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CircMTO1 inhibits cell proliferation and invasion by regulating Wnt/β-catenin signaling pathway in colorectal cancer

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Abstract. – OBJECTIVE: Circular RNAs (circRNAs) play critical roles in disease incidence. However, the roles of circRNAs in colorectal cancer (CRC) progression remain largely unknown. We explored the expression of circMTO1 in CRC and elucidated the underlying molecular mechanisms.

PATIENTS AND METHODS: Quantitative Real-time-PCR (qRT-PCR) was used to explore circMTO1 expression in CRC tissues and cell lines. The effect of circMTO1 on the biological function of CRC cells was analyzed by Cell Counting Kit-8 (CCK-8) assay, Edu assay, colony formation assay, wound-healing assay and transwell invasion assay. Gene expression and signaling pathway were detected by qRT-PCR and Western blot.

RESULTS: QRT-PCR showed that circMTO1 expression was significantly decreased in CRC tissues and cell lines compared with adjacent non-tumor tissues and human normal colon epithelial cell line (FHC), respectively. Patients with low circMTO1 expression were correlated with advanced TNM stage, lymph node metastasis, and poor overall survival. Function assays demonstrated that circMTO1 inhibition promoted CRC cells proliferation and invasion ability in vitro. In addition, we showed that circMTO1 inhibition could promote CRC progression via activating Wnt/β-catenin signaling pathway.

CONCLUSIONS: We showed that circMTO1 could act as a tumor suppressor affecting the growth and invasion of CRC cells via regulating Wnt/β-catenin signaling pathway, providing a novel potential biomarker and therapeutic target for CRC treatment.

Key Words:
Colorectal cancer, circMTO1, Wnt/β-catenin.

Introduction

Colorectal cancer (CRC) is the third most common malignant neoplasm and the fourth leading cause of cancer-related death worldwide[1]. Despite remarkable progress in early detection and treatment, the prognosis of CRC patients with advanced stage is still poor[2,3]. Therefore, it is urgent to elucidate the molecular mechanism underlying CRC carcinogenesis to identify more effective diagnostic strategies and potential therapeutic targets. Circular RNAs (circRNAs) are a novel class of long non-coding RNA which produced co-transcriptionally by the spliceosome at the expense of canonical mRNA isoforms, forming a head-to-tail backsplice to make exons join in a non-linear order[4]. The dysregulation of circRNAs has been identified in many types of malignancy, which greatly affected the progression of human cancer[5,6]. For example, Zong et al[7] showed that circRNA_102231 was increased and could act as a potential biomarker and therapeutic target for lung cancer patients. Zhou et al[8] found that Circular RNA hsa_circ_0008344 regulated glioblastoma cells proliferation, migration, invasion, and apoptosis. Zhao et al[9] revealed that circFADS2 could regulate lung cancer cells proliferation and invasion via acting as a sponge of miR-498. However, the roles of circMTO1 in CRC progression remain unclear. In the present study, we explored the roles of circMTO1 in CRC progression. Our data showed that circMTO1 expression was significantly decreased and associated with advanced TNM stage, lymph node metastasis, and poor overall survival of CRC patients. CircMTO1 inhibition promoted CRC cells proliferation and invasion ability *in vitro*. In addition, our findings indicated that circMTO1 inhibition could activate Wnt/β-catenin signaling pathway. Overall, our study demonstrated that promotion of circMTO1 could be effective in the treatment of CRC.

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Patients and Methods

Patients and Tissue Samples

63 pairs of CRC tissues and adjacent non-tumor tissues were collected from the Huaihe Hospital of Henan University between December 2014 and December 2016. None of these patients received preoperative chemotherapy or radiotherapy before surgery. All specimens were immediately cryopreserved in liquid nitrogen and stored at -80°C for standby application. Written informed consent was obtained from all patients. The study was performed with the approval of the Human Experimentation and Ethics Committee of the Huaihe Hospital of Henan University.

Cell Culture and Transfection

Human CRC cell lines (SW480, SW620, HT-29 and HCT-116) and human normal colon epithelial cell line (FHC) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), and were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in a humid incubator with 5% CO_2 at 37°C. Si-circMTO1, and the empty vectors (si-NC) were obtained from GenePharma (Shanghai, China). Cell transfection was carried out using Lipofectamine 2000 reagent (Life Technologies Corporation, Carlsbad, CA, USA) according to the protocol provided by the manufacturer.

RNA Extraction and qRT-PCR Analysis

The total RNA of CRC tissues and cells was extracted by the TRIzol reagent (Life Technologies Corporation, Carlsbad, CA, USA). After the reverse transcription of RNA conducted by using PrimeScript II 1st Strand cDNA Synthesis Kit (TaKaRa, Otsu, Shiga, Japan), qRT-PCR was performed for the obtained cDNA through SYBR Premix Ex Taq (TaKaRa, Otsu, Shiga, Japan). GAPDH was the internal control for the detection of circMTO1. The expression level of circMTO1 was calculated using the $2^{-\Delta\Delta Ct}$ method. The following primers were used for qRT-PCR: circMTO1, forward 5'-GAGCTGTAGAAGATCTTATTC-3'; reverse 5'-CACAGGCCATCCAAGGCATC-3'; GAPDH, forward 5'-GGGAGCCAAAAGGGTCAT-3', reverse 5'-GAGTCCTTCCACGATACCAA-3' [10].

Cell Viability and Colony Formation Assays

CRC cells proliferation was assessed by Cell Counting Kit-8 assay (CCK-8) (Beyotime Biotechnology, Shanghai, China) and EdU assay. For CCK-8 assay, cells were plated into the 96-well plate and culture for 24, 48, 72 and 96 h. The absorbance at 450 nm was detected by a microplate reader. For EdU assay, cells were cultured in DMEM with EdU labeling for 4 h; then cells were fixed, permeated, and stained with EdU antibody. Cell nuclei were labeled by Hoechst 33342 (RiboBio, Shanghai, China). Lastly, the treated cells were observed by laser scanning microscope. For colony formation assay, cells were seeded into 6-well plates and cultured for two weeks. Then, the cell colonies were stained with crystal violet and counted.

Wound-Healing Assay

CRC cells were seeded in the 6-well plate and when grown to approximately 90% confluence, the wound was generated by scraping the cells using a 200 μl tips. The cells were washed with PBS to remove the debris and cultured with fresh Dulbecco's Modified Eagle Medium (DMEM) medium containing 10% fetal bovine serum (FBS). After 24 h, the wound healing of different groups was observed with the microscopy.

Transwell Invasion Assay

CRC cells were seeded into Transwell chambers (8.0 μm pore size, BD Biosciences, San Jose, CA, USA) coated with Matrigel (Franklin Lakes, NJ, USA). The cells were seeded into a serum free medium in upper chamber, and 20% FBS was added to the lower chamber. After 24 h culture, the cells on the upper surface were scraped off, and Diff-Quik was used for fixing and staining the invasive cells, which attached on the lower surface. After that, we choose five random fields to observe and count the invading cells.

Western Blotting

Total proteins were extracted from CRC cells using RIPA Lysis Buffer (Beyotime Biotechnology, Shanghai, China). Lysates of total protein were separated by a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was blocked by 5% skim milk in TBST at room temperature for 2 h, followed by incubation with the primary antibodies (Cell Signaling Technology, Danvers,

MA, USA) at 4°C overnight. After washing, the membrane was incubated with HRP-conjugated secondary antibody (Cell Signaling Technology, Danvers, MA, USA) at room temperature for 1 h. Proteins were visualized using ECL reagents (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instruction.

Statistical Analysis

All experiments were repeated for three times independently. GraphPad Prism version 5.0 software (La Jolla, CA, USA) was used for statistical analysis. Data is shown as mean ± standard deviation (SD). The differences between groups were analyzed by One-way ANOVA test followed by least significant difference (LSD). $p < 0.05$ suggested that the difference was statistically significant.

Results

CircMTO1 was Downregulated in CRC

The circMTO1 expression in CRC cell lines and tissues was determined by qRT-PCR assay. Results showed that circMTO1 expression was distinctly decreased in CRC cell lines (SW480, SW620, HT-29 and HCT-116) compared to human normal colon epithelial cell line (FHC) (Figure 1A). Further results of qRT-PCR showed that the expression of circMTO1 was significantly decreased in CRC tissues compared with adjacent non-tumor tissues (Figure 1B). Moreover, we showed that low circMTO1 expression was associated with CRC patients with advanced TNM stage and lymph node metastasis (Figure 1C and 1D). In addition, Kaplan-Meier survival analysis showed that low circMTO1 expression was significantly correlated with poor overall survival of CRC patients (Figure 1E).

CircMTO1 Inhibited CRC Cells Proliferation

To investigate its biological functions in CRC, si-circMTO1 and si-NC were transfected in CRC cells. QRT-PCR showed that circMTO1 expression was significantly decreased in SW620 cells transfected with si-circMTO1 compared to si-NC group (Figure 2A). CCK-8 assay and 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay showed that circMTO1 inhibition signifi-

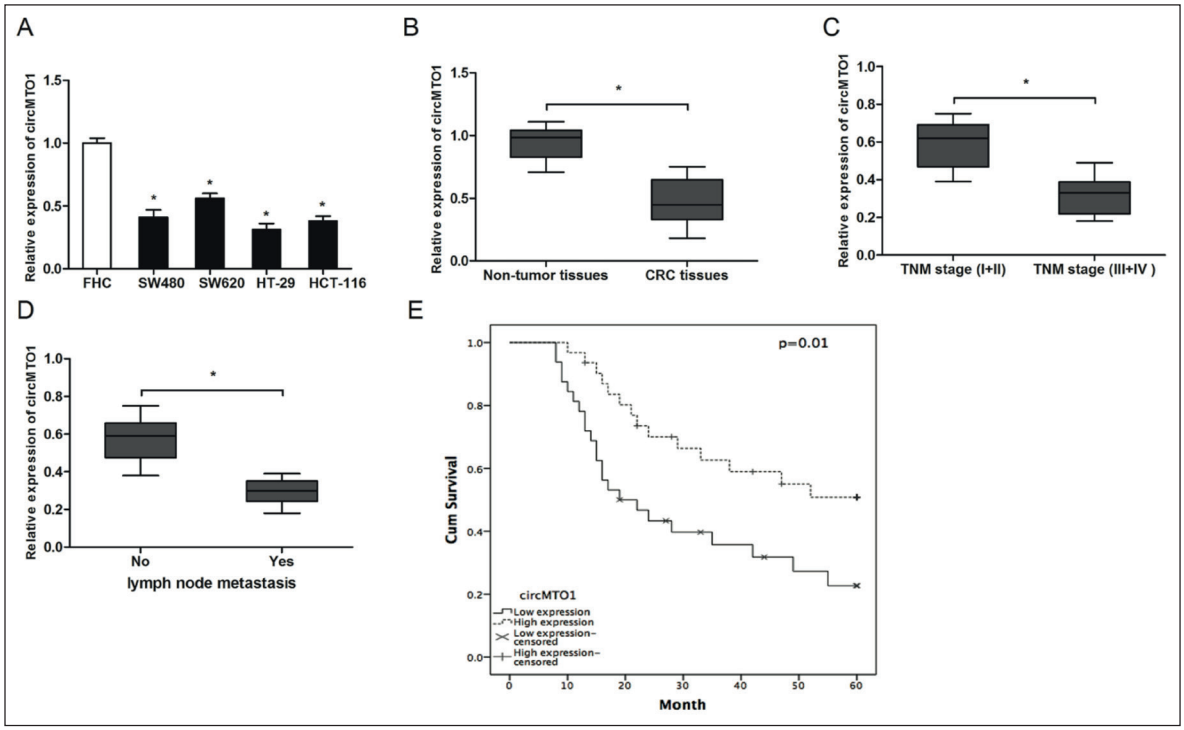

Figure 1. CircMTO1 was decreased in CRC. ***A*,** CircMTO1 expression in CRC cell lines (SW480, SW620, HT-29 and HCT-116) and human normal colon epithelial cell line (FHC) was determined by qRT-PCR. ***B***, QRT-PCR was performed to determine circMTO1 expression in CRC tissues and adjacent non-tumor tissues. ***C-D*,** Low circMTO1 expression was significantly associated with CRC patients with advanced TNM stage and lymph node metastasis. ***E*,** Kaplan-Meier analysis showed that low circMTO1 was associated with poor overall survival of CRC patients (*$p < 0.05$).

cantly increased the proliferation ability of CRC cells compared to si-NC group (Figure 2B, C). In addition, colony formation assay suggested that circMTO1 inhibition significantly induced colony numbers in CRC cells compared to si-NC group (Figure 2D). Furthermore, flow cytometry analysis showed that circMTO1 inhibition decreased CRC cells in G0/G1 phase compared to si-NC group (Figure 2E). Taken together, we suggested that circMTO1 could reduce CRC cells proliferation ability by regulating cell cycle process.

CircMTO1 Inhibited CRC Cells Invasion

Next, we explored the effects of circMTO1 on CRC cells migration and invasion ability. Wound healing assay showed that circMTO1 inhibition significantly increased CRC cells migration ability compared to si-NC group (Figure 3A). Transwell invasion assay showed that circMTO1 knockdown induced the invasion ability of CRC cells compared to si-NC group (Figure 3B, $p < 0.05$). These data indicated that circMTO1 could suppress CRC cells invasion ability *in vitro*.

CircMTO1 Inactivated Wnt/β-Catenin Signaling Pathway

Wnt/β-catenin signaling pathway play essential roles in the regulation of tumor cell proliferation and invasion[11]. However, whether circMTO1 affects Wnt/β-catenin signaling is still unclear. Thus, in the present study, β-catenin expression and a few of the downstream genes of the Wnt/β-catenin signaling pathway were detected by qRT-PCR and Western blot. QRT-PCR showed that circMTO1 inhibition induced the mRNA expression of β-catenin, c-myc, and cyclin D1 in CRC cells (Figure 4A). Western blot assay also showed that the protein levels of β-catenin, c-myc, and cyclin D1 were significantly increased in cells transfected with si-circMTO1 (Figure 4B).

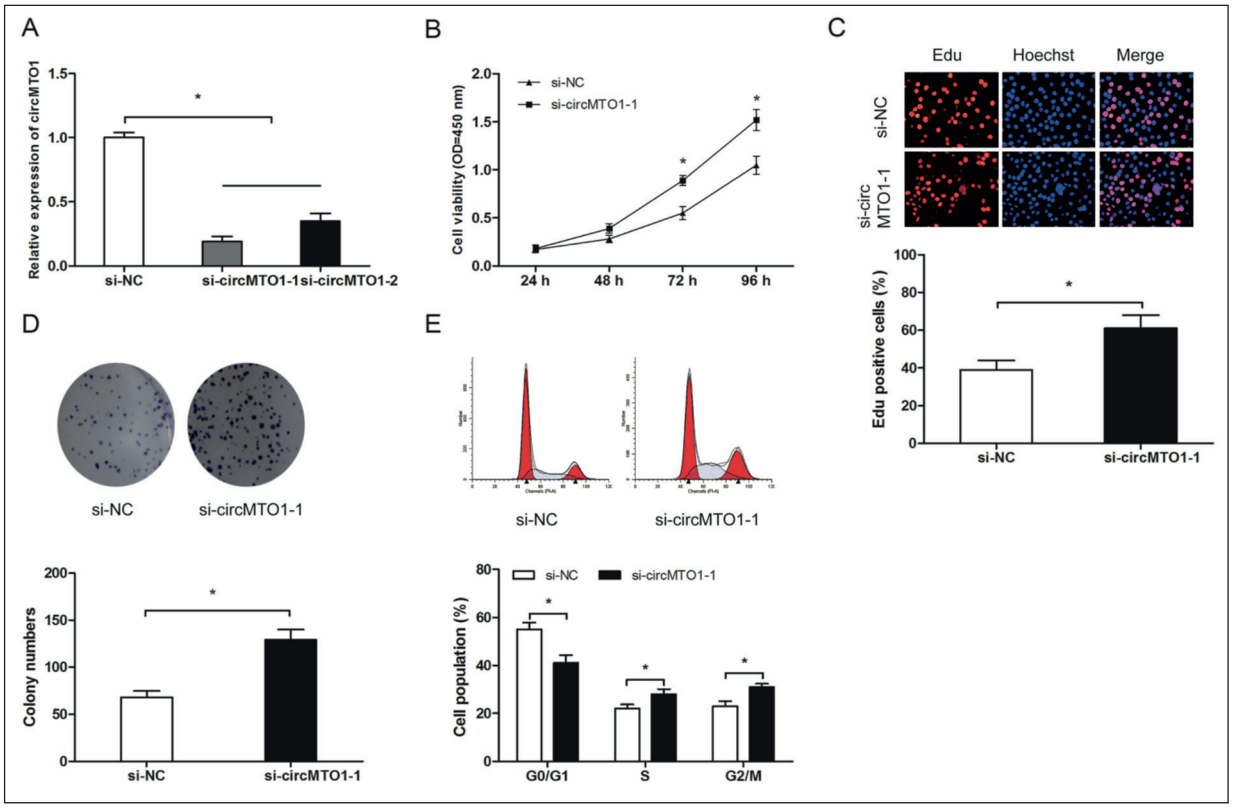

Figure 2. CircMTO1 inhibition increased the proliferation of CRC cells. ***A*,** CircMTO1 expression in SW620 cells transfected with si-circMTO1 or si-NC was determined by qRT-PCR. ***B-C*,** CCK-8 assay and EdU assay revealed that circMTO1 down-regulation increased CRC cells proliferation ability. ***D*,** Colony formation assay demonstrated that circMTO1 inhibition significantly induced colony numbers in CRC cells. ***E*,** Flow cytometry analysis showed that circMTO1 inhibition decreased CRC cells in G0/G1 phase compared to si-NC group (*$p < 0.05$).

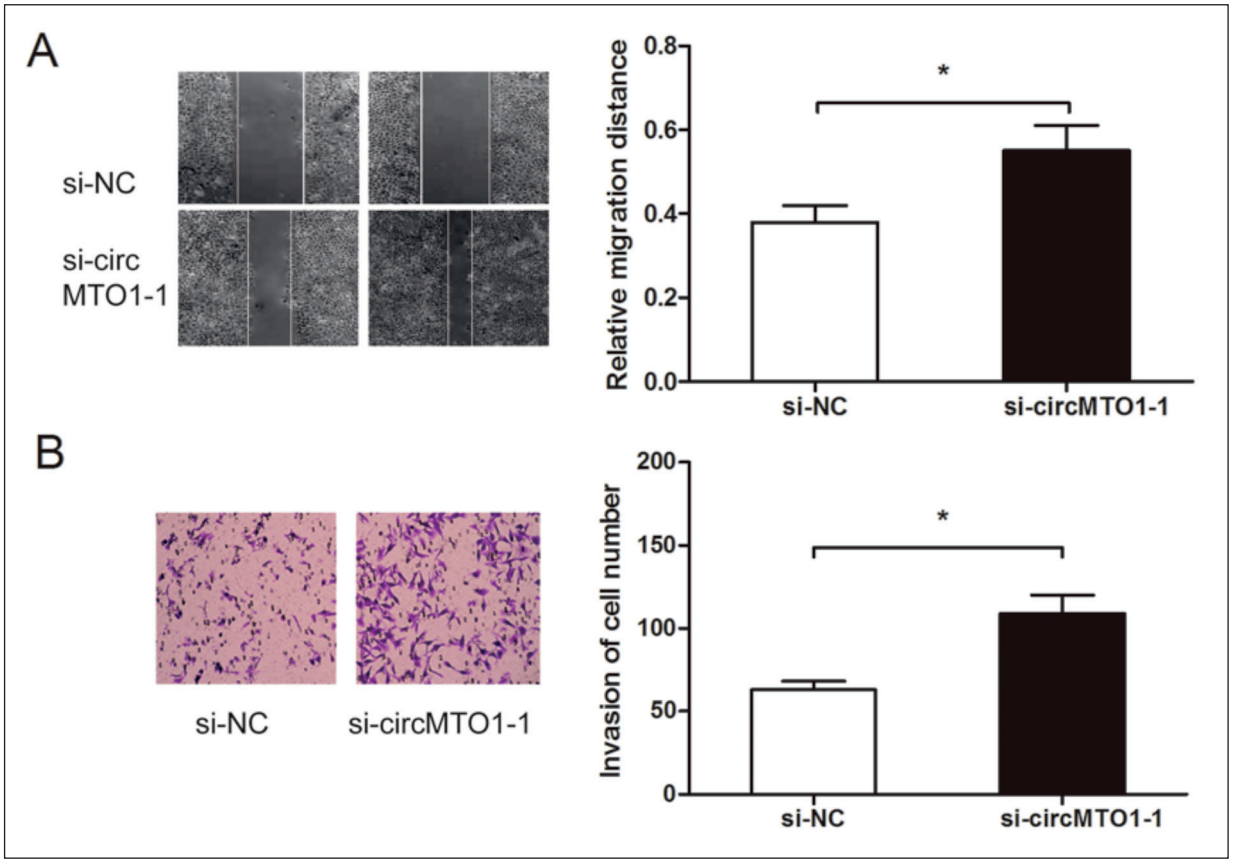

Figure 3. CircMTO1 inhibition increased CRC cells migration and invasion. ***A*,** Wound-healing assay showed that circMTO1 down-regulation increased the migration ability of SW620 cells. ***B*,** Transwell invasion assay showed that circMTO1 suppression evaluated the invasion ability of SW620 cells (*$p < 0.05$).

Discussion

Colorectal cancer (CRC) is still a malignant disease with unsatisfactory curative effects in clinical practice. Activating of invasion and metastasis is one of the hallmarks of cancer, which is closely associated with poor prognosis[12]. So it is an urgent need to explore the molecular mechanism involved in CRC progression. Recent studies revealed that circRNAs play important regulatory roles in gene expression and might account for initiation and development of tumor, including CRC[13]. For example, Wang et al[14] showed that decreased expression of hsa_circ_001988 in

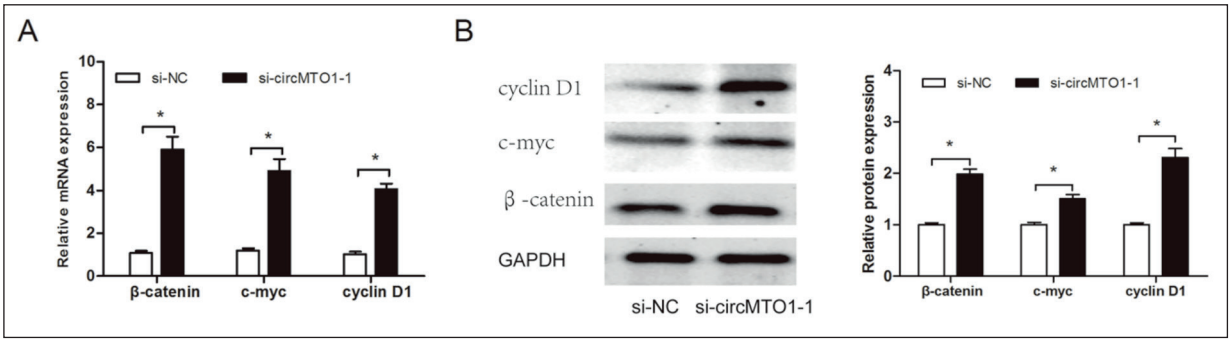

Figure 4. CircMTO1 inactivated Wnt/β-catenin signaling pathway in CRC. ***A*,** Effects of si-circMTO1 on the mRNA expression of β-catenin, c-myc and cyclin D1 in SW620 cells. ***B*,** Effects of si-circMTO1 on β-catenin, c-myc and cyclin D1 protein levels in SW620 cells (*$p < 0.05$).

CRC could act as a novel potential biomarker and novel target. Zhang et al[15] showed that silencing of hsa_circ_0007534 suppressed proliferation and induced apoptosis in CRC cells. Zeng et al[16] found that circHIPK3 could promote CRC cells growth and metastasis by sponging miR-7. However, the roles and the underlying mechanisms of circRNAs in CRC remain unclear. CircMTO1 was first be found by Cao et al[10], they showed that circMTO1 expression was significantly down-regulated and correlated with poor prognosis of hepatocellular carcinoma (HCC) patients. Furthermore, they showed that circMTO1 suppressed HCC growth by the sponge activity on miR-9 and upregulation of p21 expression. However, the expression and functions of circMTO1 in CRC are still unclear. In the current study, qRT-PCR showed that expression of circMTO1 was distinctly decreased in SW480, SW620, HT-29 and HCT-116 cells compared to FHC cells. Further results of qRT-PCR showed that the expression of circMTO1 was significantly decreased in CRC tissues compared with adjacent non-tumor tissues. CircMTO1 downregulation was found in CRC patients with advanced TNM stage and lymph node metastasis. Moreover, Kaplan-Meier survival analysis showed that low circMTO1 expression in was significantly correlated with poor overall survival of CRC patients. These findings suggested that circMTO1 might be involved in the progression and development of CRC patients. Next, we explored the biological functions of circMTO1 on CRC cells. First, we chose SW620 cells, which showed the highest expression of circMTO1 for further study. CCK-8 assay, Edu assay, and colony formation assay showed that circMTO1 inhibition significantly increased CRC cells viability. Flow cytometry analysis revealed that circMTO1 inhibition decreased CRC cells in G0/G1 phase. Furthermore, we explored the effects of circMTO1 on CRC cells migration and invasion. Wound healing assay and transwell invasion assay showed that circMTO1 inhibition significantly increased CRC cells migration and invasion ability. Thus, these data indicated that circMTO1 could act as a tumor suppressor in CRC progression. Increasing evidence demonstrated that Wnt/β-catenin signaling pathway is involved in a variety of cellular activities, including proliferation, differentiation, metastasis, and death[17,18]. The activation/inhibition of Wnt/β-catenin signaling pathway could regulate the downstream target genes such as c-myc, and cyclin D1 in tumors[19]. Recently, scholars studies showed that circRNAs could participate in the changes in tumor biological behaviors via regulating Wnt/β-catenin signaling pathway. For example, Wu et al[20] found that overexpressing circular RNA hsa_circ_0002052 impaired osteosarcoma progression via inhibiting Wnt/β-catenin pathway by regulating miR-1205/APC2 axis. Zhu et al[21] revealed that circRNA circ_0067934 promoted tumor growth and metastasis in hepatocellular carcinoma through regulation of miR-1324/FZD5/Wnt/β-catenin axis. In the present study, we showed that the expression of β-catenin, c-myc, and cyclin D1 were induced in circMTO1 suppressed CRC cells, suggesting that Wnt/β-catenin signaling pathway might be partially related to circMTO1 suppression effective on CRC progression.

Conclusions

We found that circMTO1 was down-regulated and could suppress the proliferation and invasion of CRC cells via regulating Wnt/β-catenin signaling pathway. Our study indicated that circMTO1 might serve as a potential predictor and therapy target for the treatment of CRC patients.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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