CircCUX1 Promotes Colorectal Cancer Progression by the microRNA 500a-3p/MUC4/ β -catenin/C-MYC Axis and Is Regulated by ELAVL1

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Abstract

Background

Noncoding circRNAs becoming an increasingly prominent topic of scientific study in a variety of cancers. Nevertheless, the regulatory mechanism of circRNAs in colorectal cancer(CRC) remain have not been described in detail.

Methods

Sanger sequencing, RT-qPCR and Fluorescence in situ hybridization (FISH) were used to analyse the expression and localization of circCUX1. The biological function of circCUX1 in CRC was studied by using CCK8, colony formation, EDU, Transwell and metastasis models. In terms of mechanism, western blot, luciferase reporter gene detection, RIP, ChIRP were performed.

Results

CircCUX1 was overexpressed in both cells and tissues from CRC patients. CircCUX1 overexpression was strongly linked with clinicopathological features and prognosis of colorectal patients. Functionally, CircCUX1 promoted proliferation, migration, and invasion of CRC in vivo and in vitro. Mechanistically, circCUX1 sponged miR-500a-3p to upregulate *MUC4* expression, thereby promoting the nuclear accumulation of β -catenin, and finally promoting *C-MYC* transcription. On the other hand, circCUX1 recruited ELAVL1 to increased C-MYC stability.

Conclusions

Our study for the first time reveals the specific mechanisms by which circCUX1 regulates CRC progression through two pathways, and provides a new target for the diagnosis and treatment of CRC.

Background

Colorectal cancer (CRC) is one of the most often occurring malignant tumor of the digestive tract worldwide[1], and the number of deaths due to CRC is the second highest among all malignancies[2]. In recent years, the onset age of CRC has gradually become younger. Additionally, various risk factors, such as genetic factors lifestyle, dietary habits and environmental influences, have been shown to the occurrence and development of CRC[3]. Despite recent efforts and meaningful advances in surgical resection, radiotherapy and chemotherapy, overall long-term survival in CRC patients has not changed significantly[4]. Early diagnosis and appropriate treatment can prolong the survival and improve the quality of life of patients with CRC. Therefore, there is an urgent need for highly specific biomarkers for diagnosis and prognosis, as well as the discovery of novel therapeutic targets.

A late-model noncoding RNA (ncRNA) has been discovered, which is circular RNAs (circRNA) with a covalently closed single-stranded loop structure and the loss of their 5' end caps and 3' end poly(A) tails[5]. Unlike other linear ncRNAs, circRNAs have a unique continuous circular structure and are hardly digested by exonucleases, which makes them better stability [6]. With the rapid development of bioinformatics analysis technology and high-throughput RNA sequencing, multiple circRNAs have been found to affect in the occurrence and development of a variety of diseases [7]. CircRNAs can act as microRNA (miRNA) sponge that competitively combine miRNA to block specific mRNA translation or promote its degradation. CircRNA can also interact with some proteins to regulate downstream target function functions[8]. Recent studies have shown that circRNA exists in the cytoplasm, suggesting that circRNA can be transferred to ribosomes and participate in protein translation[9]. In summary, abnormal expression of circRNAs affects tumor progression in a variety of pathways.

The proto-oncogenes of the *C-MYC* family are composed of three genes (*C-MYC*, *L-C-MYC* and *N-C-MYC*) [10]. The most studied in this proto-oncogenes family is *C-MYC*, a special transcription factor. It affects the expression of up to 15% of genes in the body[11]. Moreover, C-MYC plays a pivotal role in maintaining normal cell function and participates in cells proliferation, differentiation and apoptosis[12]. Studies have reported that *C-MYC* expression is aberrant in most human tumors. Aberrant expression of *C-MYC* can promote the development of breast cancer, multiple myeloma and melanoma, and is in connection with unfavourable prognosis[13–15]. A recent study shows that circNOTCH1 stabilizes *C-MYC* by combining with mir-449c-5p in gastric cancer cells[16]. This study is to reveal the relationship between circRNA and *C-MYC* in CRC.

In the present study, we identified a novel circRNA that had never been reported in CRC and explored its function, potential mechanism and clinical significance. We found that hsa_circ_0132813 has aberrant expression in CRC patients and is in connection with unfavourable prognosis. Furthermore, hsa_circ_0132813 enhanced the proliferation and migration of CRC cells. Hsa_circ_0132813 could sponge miR-500a-3p and degrade the mRNA of *MUC4*, a gene that was identified to translocate β -catenin into the nucleus to promote C-MYC transcription. In addition, hsa_circ_0132813 could bind to ELAVL1 to stabilize C-MYC. In a summary, hsa_circ_0132813 could co-regulate the functional role of C-MYC to promote the progression of CRC by binding miR-500a-3p and ELAVL1.

Materials And Methods

Patient samples

From September 2015 to June 2019, 80 tissue samples were obtained from the Affiliated Hospital of Xuzhou Medical University. A total of 80 pairs of CRC tissues and peritumor tissue were included. All procedures were carried out in accordance with the guidelines laid down in the Declaration of Helsinki. All the patients did not receive radiotherapy and chemotherapy before operation. The samples were verified pathologically as CRC, and the associated pathological information was gathered. This study was approved by the human research ethics committee of the Affiliated Hospital of Xuzhou Medical

University. All the patients signed written informed consent. The additional Table S1-2 contains extensive clinicopathological data.

Cell lines and cell culture

All cell lines used in this study (FHC, HCT116, LOVO, SW-480, SW-620, DLD-1 and HT-29) were purchased from the American type culture collection (ATCC). FHC, LOVO, DLD-1 and HT-29 cells were cultured in RPMI 1640 medium (L1210KJ, BasalMedia, China). HCT116 cells were cultured in McCoy's 5A medium (KeyGEN, Nanjing, China). SW-480 and SW-620 were cultured in L15 medium (KGM41300, KeyGEN, China). All media were additionally contained antibiotics (1% penicillin/streptomycin) and 10% fetal bovine serum (FBS Gibco, NY, USA). All cells were cultured in incubators at 37°C, 5% CO₂ and 100% humidity.

Cell transfection

siRNAs targeting circCUX1 (si-circCUX1) and negative control (siCtrl), ELAVL1 siRNA (si-ELAVL1), miR-500a-3p mimics, inhibitor and its negative control (NC-miR) were all bought from GeneCopoeia (Guangzhou, China). CircCUX1, *MUC4* and *C-MYC* was cloned into the pcDNA3.1(+) vector after being amplified from human cDNA (Invitrogen, Shanghai, China). SiRNAs were transfected by silencerselect lipid reagent (1027685, bio-rad, USA), plasmids were transfected by lipofectamine 2000 reagent (GK20005, invitrogen, USA). Lentiviruses were produced by Gene Pharma and transfected according to the manufacturer's instructions for a stable transfection method. Lentiviruses were produced by Gene Pharma and transfected according to the manufacturer's instructions for a stable transfection method. At 24–48 hours after transfection, cells were used for follow-up experiments. The additional Table S4 contained the sequences of oligonucleotides used in this study,

Cell invasion, migration, wound healing, proliferation and colony formation assays

The invasion assays were performed by using Transwell cells (Corning, New York, USA) and Transwell inserts (Corning Incorporated, USA). The difference between the invasion experiment and the migration experiment was that Matrigel (BD Biosciences, USA) was added in the upper chamber insert. The transfected cells were suspended in 200 µl serum-free corresponding medium and inoculated in the upper chamber insert, then corresponding medium with 20% serum was added to the lower chamber. After 24 or 48 hours, the invaded cell and migrated cells on the lower surface of the membrane were fixed with 4% paraformaldehyde (VIH100, VICMED, China) and stained with 0.1% crystal violet for 30 minutes. The cells were calculated under an Olympus microscope. Cell proliferation was monitored by the Cell Counting Kit-8 (CCK-8) assay (APExBio), OD450 values were measured after transfected for 0, 24, 48, 72 and 96 hours. For the wound healing assay, the transfected CRC cells were inoculated in 6-well plates, and when the cells grew to about 80% density, the cell monolayer was scraped with the tip of a 200 µL pipette. For the colony formation experiment, transfected CRC cells of different groups were inoculated into petri dishes. Two weeks later, the cells were fixed with 4% paraformaldehyde, then stained with 0.1% crystal violet and observed under a microscope (Olympus, Tokyo, Japan).

Quantitative realtime PCR (RT-qPCR)

Total RNA was extracted from tissues and cells by TRIzol reagent (TaKaRa, China). After the RNA concentration was determined, the reverse transcription reaction was carried out by using the Prime Script™ RT kit (TaKaRa, China) and gDNA eraser (R123-01, Cwbio, China). GAPDH, 18S and U6 RNA was selected as the internal control. $2^{-\Delta\Delta CT}$ method was used for relative quantification. Real time PCR was carried out on LightCycler96 real time quantitative PCR instrument (05815916001, Roche, Switzerland) independently in triplicate. The primers used for amplification are described in the additional Table S3.

RNA Stability

To test the stability of RNA in LOVO cells and SW480 cells, actinomycin D (GLP BIO) was added into the cells. Total RNA was extracted by TRIzol reagent (TaKaRa, China), and analyzed by real-time PCR, normalized to GAPDH. T1/2 of mRNA was calculated.

Fluorescence in situ hybridization (FISH)

The Cancer cell lines was first washed, fixed and permeable by using phosphate buffer salt solution (PBS), 4% formaldehyde and permeable solution. Then the cells were infiltrated with Triton X-100. Similarly, The RNA FISH probe were added to fixed cells under dark condition according to manufacturer's guidelines. Similarly, DNA was stained with DAPI-Antifade under dark conditions. Finally, confocal microscopy was used to acquire the images. The RNA FISH probe was designed and synthesized by Sangon Biotechnology (Shanghai, China).

RNA immunoprecipitation (RIP)

The RIP kit (P0101, GENESEED, China) was used for RIP analysis experiments. Firstly, CRC cells were lysed. Next, RIP-class antibodies were incubated with magnetic beads at 4°C for 6–8 hours. Then, the magnetic beads of the specific RNA protein complex are separated and purified to extract the coprecipitated RNA according to the manufacturer's instructions. Finally, the extracted RNA was analyzed by RT-PCR.

Chromatin isolation by RNA purification (ChIRP)

ChIRP analysis was performed manufacturer's instructions (BersinBio™, China). In short, CRC cells at a time were cleaved in a complete lysate and the DNA was then cleaved into small pieces by ultrasound. Lysates were oscillated with biotin-labeled Circux1 or control probe at 37°C for 4 h. In the end streptavidin magnetic beads were used to extract the probe, and the binding proteins were separated and analyzed by mass spectrometry.

Double luciferase reporter gene assay

293T cells were grafted into 24-well plates, and negative control mimics or MiR-500a-3p mimics were co-transfected into 293T cells with reporter plasmids using Lipofectamine 2000. At 48 h after transfection,

luciferase reporter gene analysis was performed using the dual luciferase reporter gene analysis system (Promega). Luciferase activity was normalized to luciferase activity in kidney.

Lentiviral transduction and tumour xenograft experiments

Female BALB/c nude mice (3 to 5 weeks of age) were purchased from HFK Bioscience (Beijing, China). The Ethics Committee for Animal Experiments of Xuedical University approved all animal experiments. In the subcutaneous tumor xenotransplantation experiment, HCT116 cells with stable transfection of OE-circCUX1 or OE-NC and LOVO cells with stable transfection of sh-circCUX1 or sh-NC were subcutaneously inoculated into nude mice. The size and weight of subcutaneous tumors in BALB/c nude mice were measured every three days, and the mice were euthanized 20 days later. In the transfer experiment, mice were injected with fluorescein-labeled cells via the tail vein, and the mice were euthanized 60 days later. Finally, Immunohistochemical staining and HE staining were performed on subcutaneous tumors and lung metastases in nude mice, all animal experiments complied with the Policy of Xuzhou Medical University on the Care and Use of Laboratory Animals.

Immunohistochemical staining

The tumor tissue was preserved with 4% formalin and paraffin, and the tissue was cut into 5 micron sections and treated overnight with primary antibody at 4°C. The next day, goat anti-rabbit IgG was incubated for 1 hour at room temperature. These images are captured using Olympus microscope (Tokyo, Japan).

Statistical analysis

SPSS 17.0 (IBM, Armonk, NY, USA) and GraphPad Prism V9.0 (GraphPad Software, La Jolla, CA, USA) was performed to analyse statistical analysis of the data and the picture data. The data are expressed as mean $\pm$ SD or mean $\pm$ SEM. One-way ANOVA or student t-test was determined the significance of the difference. Pearson's correlation coefficient detected the correlation analysis. The Overall survival (OS) were drawn using the Kaplan Meier method and compared using the log-rank tests. $P < 0.05$ was considered statistically significant.

Results

Clinical implication of circCUX1 levels in CRC patients

To investigate the role of circCUX1 in the clinical implication of CRC in CRC, 80 pairs of CRC tissues and matched noncancerous tissues were selected to measure their expression levels by RT-qPCR. The results indicated that circCUX1 was overexpressed in CRC tissues compared to noncancerous tissues (Fig. 1A). FISH staining assays in tissue also identified that the expression of circCUX1 was prominently in CRC tissues (Fig. 3I). 80 patients were divided into two groups by the median RT-qPCR results, and then analyzed the clinical data to study clinical implication of circCUX1 levels in CRC patients. Clinical data from Cohort S1 identified that Patients with high circCUX1 expression had greater tumor size ($P = 0.0056$),

positive tumor invasion ($P=0.0033$), advanced lymphatic metastasis ($P=0.0105$) and higher TNM stage ($P=0.0363$), but it had nothing to do with age or sex (Additional file 2: Table S1). Kaplan–Meier survival analysis revealed that circCUX1 was relevant to OS in CRC patients (Fig. 1H). Univariate Cox regression analysis identified that the important risk factors for OS were tumor invasion, lymphatic metastasis and circCUX1 expression. Multivariate Cox regression analysis identified that circCUX1 expression could act as an independent risk factor for OS ($P=0.031$; HR, 2.229; 95% CI, 1.078–4.610) in CRC patients (Additional file 2: Table S2). In conclusion, these findings imply that circCUX1 expression may be favorably linked with the progression of CRC.

Characterization of circCUX1 in CRC

Exon 2 and intron 2 in the CUX1 gene (chr7: 101559394–101559787) cyclized to form hsa_circ_0132813. It was 393 bp in length. Then, Sanger sequencing confirmed RT-PCR products of circCUX1 (Fig. 1C). CircCUX1 was tested in LOVO and SW480 by RT-PCR using divergent primers, and its cDNA was detected while the genomic DNA could not be detected (Fig. 1D). The closed continuous loop structure of circRNA was more stable than the structure of the parental linear genes with RNase R treatment (Fig. 1E). Similarly, the RT–qPCR results confirmed that the half-life of circCUX1 compared with that of CUX1 is longer after actinomycin D was applied (Fig. 1F). Moreover, we determined that the subcellular localization of circCUX1 is mainly in the cytoplasm through FISH assays in LOVO and SW480 cells (Fig. 1G). To sum up, we demonstrated that circCUX1 was a highly stable circRNAs and was mainly distributed in the cytoplasm.

CircCUX1 promotes CRC cells metastasis and proliferation in vivo

To investigate the influence of biological behavior of circCUX1 expression on biological behavior of CRC cells, we first evaluated that circCUX1 was overexpressed in CRC cells lines (LOVO, SW480, HT29, SW620, DLD1 and HCT-116) than in normal colon cell line (FHC) by RT–qPCR assays (Fig. 1B). LOVO, SW480 and HCT116 cells were selected for the following in vitro cell experiments. We designed and synthesized two siRNAs (si-circCUX1#1 and si-circCUX1#2) and an overexpression plasmid (pcDNA3.1 circRNA CUX1). Due to the analysis of clinical data, circCUX1 was found to be associated with distant metastasis, lymph node metastasis and tumor invasion. We hypothesized that circCUX1 could promote CRC cells metastasis. Subsequently, we verified whether it has an impact on migration and invasion ability through transwell assays and wound healing assays. Compared to the control groups, circCUX1-silenced CRC cells were inhibited the migratory and invasive abilities. In contrast, HCT-116 cells overexpressing circCUX1 caused increased the migratory and invasive abilities (Fig. 2A–B). We then investigated the effect of circCUX1 on the proliferation of CRC cells. CCK-8, colony formation assays and EdU assays indicated that circCUX1-silenced CRC cells were markedly suppressed proliferation abilities. On the contrary, the CRC cells in the overexpressed group showed the opposite results (Fig. 2C–E). Therefore, we demonstrated that circCUX1 promotes CRC cells metastasis and proliferation in vivo.

C-MYC mediates the carcinogenic effect of circCUX1 in CRC cells

To discover the specific molecular mechanism of circCUX1-mediated carcinogenesis in CRC, RNA-seq was performed on noncancerous controls and circCUX1 knockdown LOVO cells. In all, 703 genes were upregulated and 604 genes were downregulated when circCUX1 was knocked down (Fig. S1B). Moreover, we found that one of significant differences genes, C-MYC, has been confirmed to be a carcinogene in many cancers. We decided to study C-MYC as our target gene. Thus, western blot analysis revealed that the expression of C-MYC is poor in circCUX1-silenced CRC cells, while a high level of circCUX1 promoted its expression (Fig. S1A). An C-MYC overexpression vector was constructed in sh-circCUX1 cells to perform several rescue experiments. As a result, circCUX1 silencing reversed the promotion of CRC proliferation, migration and invasion caused by C-MYC overexpression (Fig. 3A-H). In addition, FISH staining assays in tissue also identified that the expression of C-MYC was prominently in CRC tissues (Fig. 3I). Collectively, *C-MYC* mediates the carcinogenic effect of circCUX1 in CRC cells.

CircCUX1 acts as a sponge of miR-500a-3p

Next, we explored the sponge function of circCUX1 and miRNA. By cross-analyzing different databases (circBank, BIOINF and starBase), we predicted four potential miRNAs (miR-188-5p, miR-500a-3p, miR-501-3p and miR-502-3p) that could bind to circCUX1 (Fig. 4A). Among these candidate miRNAs, only miR-500a-3p was increased after circCUX1 was knocked down and decreased after overexpression in CRC cells (Fig. 4B, Fig. S1C-E). Furthermore, we identified the putative potential binding site between circCUX1 and miR-500a-3p by searching starBase (<https://starbase.sysu.edu.cn/>). A dual-luciferase reporter assay confirmed the existence of this potential binding site. Based on their potential binding sites, we constructed the wild type (WT) and mutant(MUT) gene sequences. We co-transfected circCUX1 WT or MUT with miR-NC or mir-500a-3p mimics. The results revealed that the relative luciferase activity of circCUX1 WT in the miR-500a-3p overexpression group was significantly lower compared to miR-NC, while there was no similar change in circCUX1 MUT (Fig. 4C). Therefore, these results demonstrated that circCUX1 could bind to miR-500a-3p.

Downstream target of miR-500a-3p is MUC4

To explore the potential mechanisms of mir-500a-3p and its downstream target genes. We studied the sequencing results and the downstream target genes of miR-500a-3p by cross-analyzing TargetScan7.2 (<http://www.targetscan.org/>) and miRWalk3.0 (<http://mirwalk.umm.uni-heidelberg.de/>), and we discovered a potential target gene *MUC4*. *MUC4* is a transmembrane glycoprotein, that is abnormally expressed in pancreatic cancer carcinoma[17]. To further elucidate the regulatory effect of miR-500a-3p on *MUC4*, RT-qPCR and Western blotting were performed. As expected, downregulating miR-500a-3p enhanced the expression of *MUC4* mRNA and protein. Conversely, overexpression of miR-500a-3p revealed the opposite result (Fig. 4D, E). Next, we identified the putative potential binding site between *MUC4* and miR-500a-3p by searching TargetScan, and dual-luciferase reporter assay verified the potential binding sites. The results indicated that mir-500a-3p overexpression group significantly inhibited the relative luciferase activity of *MUC4* WT, while there was no difference in *MUC4* MUT (Fig. 4F). In conclusion, the downstream target of miR-500a-3p is *MUC4*.

MUC4 promotes C-MYC transcription by inducing β -catenin nuclear translocation

MUC4 has been reported to induce nuclear translocation of β -catenin to promote pancreatic cancer growth, metastasis, and angiogenesis, but the mechanism of action in CRC remains unclear[18]. Moreover, β -catenin has been shown to enter the nucleus from the cytoplasm to regulate the transcription of related genes (such as C-MYC, cyclinD1, WISP, etc.) [19, 20]. First, RT-qPCR and Western blot results indicated that downregulating circCUX1 suppressed *MUC4* mRNA and protein expression. Conversely, overexpression of circCUX1 showed the opposite result (Fig. 4G). We hypothesized that circCUX1 promoted the nuclear accumulation of β -catenin by upregulating *MUC4* expression through the sponge miR-500a-3p. To confirm this hypothesis, rescue experiments were conducted. Western blot results indicated that the nuclear translocation of β -catenin was suppressed in circCUX1-silenced CRC cells, whereas this suppression was then reversed when *MUC4* was overexpressed (Fig. 4H). Collectively, these results suggested that circCUX1 promotes nuclear accumulation of β -catenin through upregulation of *MUC4* expression by sponging miR-500-3p.

CircCUX1 enhances the stability of C-MYC mRNA by recruiting ELAVL1

Recently, emerging studies have shown the pivotal roles of circRNA-RNA-binding protein (RBP) interactions in tumor progression, they act as protein sponges or scaffolds to mediate complex biological functions. Using starBase, RBPDP and RBPmap, two potential RBPs (FUS and ELAVL1) that could bind to circCUX1 were obtained (Fig. 5A). Although FUS is a multifunctional DNA/RNA binding protein, FUS and circCUX1 were not combined through an RNA immunoprecipitation (RIP) assay (Fig.S1F). Previous studies have shown that circCUX1 is prominently located in the cytoplasm. We assumed that circCUX1 was bound to ELAVL1 in the cytoplasm.

First, FISH-IF staining indicated circCUX1 and ELAVL1 were co-located in the cytoplasm of CRC cells (Fig. 5B). Furthermore, ELAVL1 is an RBP that binds to mRNAs and increases their stability[21]. Then, we used RIP and chromatin isolation by RNA purification (ChIRP) assays to determine that circCUX1 could physically interact with ELAVL1 in LOVO and SW480 cells (Fig. 5D-E). Additionally, GEPIA database (GEPIA, <https://gepia.cancer-pku.cn/>) predicted that circCUX1 was positively associated with C-MYC expression (Fig. 5C). Next, RIP assays indicated that ELAVL1 could interact with C-MYC (Fig. 5F), and the mRNA stability assay revealed that ELAVL1 positively regulated the half-life of C-MYC mRNA (Fig. 5H). Additionally, we found that the stability of C-MYC decreased under circCUX1 inhibition (Fig. 5G). Moreover, overexpression of ELAVL1 rescued the stability of C-MYC in CRC cells after circCUX1 interference (Fig. 5I). In summary, these assays indicated that circCUX1 interacted with ELAVL1 to stabilize C-MYC.

CircCUX1 facilitates tumor growth and metastasis in vivo

To further confirm the existence of circCUX1 in vivo tumor proliferation relationship, lentivirus overexpressed cell lines (OE-circCUX1), lentivirus knockdown cell lines (sh-circCUX1) and their negative control cell lines (sh-NC and OE-NC) were constructed. And these cells were injected into the axilla of

BALB/ C nude mice. The weight and volume of tumors were a substantial reduction in the sh-circCUX1 group but significantly augmented in the OE-circCUX1 group (Fig. 6A-C). Nevertheless, immunohistochemistry (IHC) staining was performed on some subcutaneous tumors to determine the effect of circCUX1 on Ki67 and downstream related protein expression. Compared with that in the OE-NC group, the expression of C-MYC and Ki67 levels in the OE-circCUX1 group was significantly increased, whereas the results were completely reversed in the sh-NC group (Fig. 6D-E). To examine the metastatic ability of circCUX1, we established a mouse lung metastasis model. First, the sh-NC and sh-circCUX1 vectors with luciferase were stably transferred into LOVO cells, and then they were injected into the tail veins of BALB/c nude mice. Two months after injection, tumor metastasis was observed by using IVSI imaging system. The mice were then sacrificed and their lung tissues were stained with H&E. The results showed the sh-circCUX1 group had a substantial reduction in lung metastasis compared to the sh-NC group (Fig. 6F-G). In conclusion, circCUX1 enhanced the proliferation and metastasis ability of CRC cells in vivo.

Discussion

Recently, several studies have reported the carcinogenic effects of circCUX1 in neuroblastoma and hypopharyngeal squamous cell carcinoma, but the exact role of circCUX1 in CRC remains unclear[22, 23]. Our current studies identified circCUX1 as a key target of malignant behavior in CRC cells. We found that circCUX1 was highly expressed in CRC tissues and promoted tumor cell proliferation and metastasis. The expression of circCUX1 was highly correlated with the clinical characteristics of CRC patients, such as tumor size, pathological staging, lymph node metastasis, and distant metastasis. This finding suggested that circCUX1 could be a potential novel biomarker for predicting the prognosis of and determining a treatment for CRC.

A large number of studies have shown that circRNAs contribute to the progression of CRC through multiple mechanisms involved in gene expression and regulation[24]. Firstly, we found a gene *MUC4* through database analysis and sequencing results. *MUC4* is a membrane-anchored glycoprotein commonly expressed in epithelial tissues[25]. Its overexpression has been shown to be closely related to the aggressiveness of various cancers[26–28]. Furthermore, nuclear translocation of related proteins promoted by circRNAs has rarely been reported[29]. For example, circSPARC has been shown to promote the nuclear translocation of p-STAT3 and activate the JAK/STAT signaling pathway[30]. Recently, *MUC4* was demonstrated to induce nuclear β -catenin translocation[18]. A dual-luciferase reporter assay also verified this result. A large number of studies have proved that β -catenin regulates C-MYC transcription in the nucleus, thus linking it to our target gene to form a complete pathway. Taken together, we modulated the function of the downstream target *MUC4* by using circCUX1 as a sponge for miR-500a-3p, which thus further enabled β -catenin to undergo nuclear translocation and activated C-MYC transcription to influence CRC progression.

In recent years, the role of RBP in the pathogenesis and progression of CRC has attracted more and more attention. Simultaneously, circRNAs and RBPs can form specific circRNA-protein complexes (circRNPs)

[31], which subsequently affect protein function[32]. According to a previous paper, ELAVL1 plays a role in regulating gene expression by stabilizing mRNA[33–36]. ELAVL1 stabilizes mRNA by binding to the 3'UTR region of mRNA.[36, 37]. In this study, ELAVL1 was found to be a potential RBP of circCUX1 through several databases. After that, RIP and ChRIP experiments verified that circCUX1 was directly related to ELAVL1, and actinomycin experiments showed that ELAVL1 could stabilize C-MYC mRNA and promote CRC progression. In conclusion, circCUX1 enhanced C-MYC stability by binding to ELAVL1.

In general, it was very rare for circRNAs in this article to be reported to affect downstream targets through two mechanisms. Most of the studies were influenced by have described a single mechanism, such as autophagy, metabolism, or methylation. Our two pathways, one that affected the stability of circCUX1, and the other that promoted molecular transport, were novel. We also found that in the process by which circRNA affects stability, ELAVL1 remained unchanged after we knocked down circCUX1. However, we interfered with or overexpressed circCUX1 and ELAVL1 respectively, and the stability of C-MYC changed accordingly. We suspect that this was a synergistic effect of circCUX1 and ELAVL1, but the specific mechanism needs to be further studied.

Conclusions

In summary, we found that circCUX1 promotes the development of CRC through two pathways. On the one hand, circCUX1 promotes β -catenin nuclear translocation by enhancing *MUC4* expression by sponging miR-500a-3p, and then β -catenin further promotes C-MYC transcription. On the other hand, circCUX1 recruits ELAVL1 to increase C-MYC stability. These results suggest that circCUX1 regulates C-MYC levels through two different mechanisms. Therefore, we consider circCUX1 to be a novel diagnostic and therapeutic target for CRC.

Abbreviations

CRC
Colorectal cancer
circRNA
Circular RNA
ncRNA
noncoding RNA
miRNA
MicroRNA
siRNAs
Short interfering RNAs
OS
Overall survival
WB
Western blot

CCK-8
Cell Counting Kit-8
FISH
Fluorescence in situ hybridization
RT-qPCR
Quantitative real-time PCR
RIP
RNA immunoprecipitation
ChRIP
Chromatin Isolation by RNA Purification
H&E
Hematoxylin and eosin
IHC
Immunohistochemical staining
RBP
RNA-binding protein
WT
Wild-type
MUT
Mutated- type

Declarations

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Authors' contributions

JS, YZ and JYZ conceived the study and participated in the study design. JYZ and XDS performed cell culture, RT-qPCR, RNA stability, Western blot, RIP, ChRIP and Double luciferase reporter gene assay. JYZ, JQW and HS performed all animal experiments. Bioinformatics analysis and IHC staining were performed by JYZ and LZ. TJ and HG contributed to clinical sample collection, follow-up and clinical data analysis. Primer design and plasmid construction were carried out for JYZ and XDS. JYZ , XDS and JQW

conducted data analysis, sorted out the charts and wrote the manuscript. The final draft read and approved by all authors.

Ethics approval and consent to participate

All participants provided written informed consent, and the study was approved by the ethics committee of The Affiliated Hospital of Xuzhou Medical University. All animal experiments complied with the Policy of Xuzhou Medical University on the Care and Use of Laboratory Animals.

Consent for publication

All authors agree to publication of the article.

Competing interests

There are no competing interests between all authors.

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Figures

Figure 1

Elevated levels and characteristics of circCUX1 in CRC. **A.** RT-qPCR analysis of circCUX1 in 80 pairs of CRC and peritumor tissues. **B.** RT-qPCR analysis of circCUX1 in six CRC cell lines (LOVO, SW480, SW620, HCT-116, DLD1 and HT29) compared with normal colorectal epithelium cell lines. **C.** Schematic illustration indicated the circularization of CUX1 exon 2 and intron 2 to form circCUX1 and validation by Sanger sequencing. **D.** The cDNA and gDNA amplification products of circCUX1 were detected by agarose

gel electrophoresis . **E.** RT-qPCR analysis of the relative RNA levels of circCUX1 and CUX1 mRNA in CRC cells treated with RNase R. **F.** Timecourse of circCUX1 and *CUX1* mRNA in LOVO and SW480 cells treated with actinomycin D. **G.** FISH assay show the locational distribution of circCUX1 in CRC cells. **H.** Kaplan–Meier analysis showed increased levels of circCUX1 were substantially associated with a decrease in OS. (n=80, P=0.0013) * P < 0.05, ** P < 0.01, and *** P < 0.001.

Figure 2

CircCUX1 promotes CRC cells metastasis and proliferation in vivo. **A.** The migration and invasion abilities of CRC cells after various treatments were detected by Transwell assays. **B.** The wound healing assay was performed to assess the migration of CRC cells. **C.** CCK-8 assays were performed to assess the proliferation of CRC cells. **D.** EdU assays were performed to assess the proliferation of CRC cells. **F.** The colony formation assay was performed to assess the proliferation of CRC cells. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Figure 3

C-MYC mediates the carcinogenic effect of circCUX1 in CRC cells. **A-D.** Transwell assays and the wound healing assay demonstrated that silencing circCUX1 decreased the migration and invasion abilities of cells, while cotransfection of OE-*C-MYC* eliminated this effect in CRC cells. **E-H.** CCK-8 assay, EdU assays and colony formation assay showed that silencing circCUX1 decreased proliferation activity, while cotransfection of OE-*C-MYC* eliminated this effect in CRC cells. **I.** FISH-IF staining assays demonstrated that circCUX1 was prominently overexpressed in CRC tissues(200X). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Figure 4

CircCUX1 regulates *MUC4* expression by targeting miR-485-3p and promotes *C-MYC* transcription by inducing β -catenin nuclear translocation. **A**. The Venn diagram showed the miRNAs predicted to bind circCUX1 in the three databases. **B**. RT-qPCR showed that miR-500a-3p could bind to circCUX1 in LOVO and HCT-116 cells. **C**. Luciferase activity assay showed that circCUX1 interacted with miR-500a-3p in 293T cells. **D-F**. RT-qPCR and Western blot assays were used to detect *MUC4* levels in CRC cells after

various treatments. **G.** Luciferase activity assay showed that *MUC4* interacted with miR-500a-3p in 293T cells. **H.** Western blotting assays were used to detect *MUC4* levels after nuclear-cytoplasm separation in LOVO cells. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Figure 5

CircCUX1 enhances the stability of *C-MYC* mRNA by recruiting ELAVL1. **A.** The Venn diagram showed two potential RBPs predicted to bind circCUX1 in the three databases. **B.** FISH-IF staining indicated that the colocalization of circCUX1 and ELAVL1 was mainly in the cytoplasm. **C** and **D.** RIP and ChIRP assays to determine whether circCUX1 could physically interact with ELAVL1 in LOVO and SW480 cells. **E.** GEPIA predicted that ELAVL1 and *C-MYC* expression were positively correlated. **F.** RIP assay to determine whether *C-MYC* could physically interact with ELAVL1 in LOVO and SW480 cells. **G.** RT-qPCR was used to detect *C-MYC* levels in circCUX1-knockdown CRC cells treated with actinomycin D at a specified time point. **H.** RT-qPCR was used to detect *C-MYC* levels in ELAVL1-knockdown CRC cells treated with actinomycin D at a specified time point. **I.** RT-qPCR results demonstrated that the level of *C-MYC* in CRC cells transfected with sh-circCUX1 was increased when OE-ELAVL1 was cotransfected. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Figure 6

CircCUX1 facilitates tumor growth and metastasis in vivo. **A**. Images of subcutaneous tumors in nude mice in the interference group and the overexpression group. **B and C**. Analysis of subcutaneous tumor with respect to the weight and volume in different groups. **D and E**. IHC staining showed *C-MYC* and Ki67 levels in the subcutaneous tumors. **F and G**. After LOVO cells stably transfected with sh-circCUX1 were injected into the tail vein of nude mice, in vivo fluorescence imaging and H&E staining of pulmonary

metastatic nodules were observed. **H.** Schematic representation showing the roles of circCUX1 in the promotion of CRC. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

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