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Hsa_circ_0005075 predicts a poor prognosis and acts as an oncogene in colorectal cancer via activating Wnt/β-catenin pathway

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Abstract. – OBJECTIVE: Emerging evidence has demonstrated vital regulation of circular RNAs (circRNAs) on tumorigenesis and progression of tumors. Abnormally expressed circRNAs hsa_circ_0005075 (circ_0005075) has been reported in several tumors. In this study, we aimed to explore the expression profiles, prognostic value, and potential function of circ_0005075 in colorectal cancer (CRC)

PATIENTS AND METHODS: RT-PCR was used to detect the expression of circ_0005075 in both CRC tissues and cell lines. The associations between circ_0005075 expression and clinicopathological features and clinical prognosis were statistically analyzed. The effects of circ_0005075 on CRC cells were evaluated by Cell Counting Kit-8 (CCK-8), colony formation assays, Flow cytometer, and transwell assays. Western blot was performed to explore whether circ_0005075 modulated the Wnt/β-catenin pathway.

RESULTS: We observed that circ_0005075 expression was significantly up-regulated in both CRC tissues and cell lines. Then, clinical assays indicated that high circ_0005075 expression was significantly associated with histology/differentiation, depth of invasion, advanced TNM stage, and shorter overall survival and disease-free survival of CRC patients. Cellular studies indicated that circ_0005075 can enhance the proliferation, migration, and invasion capacities of CRC cells, thereby promoting tumor progression. Further mechanistic investigation showed that circ_0005075 displayed its tumor-promotive roles through activating Wnt/β-catenin pathway.

CONCLUSIONS: Our present data revealed circ_0005075 as a potential molecular marker and target for the prognosis and treatment of CRC patients

Key Words
Hsa_circ_0005075, Colorectal cancer, Prognosis, Metastasis, Wnt/β-catenin pathway.

Introduction

Colorectal cancer (CRC) is one of the most common malignancies worldwide, with more than half a million deaths in 2016[1]. In China, CRC has significantly increased due to changes in dietary patterns and physical activity in recent years[2]. Unfortunately, the ages of patients are becoming increasingly younger. Despite the introduction of early diagnosis and treatment options in recent years, 40-50% of patients are likely to develop local or distant tumor recurrences, and long-term survival is very poor[3,4]. In addition, a high propensity for metastasis is also the main cause of high mortality[5]. Thus, identification of novel prognostic markers of metastasis and understanding the potential mechanism of CRC metastasis would be useful for the management of CRC patients.

Circular RNAs (circRNAs) are a class of non-coding RNAs with a lack of capability for protein coding and characterized by a stable and covalently closed continuous loop without 5'-3' polarity and a polyadenylated tail[6]. Previously, non-coding RNAs termed as "junk molecule" were identified. However, a lot of evidence show that circRNAs are involved in various cells progress[7,8]. Besides, growing researchers have reported that circRNAs are involved in carcinogenesis of various tumors, such as cervical cancer, breast cancer, osteosarcoma, and CRC[9-12]. CircRNAs could act as microRNAs sponge to regulate gene expression, and thus acted as oncogenes or tumor suppressors according to the types of tumors[13,14]. In addition, because of the stable structure of circRNAs compared to non-coding RNAs, they would be the ideal potential biomarkers for diagnosis and prognosis of tumors patients[7]. However, up to date, the biological function of most circRNAs remains largely unclear.

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Shang et al[15] firstly identified a novel circRNA circ_0005075, which was abnormally expressed in hepatocellular carcinoma. Subsequently, functional findings by Li et al[16] further indicated that circ_0005075 acted as a tumor promoter in hepatocellular carcinoma. The studies of circ_0005075 in tumors were limited and, to our best knowledge, its expression and potential function in CRC have not been investigated. In this study, for the first time, we provided important data that circ_0005075 was highly expressed in CRC and it could be used as a novel prognostic biomarker and served as an oncogene in the progression of CRC.

Patients and Methods

Patients and Tissue Samples

The clinical tissue samples from a total of 145 patients with CRC undergoing surgery were obtained from Sichuan Cancer Hospital & Institute between March 2009 and April 2011. Before the resection, the patients did not receive any neoadjuvant therapy. The tissues were preserved at -80˚C after freezing using liquid nitrogen. The clinical information of the patients included age, gender, and TNM stage was summarized in Table II. The consent was acquired from all the subjects, and this study was approved by the Ethics Committee of Sichuan Cancer Hospital & Institute, Chengdu, Sichuan, China.

Cell Lines and Cell Transfection

Human normal intestinal epithelial cell line (HCO) and six CRC cell types (SW480, SW620, HT29, HCT116, SW1116, and LOVO), which were cultured in RPMI-1640 medium (Sangon, Songjiang, Shanghai, China) supplemented with 10% FBS, were obtained from YiHui Biotechnology Co., Ltd. (Minhang, Shanghai, China). For cell transfection, we used a HyFect transfection reagent (JunFeng Biotech, Hangzhou, Zhejiang, China) to transfect small interfering RNAs (siRNAs) into CRC cells. The siRNAs against circ_0005075 (siRNA#1, siRNA#2) and negative control siRNAs (si-NC) used in this study were all designed and synthesized by Transheep Biotechnology Co., Ltd. (Suzhou, Jiangsu, China).

Real-Time PCR Assays

We used an Eastep RNA isolation kit (Lab Biotechnology, Nankai, Tianjin, China) to extract the total RNA of CRC cells. After the total RNA was reversely transcribed into cDNA by using a Golden RT MasterMix kit (Haigene, Haerbin, Heilongjiang, China), the qRT-PCR assays were performed using a qRT-PCR detection kit (Kanglang Biotechnology, Minhang, Shanghai, China). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The expression levels of β-catenin, cyclin D1, c-myc, and circ_0005075 were measured as relative fold changes based on $2^{-\Delta\Delta Ct}$ method. The primers used in the present study were listed in Table I.

Cell Counting Kit-8 (CCK-8) Assay

Briefly, we firstly transfected circ_0005075 or control siRNAs into HTC116 and LOVO cells. Subsequently, the cells (2×10^3 cells per well) were seeded into 96-well plates. At 48 h, 72 h and 96 h, each well was added with 10 μl CCK-8 reagents (ExcellBio Technology, Taicang, Jiangsu, China). Then, the absorbance at 450 nm was examined by a microreader (SM600; Yanhui Biotechnology, Jiading, Shanghai, China).

Colony Formation Assay

In brief, after circ_0005075 or control siRNAs were transfected into HTC116 and LOVO cells, the cells were collected and re-plated into 6-well plates at a density of about 500 cells per well. The cells were cultured for two weeks until the cell colonies were observed. Then, 0.1% crystal violet (Labgene, Guangzhou, Guangdong, China) was utilized to stain the colonies. The number of HTC116 and LOVO cell colonies was then counted using a microscope (PRECISE, Dongcheng, Beijing, China).

Table I. The sequences of primers for RT-PCR used in this study.

Names	Sequences (5′-3′)
Hsa_circ_0005075: Forward	CAAATCTTGCGGCAACGC
Hsa_circ_0005075: Reverse	GCGGGAGTGAAGATTCGA
GAPDH: Forward	GGAGCGAGATCCCTCCAAAAT
GAPDH: Reverse	GGCT GTTGTCATACTTCTCATGG

Table II. Correlation between hsa_circ_0005075 expression and clinicopathological features of colorectal cancer.

Clinicopathological features	No. of cases	hsa_circ_0005075 expression		*p*-value
		High	Low	
Age				NS
≤ 60	67	32	35	
> 60	78	40	38	
Gender				NS
Male	95	45	50	
Female	50	27	23	
Tumor size				NS
≤5 cm	94	42	52	
>5 cm	51	30	21	
Histology/differentiation				0.011
Well + Moderate	97	41	56	
Poor	48	31	17	
Depth of invasion				0.016
T1 + T2	102	44	58	
T3 + T4	43	28	15	
TNM stage				0.030
I-II	97	42	55	
III-IV	48	30	18	
Tumor site				NS
Colon	74	34	40	
Rectum	71	38	33	

Cell Apoptosis Analysis

The apoptosis of HTC116 and LOVO cells after treatment with circ_0005075 or control siRNAs were examined by an Annexin V Apoptosis analysis kit (MultiSciences Biotechnology, Hangzhou, Zhejiang, China). In short, the treated cells were collected, washed with phosphate-buffered saline (PBS), and sequentially stained with 15 μl annexin V-FITC/propidium iodide mixture buffer for 15 min in the dark. Thereafter, the percentage of apoptotic HTC116 and LOVO cells was measured by a flow cytometer (Attune NxT, Thermo Fisher Scientific Inc., Pudong, Shanghai, China)

Western Blot Assays

The HTC116 and LOVO cells after siRNAs treatment were collected and sequentially lysed with lysis buffer for Western blot (GeneLife Biotechnology, Mentougou, Beijing, China). Dodecyl sulfate, sodium salt (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) was then applied to separate the proteins of cell lysates, which was sequentially transferred to polyvinylidene difluoride (PVDS) membranes. Subsequently, the membranes were blocked using PBS/Tween 20 containing 5% BSA and probed with primary antibodies at 37°C. After 24 h, the membranes were incubated with corresponding secondary antibodies, following visualized using a super ECL luminescence reagent (ABSIN Biotechnology, Pudong, Shanghai, China). The anti-β-catenin, anti-Cyclin D1, and anti-c-Myc antibodies were all purchased from Cell Signaling Technology Co., Ltd. (Beverly, MA, USA); the anti-vimentin, anti-GAPDH, and anti-N-cadherin antibodies were all obtained from Wuhan BOSTER Biotechnology Co., Ltd. (BOSTER, Wuhan, Hubei, China).

Wound Healing Assays

Briefly, circ_0005075 or control siRNAs were firstly transfected into HTC116 and LOVO cells. Afterward, the cells were maintained in 12-well plates until the cell confluence reached 100%. Then, a 200 μl sterilized pipette tip was applied to scratch the cells to generate the wounded areas. After washing with PBS three times, a microscope (PRECISE, Dongcheng, Beijing, China) was employed to take photographs of the wounded areas.

Transwell Invasion Assay

HTC116 and LOVO cells transfected with circ_0005075 or control siRNAs were digested with trypsin and collected. Then, the treated cells were resuspended in 100 μl culture medium without FBS and sequentially added into the

upper sides (pre-treated with 70 µl Matrigel) of the transwell inserts (R&S Biotechnology, Pudong, Shanghai, China). In addition, complete cell culture medium with 15% FBS was filled with the lower chambers. Twenty-four hours later, the invaded cell on the lower sides of the transwell inserts was stained with 0.1% crystal violet and sequentially observed using a microscope (PRECISE, Dongcheng, Beijing, China).

Statistical Analysis

The meeasurement data were evaluated by a paired two-tailed *t*-test or one-way ANOVA. Tukey's post-hoc test was used to validate the ANOVA for comparing measurement data between groups. Survival curves were assessed by the Kaplan-Meier methods and analyzed using the log-rank test. The Cox proportional hazards model for multivariate survival analysis was used to assess predictors related to survival. SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) was applied to perform all the analysis. The *p*-value of less than 0.05 was considered statistically significant.

Results

Circ_0005075 Is Highly Expressed in Both CRC Tissues and Cell Lines

To explore the potential effect of circ_0005075 in CRC progression, we first detected the expression of circ_0005075 in 145 pair of CRC tissues and matched normal colorectal tissues. As shown in Figure 1, we found that circ_0005075 expression was significantly up-regulated in CRC tissues compared to matched normal colorectal tissues ($p<0.01$). Then, we also observed that CRC patients with advanced TNM stage exhibited higher levels of circ_0005075 (Figure 1B). We also examined circ_0005075 expression in six human CRC cell lines and a normal colonic cell line (GCO) by qRT-PCR, finding that circ_0005075 expression was significantly up-regulated in CRC cell lines (Figure 1C). Taken together, our results firstly demonstrated that circ_0005075 was highly expressed in CRC and may be involved in the progression of CRC.

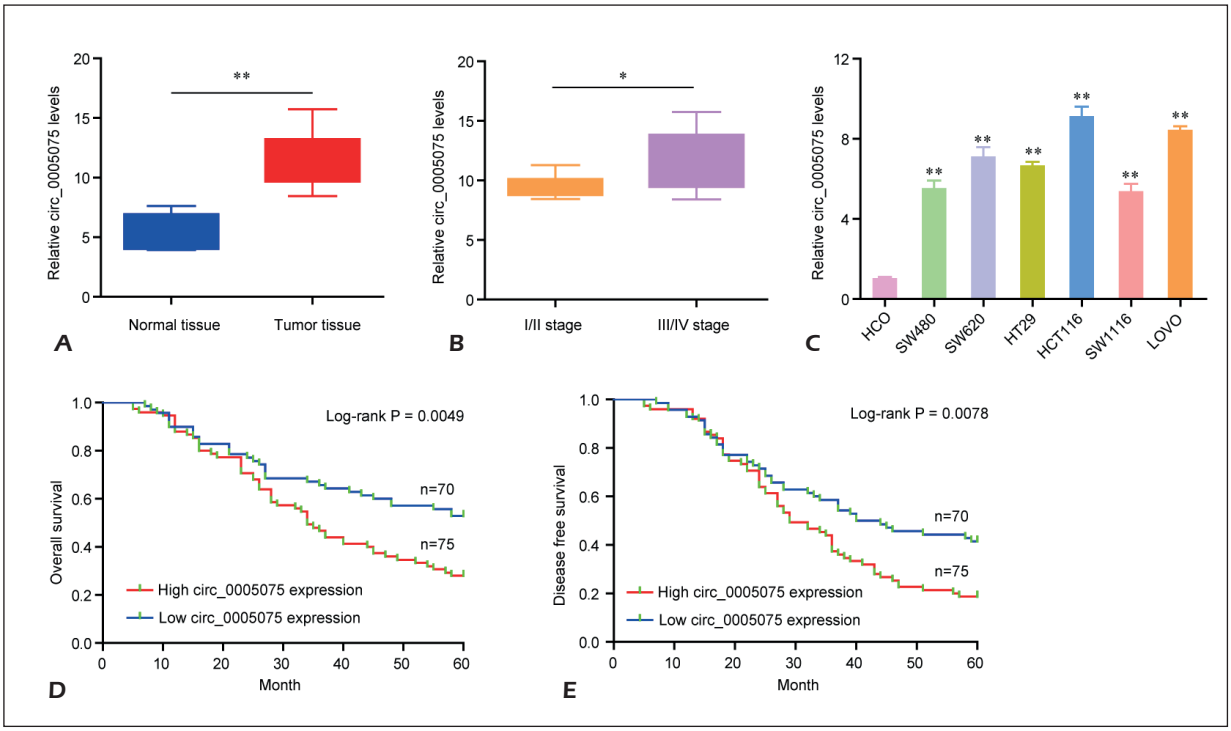

Figure 1. Circ_0005075 was highly expressed in CRC and associated with poor prognosis. **A**, Circ_0005075 expression was tested in both normal tissues and CRC tissues by qRT-PCR. **B**, CRC tissues with advanced stages displayed a higher level of circ_0005075. C, Circ_0005075 in six CRC cell lines and one normal cell by RT-PCR. **C-D**, Kaplan-Meier analysis and log-rank test indicated the 5-year overall survival and disease-free survival rate of CRC patients with high or low circ_0005075 expression level. *$p<0.05$, **$p<0.01$.

Table III. Multivariate analyses for disease-free survival and overall survival by Cox regression model.

Variable	Overall survival			Disease-free survival		
	HR	95% CI	*p*	HR	95% CI	*p*
Age	1.542	0.781-2.134	0.144	1.327	0.894-2.443	0.118
Gender	1.682	0.923-2.321	0.167	1.347	0.725-2.071	0.133
Tumor size	1.415	0.815-2.554	0.121	1.329	1.023-2.334	0.105
Tumor site	1.328	0.792-2.563	0.438	1.128	0.834-2.328	0.322
Histology/differentiation	3.542	1.326-4.668	0.006	3.833	1.548-5.226	0.002
Depth of invasion	3.126	1.428-4.432	0.011	3.279	1.672-4.679	0.006
TNM stage	3.256	1.214-4.327	0.016	3.774	1.457-4.826	0.008
hsa_circ_0005075 expression	3.237	1.479-5.158	0.003	3.452	1.638-5.438	0.001

Relationship Between Circ_0005075 Expression and the Prognosis of CRC Patients

Nest, we analyzed the potential clinical significance of circ_0005075 expression in CRC patients, and all patients were divided into a high circ_0005075 expression group and a low circ_0005075 expression group using the median circ_0005075 expression. As shown in Table II, our results indicated that high circ_0005075 expression was significantly associated with histology/differentiation ($p = 0.011$), depth of invasion ($p = 0.016$), and advanced TNM stage ($p = 0.030$). However, there was no correlation between circ_0005075 expression and other clinical parameters. Furthermore, the Kaplan-Meier survival analysis was used to investigate the association between circ_0005075 expression and the prognosis of CRC patients and the results showed that up-regulated circ_0005075 expression was significantly associated with poorer overall survival ($p = 0.0049$, Figure 1D) and disease-free survival ($p = 0.0078$, Figure 1E) of CRC patients. More importantly, multivariate analysis evidenced that circ_0005075 expression level was independently associated with the overall survival (HR = 3.237, 95% CI: 1.479-5.158, $p = 0.003$) and disease-free survival (HR = 3.452, 95% CI: 1.638-5.438, $p = 0.001$) (Table III).

Effects of Circ_0005075 on Cellular Proliferation of CRC Cells

Considering that circ_0005075 was highly expressed in CRC and predicted poor prognosis, we next aimed to study the biological function of circ_0005075 in CRC cells. We firstly synthesized siRNAs against circ_0005075 (siRNA#1 and siRNA#2) and transfected them into HTC116 as well as LOVO cells. The expression of circ_0005075 was significantly changed in these cells after transfection with circ_0005075 siRNAs using qRT-PCR detection (Figure 2A). We next performed CCK-8 assays to evaluate the affection of circ_0005075 knockdown on cell cellular growth. The data revealed that the cell viability of HTC116 and LOVO cells was evidently impeded when circ_0005075 was knocked down (Figure 2B). In addition, the colony formation assay showed that the silence of circ_0005075 remarkably depressed the colony formation abilities of HTC116 and LOVO cells (Figure 2C). Besides, cell apoptosis detected by flow cytometry demonstrated that circ_0005075 knockdown could markedly accelerate the apoptosis of HTC116 and LOVO cells (Figure 2D). Therefore, our data indicated that the depression of circ_0005075 suppressed the proliferation of CRC cells and enhanced cell apoptosis.

Circ_0005075 Modulated the Metastatic Potentials of CRC Cells

We further evaluated whether circ_0005075 affected the migration and invasion of CRC cells using wound healing and transwell invasion assays. After treatment with circ_0005075 siRNAs, the wounded areas of HTC116 and LOVO cells were remarkably wider than that of cells transfected with negative control siRNAs (si-NC), which implied that the silence of circ_0005075 reduced the cell migratory capacity (Figure 3A and B). Besides, the results of transwell invasion assays suggested that circ_0005075 siRNAs treatment notably impaired the invasive ability of HTC116 and LOVO cells (Figure 3C). Additionally, Western blot analysis revealed that N-cadherin and vimentin, which were two epithelial-mesenchymal transition (EMT) associated molecules, were significantly inhibited by transfection of circ_0005075 siRNAs in HTC116 and LOVO

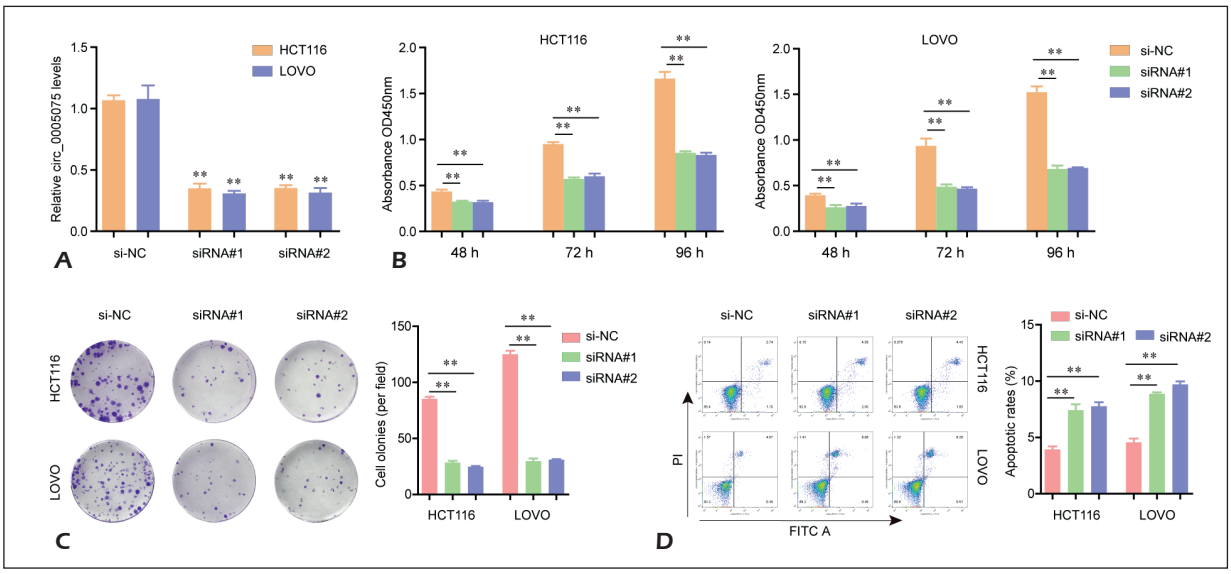

Figure 2. Knockdown of circ_0005075 affected the proliferation and invasion of HTC116 and LOVO cells. **A**, The relative circ_0005075 levels in HTC116 and LOVO cells after transfecting with circ_0005075 siRNAs (siRNA#1, siRNA#2) and negative control siRNAs (si-NC) were detected by qRT-PCR assays. **B**, CCK-8 assays measured the HTC116 and LOVO cell proliferation at 48 h, 72 h and 96 h. **C**, The colony formation assays detected the colony formation abilities of HTC116 and LOVO cells after transfection with circ_0005075 siRNAs (Magnification, 10×). **D**, Flow cytometry examined the cell apoptosis and silence of circ_0005075 promoted cell apoptosis. *p<0.05, **p<0.01.

cells (Figure 3D). Collectively, these data validated that circ_0005075 could affect the metastatic potentials of CRC cells via EMT pathway.

Knockdown of Circ_0005075 Suppressed the Activation of the Wnt/β-Catenin Signaling Pathway in CRC Cells

To uncover the downstream molecular mechanisms by which circ_0005075 modulated the development and progression of CRC, we next conducted qRT-PCR and Western blot assays to detect the expression of molecules involved in Wnt/β-catenin signaling pathway. After transfection of circ_0005075 siRNAs, the mRNA levels of β-catenin, cyclin D1, and c-myc in HTC116 and LOVO cells were remarkably reduced in contrast with si-NC-transfected control cells (Figure 4A). In addition, Western blot analysis was also performed, and the results indicated that circ_0005075 siRNAs transfection induced a significant decrease of β-catenin, cyclin D1, and c-myc protein levels in HTC116 and LOVO cells, which was consistent with the results of qRT-PCR assays (Figure 4B). Taken together, these data demonstrated that repression of circ_0005075 inhibited the activation of the Wnt/β-catenin signaling pathway in CRC cells.

Discussion

CRC is one of the major causes of cancer death throughout the world. Up to date, the clinical prognosis of CRC remains poor and those patients diagnosed with late-stage CRC have a poor survival rate of 13.1%[17,18]. Improved methods for early detection and prediction of prognosis of CRC are essential for increasing survival. In clinical practice, colonoscopy has been used to diagnose CRC for many years. However, its invasive character limited its use in early diagnosis[19,20]. In addition, several clinicopathological characteristics of CRC patients such as TNM stage and lymph node metastasis have been used to predict the clinical prognosis and guide individualized treatment. However, their specificity and sensitivity are relatively low[21]. Therefore, the identification of novel biomarkers for CRC is urgent. Recently, non-coding RNAs were reported to have potential as a biomarker for tumors patients. Several evidence[22,23] suggested that miRNAs and long noncoding RNAs were frequently dysregulated in tumor tissues and involved in the progression of tumors. Another non-coding RNAs, circRNAs, have emerged as a novel regulator in tumorigenesis and progression of tumors[24]. The enormous potential of circRNAs as a novel biomarker and therapeutic targets encouraged us to explore their biological function.

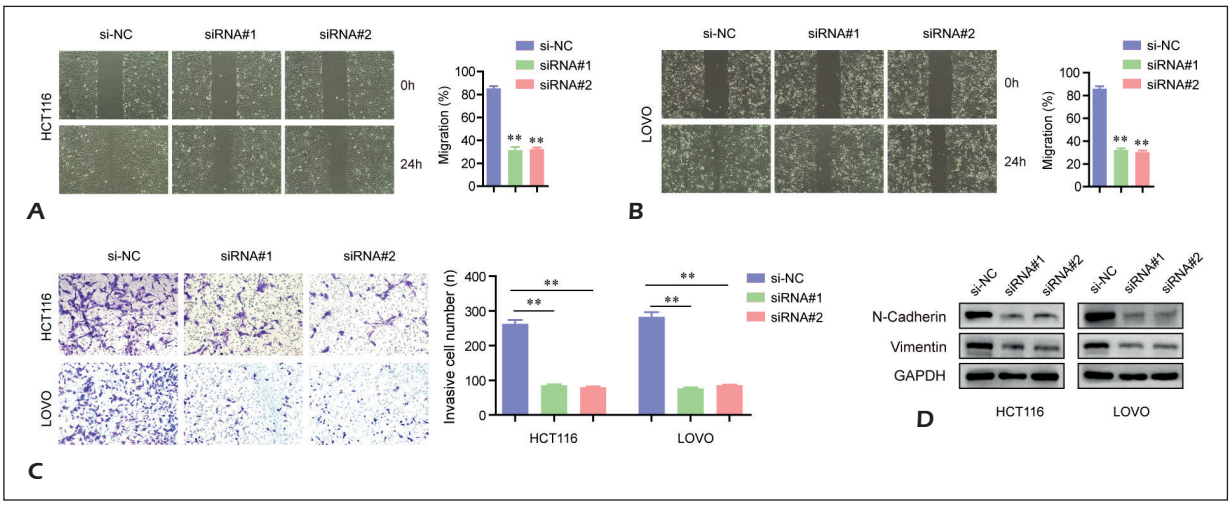

Figure 3. The depression of circ_0005075 inhibited the invasion and migration of HTC116 and LOVO cells. **A** and **B**, Knockdown of circ_0005075 reduced the migration of HTC116 and LOVO cells (Magnification, 10×). **C**, The invasive number of HTC116 and LOVO cells was decreased after the cells were transfection of circ_0005075 siRNAs (Magnification, 40×). **D**, Western blot assays evaluated the protein levels of N-cadherin and vimentin, and silence of circ_0005075 reduced the expression of N-cadherin and vimentin. *p<0.05, **p<0.01.

Circ_0005075 was a newly identified circRNA. Li et al[16] showed that circ_0005075 was highly expressed in hepatocellular carcinoma and its overexpression could promote the proliferation, migration, and invasion of hepatocellular carcinoma cells by targeting miR-431, suggesting that circ_0005075 acted as an oncogene in hepatocellular carcinoma. In this study, we also found that circ_0005075 expression was significantly up-regulated in both CRC tissues and cell lines. Interestingly, CRC tissues with advanced stages displayed a high level of circ_0005075, indicating that circ_0005075 may act as a positive regulator in the clinical progression of CRC. Then, we explored the clinical significance of circ_0005075 by studying the associations between circ_0005075 expression and clinicopathological characteristics, finding that high expression of circ_0005075 was significantly associated with depth of invasion and advanced TNM stage. Moreover, clinical survival assay indicated that higher levels of circ_0005075 predicted shorter overall survival and disease-free survival. Finally, the multivariate analysis clearly confirmed that the high circ_0005075 expression was a potential prognostic factor affecting both overall survival and disease-free survival in CRC patients, indicating that circ_0005075 may be used as a potential biomarker for CRC patients. However, the study populations are relatively small and larger sample sizes were further needed to confirm our findings.

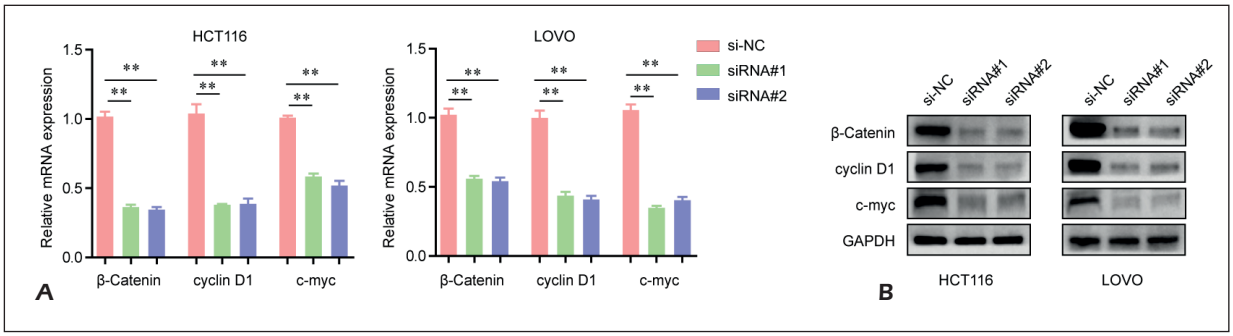

Figure 4. The effects of circ_0005075 on the activity of Wnt/β-catenin signaling pathway in HTC116 and LOVO cells. **A**, The qRT-PCR assays were utilized to examine the mRNA levels of β-catenin, cyclin D1, and c-myc in HTC116 and LOVO cells transfected with circ_0005075 siRNAs. **B**, The protein levels of β-catenin, cyclin D1, and c-myc in HTC116 and LOVO cells were decreased after the cells were transfected with circ_0005075 siRNAs. *p<0.05, **p<0.01.

Previously, several studies have reported that several circRNAs could be involved in the regulation of CRC cells proliferation, apoptosis, and metastasis. For instance, circRNA circ_0026344 was reported to be lowly expressed in CRC and acted as a tumor suppressor by decreasing the proliferation and invasion of CRC cells via sponging miR-21 and miR-31[25]. CircHIPK3, highly expressed in CRC, was found to be associated with advanced clinical stages and poor prognosis and to promote CRC growth and metastasis by sponging miR-7[26]. These results highlighted the potential of circRNAs as a novel therapeutic target for CRC. In this study, given the up-regulation of circ_0005075 in CRC, we assumed that it may function as a tumor promoter and we down-regulated its levels by using si-circ_0005075. *In vitro* assays indicated that knockdown of circ_0005075 significantly suppressed CRC cells proliferation, migration and invasion, and promoted apoptosis, suggesting that circ_0005075 acted as a positive regulator in cells progression of CRC. We also explored the association between circ_0005075 expression and EMT progress which is a criticism of tumor metastasis[27]. As expected, the down-regulation of circ_0005075 suppressed EMT progression by decreasing the levels of Vimentin and N-cadherin. Taken together, our results expanded the knowledge of circ_0005075 as an oncogenic regulator in the development of CRC.

The Wnt/β-catenin signaling pathways, also called canonical Wnt pathway, are a group of signal transduction pathways which begin with some proteins that send signals into a cell via cell surface receptors[28]. It has been highlighted that this signaling pathway acted as a critical role in the regulation of cell growth, cell development, and differentiation[29]. Dysregulated activation of this pathway can cause uncontrolled cell growth and cell metastasis. Recently, growing evidence shows that Wnt/β-catenin signaling pathways are involved in the development and progression of tumors and their potential mechanisms are also studies. Previous studies[30-33] indicated that some functional tumor-related genes, miRNAs, long noncoding RNAs, and circRNAs displayed their tumor-promotive roles or tumor-suppressive roles by modulating Wnt/β-catenin signaling pathways. In this study, in order to explore the potential mechanism by which circ_0005075 promoted progression of CRC, we focused on the association between circ_0005075 and Wnt/β-catenin signaling pathways. Our results indicated that knockdown of circ_0005075 decreased the expression levels of β-catenin, cyclin D1 and c-myc in both HTC116 and LOVO cells, suggesting that circ_0005075 displayed its tumor-promotive roles by promoting Wnt/β-catenin pathways.

Conclusions

For the first time, we reported that circ_0005075 was highly expressed in CRC and associated with an aggressive tumor phenotype and unfavorable outcome in CRC patients. Knockdown of circ_0005075 suppressed CRC cells proliferation and metastasis by modulating Wnt/β-catenin pathways. Therefore, circ_0005075 may be considered as a novel prognostic biomarker and a potential therapeutic target for CRC patients in the future.

Conflict of Interests

The Authors declare that they have no conflicts of interests.

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