Circ_0001535 Facilitates Tumor Malignant Progression by miR-485-5p/LASP1 Axis in Colorectal Cancer

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INTRODUCTION

Colorectal cancer (CRC) has the third highest rate and a common malignancy worldwide. Although early screening and radical surgery have significantly improved 5-year survival rates, most patients with CRC are still diagnosed at an advanced stage. Without effective treatments, patients with advanced diseases have a high mortality rate. Therefore, the search for new and effective treatment strategies is crucial.

Circular RNA (circRNA) is a new class of endogenous non-coding RNA. Increasingly, circRNA is differentially expressed in some malignancies, including CRC, which can be used as a potential molecular marker for tumor diagnosis, prognosis, and treatment. Furthermore, circRNA plays a key role in tumor progression by negatively regulating miRNA activity by binding to microRNA (miRNA) response elements. For instance, Wang et al. showed that circRNA circPRKDC promoted CRC cell progression by modulating miR-198 and DDR1. Liu et al. revealed that circ_100146 boosted CRC processes by sponging miR-194 and modulating APC2. Liu et al. presented that the inhibition of circ_0000231 suppressed the glycolysis and malignant behaviors of CRC cells by regulating the miR-502-5p/MYO6 pathway. A previous study reported increased hsa_circ_0001535 in CRC, but its biological remains unclear.

In this study, we verified the binding between miR-485-5p and circ_0001535 or LIM and SH3 protein 1 (LASP1) in CRC cells. Furthermore, previous studies have indicated that LASP1 was an adhesion adaptor and scaffold protein that performed an oncogenic function in multiple cancers, containing CRC. Studies have reported that LASP1 upregulation might contribute to CRC cell growth and metastasis in vitro. Hence, their effects on CRC cell proliferation, apoptosis, invasion, and stemness were detected, and their regulatory mechanism was demonstrated in CRC cells.

Background: Increasing evidence revealed that circular RNAs (circRNAs) are involved in colorectal cancer progression. However, the potential function of circ_0001535 in colorectal cancer remains unclear.

Aims: To investigate the mechanism of circ_0001535 by silencing circ_0001535 in colorectal cancer cells and nude mice.

Study Design: A cell study.

Methods: Expressions of circ_0001535, LIM and SH3 protein 1 (LASP1) mRNA, and miR-485-5p were detected by real-time quantitative polymerase chain reaction (RT-qPCR). Western blot analyses of LASP1, PCNA, cleaved caspase 3, snail 1, and OCT4 protein expression were performed. CCK-8, EdU, flow cytometry, transwell assays, and sphere formation were conducted to evaluate colorectal cancer cell proliferation, apoptosis, invasion, and stemness. Luciferase reporter assays, RNA pull-down, and RIP validated binding. A nude mice xenograft model was constructed.

Results: Circ_0001535 was significantly upregulated in colorectal tissues and cells. Circ_0001535 knockdown suppressed the malignant behavior of colorectal cells such as proliferation, invasion, stemness, and tumor growth in vivo. This knockdown also induced apoptosis by sponging miR-485-5p and upregulating LASP1 expression.

Conclusion: Circ_0001535 promotes colorectal cancer cell development by absorbing miR-485-5p and upregulating LASP1.
MATERIALS AND METHODS

Tissue Samples
Tumor samples and adjacent non-cancer tissues were collected from Second Affiliated Hospital of Xi’an Jiaotong University, and samples were stored and kept at -80 °C. No patient received radiotherapy or chemotherapy before the operation. All participants provided written informed consent for experimentation. The detailed clinical characteristics of patients are described in Table 1.

Cell and Cell Culture
At 37 °C with a 5% CO₂ incubator, ATCC (Manassas, VA, USA) offered four CRC cells (HCT116, LoVo, SW480, and SW620) and normal colorectal mucosal cell (FHC), which were grown in Dulbecco’s modified Eagle’s medium (DMEM) and 10% fetal bovine serum (FBS; Procell, Wuhan, China).

Cell Transfection
For cell transfection, lentivirus short-hairpin RNAs for circ_0001535 (sh-circ_0001535#1, sh-circ_0001535#2, and sh-circ_0001535#3), miR-485-5p mimic or inhibitor (miR-485-5p or anti-miR-485-5p), LASP1-overexpressed plasmid pcDNA-LASP1 (LASP1), and their negative controls were all bought from RiboBio. These transfections were implemented in CRC cells using lipofectamine 3000 reagent (Invitrogen).

RT-qPCR
Using a total RNA extractor (Trizol, Songon, Shanghai, China), total RNAs were prepared. Then, RNAs were reversed into cDNA according to PrimeScript RT reagent kit (Exiqon, Aarhus, Denmark). cDNA was mixed up with SYBR. Primers are shown in Table 2. Circ_0001535, miR-485-5p, and LASP1 relative expressions were measured using the 2⁻ΔΔCt method.

RNase R Treatment
Briefly, 1 µg of RNA isolated from CRC cells at 37 °C was digested with RNase R. After 15 min of incubation, RNAs were reversed-transcribed into cDNA, followed by RT-qPCR analysis.

Actinomycin D Treatment
CRC cells were exposed to 2 mg/ml actinomycin D for 6 h, 12 h, 18 h, and 24 h. Then, RNAs were isolated from CRC cells, and expressions of circ_0001535 and FAM13B mRNA were estimated by RT-qPCR.

Cell-counting kit 8 (CCK8) assay
Here, 5 x 10⁶ CRC cells were incubated for 24, 48, and 72 h. Moreover, 10 µM CCK8 reagent (Beyotime, Shanghai, China) in 96-well plates were added in cells for 4 h.

EDU Assay
After EDU buffer (Solarbio, Beijing, China) treatment, CRC cells were subjected to 1x Apollo staining solution and 4’,6-diamidino-2-phenylindole staining. Moreover, 4% formaldehyde was used to fix the cells. Then, EDU-positive cells were assessed by fluorescence microscope (Olympus, Tokyo, Japan) and counted by ImageJ software.

TABLE 1. Characteristics of the Patients with Colorectal Cancer.

| Parameters          | N = 66 |
|---------------------|--------|
| Sex                 |        |
| Male                | 35     |
| Female              | 31     |
| Age, years          |        |
| <65                 | 40     |
| ≥65                 | 26     |
| Tumor location      |        |
| Colon               | 38     |
| Rectum              | 28     |
| Tumor size, cm      |        |
| <5                  | 44     |
| ≥5                  | 22     |
| Histological grade  |        |
| Well                | 8      |
| Moderate            | 47     |
| Poor                | 10     |
| Tumor stage         |        |
| I/II                | 47     |
| III                 | 19     |
| Lymph node metastasis|     |
| Yes                 | 18     |
| No                  | 48     |

TABLE 2. Primers Sequences Used for the Polymerase Chain Reaction.

| Name      | Primers for PCR (5'-3')     |
|-----------|-----------------------------|
| circ_0001535 | Forward AGACTGTTCAAAAACCTGTGGC |
|            | Reverse GGCTGGTAGGATGCTGATGG  |
| LASP1      | Forward GGAAAAACCTTGCCCTAACAGC |
|            | Reverse TACGCCTGAAAACCTTTGCCCCT |
| miR-5691   | Forward GTATGATTGCTCTGAGCTCC |
|            | Reverse CTCAGCTGTCTGCGGTGAGGAG |
| miR-433-3p | Forward GTATGATTGCTCTGAGCTCC |
|            | Reverse CTCAGCTGTCTGCGGTGAGGAG |
| miR-485-5p | Forward GTATGATTGCTCTGAGCTCC |
|            | Reverse CTCAGCTGTCTGCGGTGAGGAG |
| GAPDH      | Forward GACAGTCGGCAGCATTCTCT |
|            | Reverse GCCGCAATACGAACAAATC   |
| U6         | Forward CTGCCTTCCGACGACA     |
|            | Reverse AACGGTCACGAAATTTGCGT |
| FAM13B     | Forward TCCATCATAGTCGCACAG   |
|            | Reverse TGTCATATCTGCGGAAGC   |
In binding buffer, CRC cells were reacted with Annexin V-FITC (Solarbio), and PI (Solarbio) was employed to stain cells for 20 min. Then, cell apoptosis was estimated by flow cytometry (Agilent, Beijing, China).

**Transwell Invasion Assay**

CRC cells were added in Matrigel-coated transwell upper chambers (Corning, Cambridge, MA, USA) with serum-free medium, and lower chambers filled with DMEM plus 10% FBS. After 48 h, invaded cells were stained and then photographed under a microscope (Olympus) to obtain the numbers of invaded cells.

**Sphere Formation Assay**

CRC cells in ultralow attachment 6-well plate (Sigma-Aldrich, Louis, MO, USA) were cultured, which included insulin (4 ng/ml), basic fibroblast growth factor (10 ng/ml), B27 (2%), and epidermal growth factor (100 ng/ml). All factors were obtained from Sigma-Aldrich. After 10 days, cells were observed under a microscope (Olympus).

**Western Blot Analysis**

Cell proteins were acquired by RIPA buffer (Sigma-Aldrich). Sodium dodecyl-sulfate polyacrylamide gel electrophoresis was used to separate proteins and proteins were transferred into PVDF membrane (Merck, Darmstadt, Hesse, Germany). After primary antibody incubation, the secondary antibody (Abcam, Cambridge, MA, USA) was added to membrane, and protein signals were visualized using ECL Kit. The following primary antibodies were bought from Abcam: anti-LASP1 (ab191022), anti-PCNA (ab18197), anti-cleaved caspase 3 (ab2302), anti-snail 1 (ab216347), anti-OCT4 (ab200834), or anti-β-actin (ab5694).

**Dual-luciferase Reporter Assay**

The sequences of wild-type (WT) and mutant-type (MUT) for circ_0001535 or LASP possessing miR-485-5p binding sites were cloned downstream pmirGLO reporter vector, which formed circ_0001535-WT, circ_0001535-MUT, LASP1-WT, and LASP1-MUT vector. Co-transfection was implemented with vectors miR-485-5p or miR-NC, followed by analysis using dual-luciferase Reporter Gen Assay Kit.

**RNA Pull-down Assay**

CRC cell RNA was co-incubated with biotinylated-circ_0001535 (circ_0001535 probe) or (oligo probe), and lysis complexes were mixed with magnetic beads. At 24 h later, miR-485-5p, miR-5691, and miR-433-3p enrichment were monitored using RT-qPCR.

**RIP**

Magna RIP Kit (Abcam) was applied for RIP. In simple terms, cell lysates were cultured with magnetic beads and an antibody against Ago2 or IgG, followed by qPCR detection.

**Apoptosis Analysis**

SW480 cells with sh-circ_0001535#1 or sh-NC (5 x 10^6 cells/0.2 ml PBS) were injected into 6-week-old mice (n = 5 per group; Vital River Laboratory, Beijing, China). The tumor volume was calculated weekly, and the tumor weight was detected after 35 days with euthanized mice. In immunohistochemical (IHC), LASP1 and Ki-67 were stained with anti-LASP1 and anti-Ki-67.

**Statistical Analysis**

GraphPad Prism 8.0 software was used to analyze data and defined using p < 0.05. Comparisons between groups were implemented using Student’s t-test and one-way analysis of variance.

**RESULTS**

**Circ_0001535 was Upregulated in CRC**

First, circ_0001535 was upregulated 3.56-fold in CRC tissues compared with adjacent non-cancer tissues (p < 0.001, Figure 1a). High circ_0001535 had a poor overall survival rate (Figure 1b). Circ_0001535 expression in CRC cells (HCT116, LoVo, SW480, and SW620) was respectively increased 4.81-fold, 3.18-fold, 4.63-fold, and 3.57-fold versus normal colorectal mucosal cells (FHC) (p < 0.001, Figure 1c). As shown in Figure 1d, circ_0001535 was located in chr5: 137320945-137324004 and formed from exons 8, 9, and 10 of FAM13B, which is 331nt long. Furthermore, circ_0001535 stability was also assessed. Circ_0001535 expression has no obvious change after being digested with RNase R (p < 0.001, Figure 1e), and circ_0001535 had a longer half-life after being treated with actinomycin D (p < 0.001, Figure 1f). In addition, subcellular fraction assay revealed circ_0001535 mainly located in the cytoplasm in CRC cells (Figure 1g). These results suggested that circ_0001535 was upregulated in CRC, with a stable circular structure.

**Circ_0001535 Silencing Inhibited CRC Cell Proliferation, Invasion, and Stemness and Promoted Cell Apoptosis**

Moreover, shRNA of circ_0001535 was used to reduce circ_0001535 content in tumor cells (p < 0.01, Figure 2a). Because of the most knockdown efficiency of sh-circ_0001535#1 (reduced by 75% in HCT116 cells; 73% in SW480 cells) among three shRNA, we selected sh-circ_0001535#1 to transfected into HCT116 and SW480 for further study. circ_0001535 knockdown obviously inhibited cell proliferation (p < 0.001, Figure 2b, c). On the contrary, circ_0001535 inhibition apparently promoted HCT116 and SW480 cell apoptosis (p < 0.001, Figure 2d). Furthermore, circ_0001535 silencing greatly hindered cell invasion and stemness ability (p < 0.001, Figure 2e, f). In addition, circ_0001535 knockdown decreased PCNA (reduced by nearly 57%), snail1 (reduced by approximately 58%), and OCT4 expression (reduced by 72% in HCT116 and 55% SW480 cells), but increased by approximately 1.7-fold cleaved caspase-3 expression in HCT116 and SW480 cells (p < 0.001, Figure 2g). Collectively, circ_0001535 deficiency suppressed CRC cell proliferation, invasion, and stemness and promoted apoptosis.
Circ_0001535 Directly Targeted miR-485-5p in CRC Cells

According to circatlas, circBank, and starbase analyses, the Venn diagram presented three miRNA (miR-485-5p, miR-5691, and miR-433-3p) that were associated with circ_0001535 (Figure 3a). The RNA pull-down assay suggested that only miR-485-5p could directly bind to circ_0001535 (p < 0.001, Figure 3b). Then, the expression of miR-485-5p was downregulated by approximately 60% in CRC tissues and 70% in cells (p < 0.001, Figure 3c, d). Their binding sequences are presented in Fig. 3e. MiR-485-5p content notably increased 21.17-fold in HCT116 cells and 22.97-fold in SW480 cells after miR-485-5p mimic introduction (p < 0.001, Figure 3f). The luciferase activity of WT-circ_0001535 decreased by 71% in HCT116 cells and 70% in SW480 cells via miR-485-5p, but there was no obvious change in the mutant group (p < 0.001, Figure 3g). Moreover, circ_0001535 and miR-485-5p enrichment in Ago2 antibodies group was increased 12.2-fold and 16.77-fold, respectively, in HCT116 cells and 12.7-fold and 15.57-fold in SW480 cells, respectively, relative to the IgG antibodies group (p < 0.001, Figure 3h). Overall, circ_0001535 was associated with miR-485-5p.
Fig. 2. Circ_0001535 knockdown inhibits malignant behavior of colorectal cancer cells.
(a) Circ_0001535 content in HCT116 and SW480 cells was assessed by RT-qPCR. (b, c) CCK-8 and EDU analysis of cell proliferation. (d) Flow cytometry analysis of the apoptotic rate. (e and f) Transwell invasion and sphere formation analysis of cell invasion and stemness. (g) PCNA, cleaved caspase-3, snail 1, and OCT4 protein levels were detected using Western blot assays. **p < 0.01, ***p < 0.001.
Furthermore, an elevation of about 2.8-fold miR-485-5p induced by knockdown circ_0001535 was partially reversed via miR-485-5p suppression ($p < 0.001$, Figure 4a). Subsequently, CCK8 and EDU assay presented that circ_0001535 deficiency-triggered proliferation inhibition was regained after co-transfection with anti-miR-485-5p ($p < 0.001$, Figure 4b, c). Flow cytometry showed that circ_0001535 absence-triggered CRC cell apoptosis was rescued by miR-485-5p suppression ($p < 0.001$, Figure 4d). Furthermore, circ_0001535 silencing inhibited cell invasion and stemness, and miR-485-5p inhibition reverted the effects ($p < 0.001$, Figure 4e, f). Circ_0001535 deficiency-mediated alteration in PCNA cleaved caspase-3, snail 1, and OCT4 protein levels, which was countervailed after decreasing miR-485-5p ($p < 0.001$, Figure 4g). To sum up, circ_0001535/miR-485-5p regulated tumor cell malignant behaviors.

**MiR-485-5p Directly Targeted LASP1 in CRC Cells**

In starbase, the target gene of miR-485-5p was LASP1 (Figure 5a). MiR-485-5p mimics markedly decreased the luciferase activity of WT-3'UTR LASP1 (reduced by 69% in HCT116 cells and 71% in SW480 cells), but did not affect the mutant group ($p < 0.001$, Figure 5b). Subsequently, the protein levels of LASP1 were drastically reduced by 66% in HCT116 cells and 58% in SW480 cells via miR-485-5p overexpression ($p < 0.001$, Figure 5c).

In addition, circ_0001535 inhibition repressed LASP protein levels, whereas miR-485-5p suppression overturned the influence ($p < 0.001$, Figure 5d). These data illustrated that circ_0001535/miR-485-5p could regulate LASP1 content in CRC cells.

**MiR-485-5p Suppressed CRC Progression by Modulating LASP1**

Some rescue experiments were conducted in tumor cells. To begin with, LASP1 content was greatly upregulated threefold in CRC...
Fig. 4. circ_0001535 modulates colorectal cancer cell malignant behaviors by absorbing miR-485-5p.
(a-g) HCT116 and SW480 cells were transfected with sh-NC + anti-NC, sh-circ_0001535#1 + anti-NC, or sh-circ_0001535#1 + anti-miR-485-5p. (a) Expression of miR-485-5p assessed by RT-qPCR. (b-f) Proliferation, apoptosis, invasion, and stemness of HCT116 and SW480 cells evaluated by the CCK8 assay, EDU assay, flow cytometry assay, transwell assay, and sphere formation assay, respectively. (g) Protein levels of PCNA, cleaved caspase-3, snail 1, and OCT4 in HCT116 and SW480 cells measured by Western blot assay. ***p < 0.001.

Fig. 5. LASP1 is the target of miR-485-5p.
(a) Binding sites between miR-485-5p and LASP1 predicted by starbase, and the mutant LASP1 constructed based on indicated sites. (b) Luciferase activities. (c, d) LASP1 protein level determined by Western blot assays; ***p < 0.001.
tissues and CRC cells (2.1-fold in HCT116 cells, 1.7-fold in LoVo cells, 2.1-fold in SW480 cells, and 1.9-fold in SW620 cells) ($p < 0.001$, Figure 6a-c). LASP1 protein level was effectively increased 2-fold in HCT116 cells and 1.99-fold in SW480 cells after transfection with pcDNA-LASP1 ($p < 0.001$, Figure 6d). miR-485-5p overexpression suppressed cell proliferation, whereas increased LASP1 abolished the effect ($p < 0.001$, Figure 6e, f). The flow cytometry assay displayed that miR-485-5p enrichment promoted cell apoptosis, but LASP1 overexpression abated the effect ($p < 0.001$, Figure 6g). Transwell assay and sphere formation showed that miR-485-5p mimics dampened cell invasion and stemness, whereas the effect was abrogated by elevating LASP1 ($p < 0.001$, Figure 6h, i). Meanwhile, miR-485-5p upregulation decreased PCNA, snail 1, and OCT4 expression and increased cleaved caspase 3 expression, while LASP1 overexpression reverted the effects ($p < 0.001$, Figure 6j). These data revealed that miR-485-5p inhibited the malignant behaviors of CRC cells by regulating LASP1 expression.

**Circ_0001535 Accelerated CRC Tumor Growth In Vivo**

As shown in Figure 7a, b, circ_0001535 knockdown repressed the tumor volume and weight, ($p < 0.01$). The IHC assay showed that circ_0001535 deficiency reduced ki-67 and LASP1 expressions (Figure 7c). In tumors with circ_0001535 and circ_0001535 inhibition, LASP1 content notably decreased, but miR-485-5p expression obviously increased ($p < 0.001$, Figure 7d). Finally,
circ_0001535 silencing downregulated LASP1 (reduced by 56%), PCNA (reduced by 59%), snail 1 (reduced by 59%), and OCT4 (reduced by 54%) protein level, but upregulated cleaved caspase 3 content (increased 1.7-fold) in nude mice tumor tissues \( (p < 0.001, \text{ Figure 7d}) \). These data illustrated that circ_0001535 accelerated CRC growth in vivo.

**DISCUSSION**

circRNA participated in tumor progression,\(^{20,21}\) especially in CRC.\(^{22}\) For example, circGLIS2 facilitated CRC cell viability and metastasis by absorbing miR-671.\(^{23}\) CircRAE1 boosted the invasion and migration of CRC cells by decreasing miR-338-3p and increasing TYRO3.\(^{24}\) Herein, reinforced circ_0001535 in CRC had poor survival. For functional studies, circ_0001535 inhibition repressed CRC cell proliferation and invasion and facilitated cell apoptosis in vitro. Meanwhile, circ_0001535 knockdown suppressed tumor growth in vivo. Besides, several studies have suggested that OCT4, a well-known transcription factor, exerts fundamental roles in stem cell self-renewal pluripotency, tumorigenesis, and somatic cell reprogramming.\(^{25,26}\) Recent reports indicated that OCT4 acted as a cancer stem cell marker, and its high expression could confer malignant and aggressive behavior to CRC.\(^{27,28}\) Herein, our data corroborated that circ_0001535 absence might impede OCT4 expression in tumor cells, implying the repression of circ_0001535 depletion on stemness of CRC cells.

circRNAs are known to function mainly as miRNAs sponges,\(^{29}\) which are bound to RNA-binding protein (RBP)\(^{30}\) and regulate gene transcription,\(^{31}\) translation of proteins,\(^{32}\) etc. In this study, we found that circ_0001535 are mainly located in the cytoplasm of CRC cells, which indicated that circ_0001535 functions as miRNA sponge. Therefore, we predicted and verified that circ_0001535 targeted miR-485-5p. MiR-485-5p was notably restrained in CRC and inhibited the malignant behaviors of CRC cells.\(^{33}\) Hu et al.\(^{34}\) presented the suppressive functions of miR-485-5p on CRC progression by directly regulating CD147. In this study, we also verified that miR-485-5p was obviously downregulated in CRC. In addition, miR-485-5p silencing restored the repression of circ_0001535 knockdown-triggered CRC cell malignant behaviors.
Those data indicated the regulatory roles of circ_0001535 and miR-485-5p on CRC progression. LASP1 expression increases in many cancers,\textsuperscript{35-37} including CRC.\textsuperscript{17} Chen et al.\textsuperscript{38} illustrated that LASP1 expression was evidently upregulated and could facilitate CRC cell growth and metastasis. Wang et al.\textsuperscript{39} found that LASP1 boosted CRC cell epithelial-mesenchymal transition (EMT) by modulating S100A4. The results of the present study were consistent with those of a previous report, and LASP1 was greatly increased in CRC. We confirmed that miR-485-5p directly targeted LASP1. Circ_0001535 could regulate LASP1 by absorbing miR-485-5p. Furthermore, LASP1 overexpression could rescue miR-485-5p mimics on CRC cell progression, indicating the promotion function of LASP1 in CRC progression.

In conclusion, circ_0001535 accelerated CRC development via the modulating miR-485-5p/LASP1 pathway. This study indicated that the targeted knockdown of circ_0001535 might be a potential therapeutic strategy for CRC.

Ethics Committee Approval: The Ethics Committee of the Second Affiliated Hospital of Xi’an Jiaotong University approved this study.

Data Sharing Statement: The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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