN, while inhibited the protein expressions of p-Akt and p-mTOR. These results indicated that knockdown of Circ-0104631 could inhibit the PTEN/Akt/mTOR pathway *in vitro*.

Knocking Down of PTEN Could Reverse the Inhibitory Effect of Circ-0104631

To further validate our hypothesis, we knocked down PTEN expression in SW480 and LoVo cells by shRNA-PTEN transfection (Figure 4A). Subsequently, we transfected shRNA-PTEN simultaneously into SW480 and LoVo cells that had silenced Circ-0104631. The effect of PTEN silencing on cell proliferation was examined by CCK8 assay and colony formation assay. The results showed that knockdown of PTEN could partially reverse the inhibitory effect of Circ-0104631 knockdown on cell growth (Figure 4B, 4C). Similarly, we also found that shRNA-PTEN

partially reversed the inhibitory effect of Circ-0104631 silencing on cell invasion. Taken together, these results indicated that highly expressed Circ-0104631 could promote the proliferation and invasion of CRC cells by regulating the PTEN/Akt/mTOR pathway.

Discussion

Gene expression and regulation abnormalities play an important role in the development of malignant tumors. Researchers discovered that many gene expression abnormalities or mutations, such as APC and KRAS, act as oncogenes in CRC. However, some genes such as P53, serve as tumor suppressor genes. These abnormally expressed genes regulate the infiltration, invasion, and metastasis of CRC by regulating downstream molecular pathways[16,17]. However, the specific pathogenesis of CRC remains unclear.

As a stable expression of closed circular RNA, Circ-RNA is abundantly present in eukaryotic transcriptome. Most of the circular RNAs are composed of exon sequences, which are conserved in different species and characterized by tissue specificity[18]. Since circular RNA is insensitive to nucleases, they are more stable than linear RNA. This characteristic leads to its considerable advantages in the development and application of novel clinical diagnostic markers[19]. Meanwhile, Circ-RNA can also act on the linear splicing competition of gene-regulated mRNA (pre-mRNA), as well as regulate the gene expression of parents. The interaction of disease-associated miRNAs suggests that circular RNA participates in disease processes by regulating miRNAs[20].

Many studies showed that circular RNAs play an important role in the development of CRC. For example, hsa_circ_0000523 acts as a miR-31 sponge to regulate the proliferation and apoptosis of CRC cells[21]. CircRNA100290 promotes CRC progression through down-regulation of FZD4[22]. Besides, CircRNA ITGA7 inhibits CRC growth and metastasis by modulating the Ras pathway and up-regulating ITGA7[23]. Here in this study, we found that Circ-0104631 was highly expressed in CRC tissues. Meanwhile, highly expressed Circ-0104631 was associated with a poor prognosis of patients with CRC. Through a series of *in vitro* experiments,

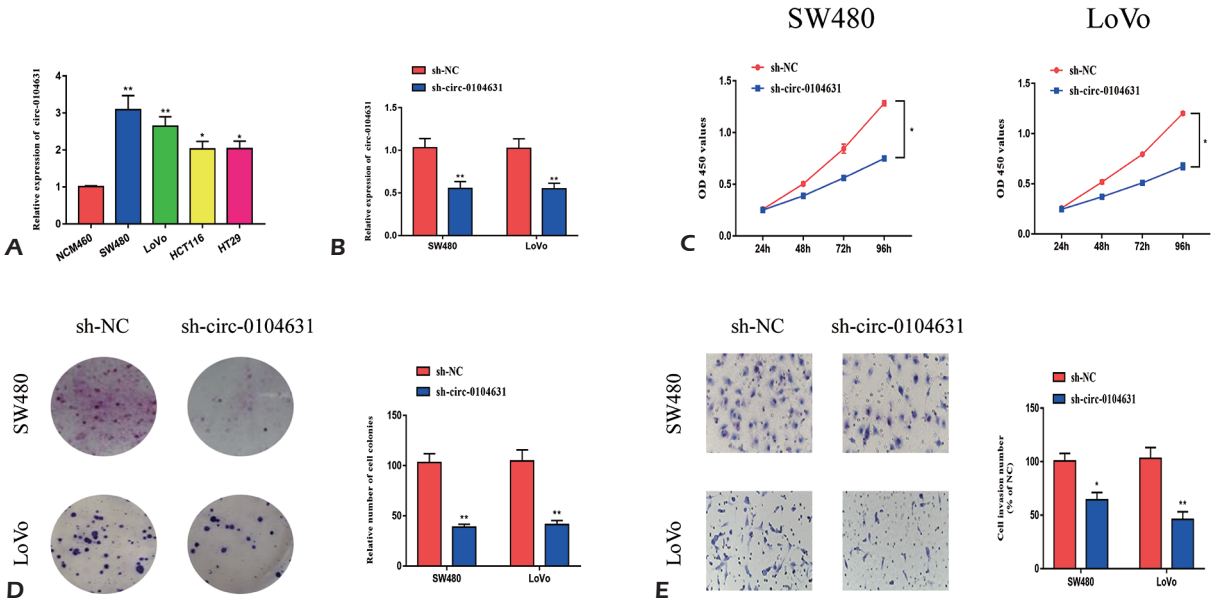

Figure 2. Knockdown of Circ-0104631 inhibited the proliferation and invasion of CRC cells. ***A,*** Expression of Circ-0104631 in normal control cells as well as CRC cells was analyzed by qRT-PCR assay. ***B,*** Circ-0104631 was silenced in SW480 and LoVo cells by shRNA transfection, and its expression was explored by qRT-PCR assay. ***C-D,*** CCK8 assay and colony formation assay showed a significant decrease in cell proliferation ability after knockdown of Circ-0104631 *in vitro*, CCK8 assay (***C***), and colony formation assay (***D***). ***E,*** Cell invasion ability was significantly weakened after knocking down Circ-0104631 in SW480 and LoVo cells. (Magnification: 20X) *p<0.05, **p<0.01.

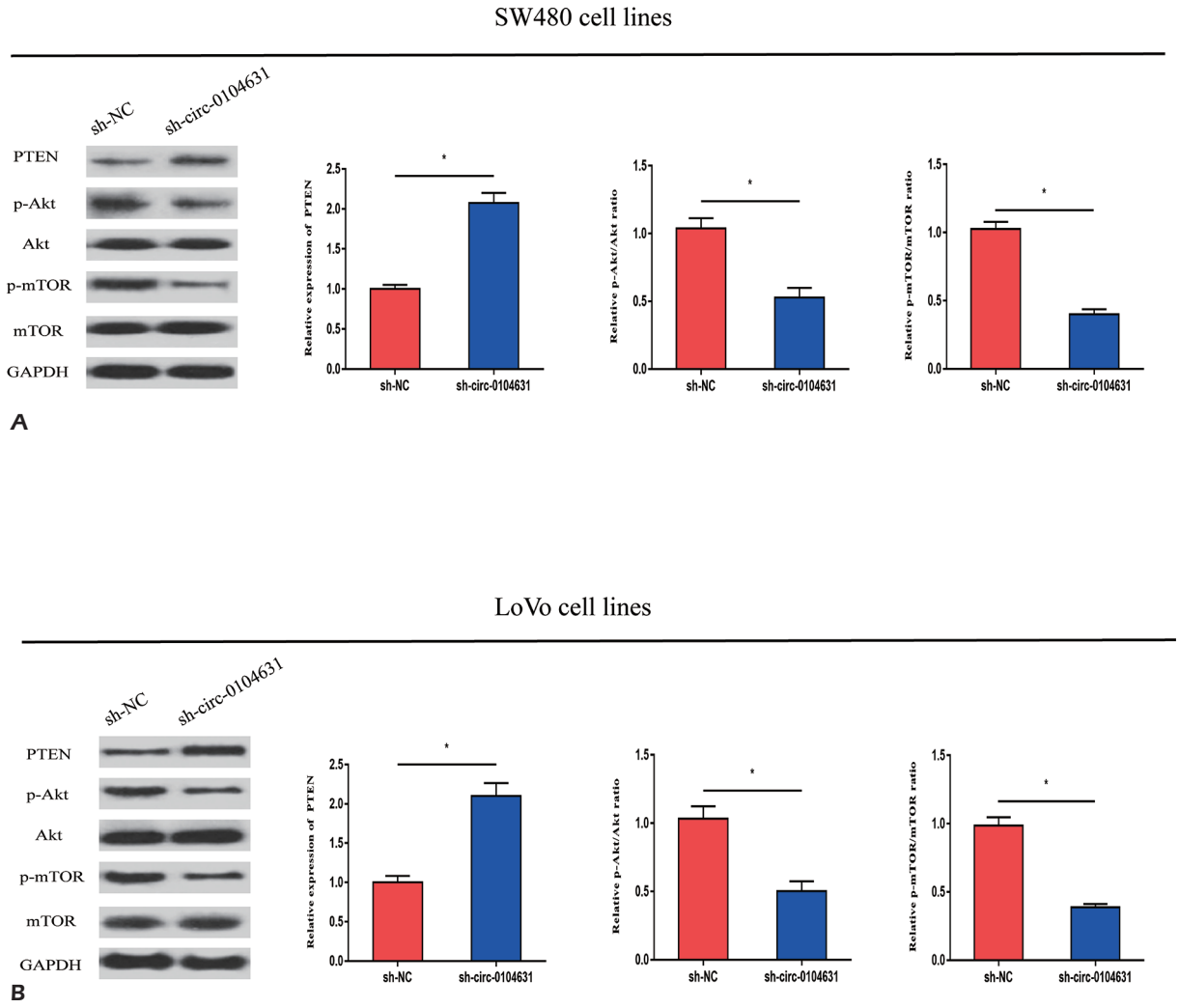

Figure 3. Knocking down Circ-0104631 could inhibit the PTEN/Akt/mTOR pathway. ***A,*** The protein expressions of PTEN, p-Akt, Akt, p-mTOR, mTOR in SW480 cells transfected with p-Circ-0104631. ***B,*** The protein expressions of PTEN, p-Akt, Akt, p-mTOR, mTOR in LoVo cells transfected with sh-Circ-0104631.*$p<0.05$.

we found that knocking down Circ-0104631 in CRC SW480 and LoVo cells significantly inhibited cell growth and metastasis.

PTEN is the first tumor suppressor gene with phosphatase activity and lipase activity discovered so far. Abnormal mutations or loss of PTEN function exist in multiple malignant tumors. At present, studies have shown that PTEN can negatively regulate the AKT/mTOR pathway, reduce the levels of p-AKT active molecules, and inhibit the abnormal expression of downstream signaling pathways. Eventually, this may induce cell apoptosis and inhibit cell proliferation[24,25]. Abnormal activation of the AKT/mTOR signaling pathway has also been reported in tumorigenesis and development. Similarly, persistent activation of the AKT/mTOR pathway has been confirmed in the development of CRC. Peng et al[26] have demonstrated that the AKT/mTOR pathway mediates tumorigenesis through inhibition of apoptosis, promotion of cell proliferation, regulation of cell cycle, promotion of tumor vascularization and malignant invasion and metastasis. As a downstream signaling molecule of P13K/AKT, mTOR has been shown to be involved in the regulation of cell growth, migration and invasion. Meanwhile, it is abnormally elevated in tumor progression, suggesting that abnormal activation of mTOR is associated with tumorigenesis[27].

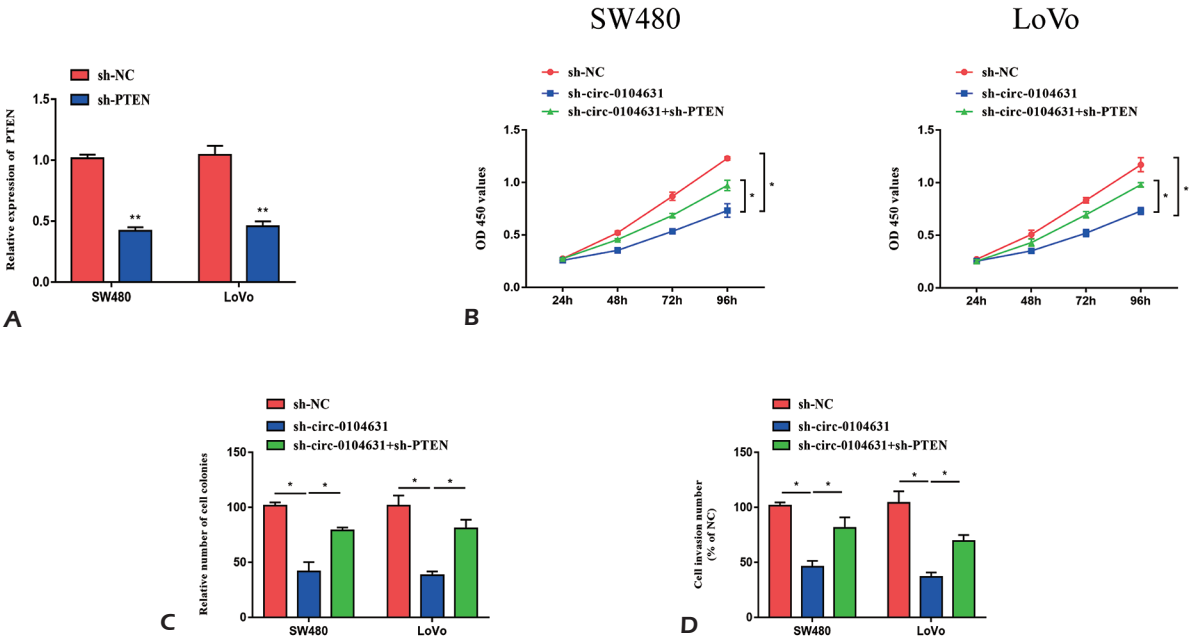

Figure 4. Knocking down PTEN reverses the inhibitory effect of Circ-0104631. ***A,*** Knockdown of PTEN in SW480 and LoVo cells by shRNA transfection. ***B, C,*** The proliferation ability of SW480 and LoVo cells simultaneously transfected with sh-Circ-0104631 and sh-PTEN was observed by CCK8 assay and colony formation assay. CCK8 assay (***B***), colony formation assay (***C***). ***D,*** Cell migration ability in SW480 and LoVo cells simultaneously transfected with sh-Circ-0104631 and sh-PTEN was observed. (Magnification: 20X) *p<0.05, **p<0.01.

Therefore, we hypothesized that Circ-0104631 might promote the progression and metastasis of CRC by inhibiting the expression of PTEN and promoting the activation of the AKT/mTOR pathway. The results showed that the protein expression of PTEN increased significantly after knockdown of Circ-0104631, while the protein expressions of p-Akt and p-mTOR decreased significantly. Subsequently, *in vitro* recovery experiments indicated that knockdown of PTEN could partially alleviate the inhibitory effect of Circ-0104631 silencing on cell proliferation and invasion.

Conclusions

We observed that circ-0104631 was highly expressed in CRC, which could promote the growth and metastasis of CRC cells through inhibitingPTEN expression and promoting the AKT/mTOR pathway. Our results also revealed that Circ-0104631 could be used as a prognostic marker for CRC.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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