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Characteristics and prognostic significance of circRNA-100876 in patients with colorectal cancer

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Abstract. – OBJECTIVE: The purpose of this study was to explore the correlation between circRNA-100876 and the clinicopathological parameters of patients with colorectal cancer (Cc).

PATIENTS AND METHODS: Quantitative real-time polymerase chain reaction (RT-qPCR) was applied to detect the circRNA-100876 expression in Cc tissues and cell lines. Overall survival analysis was carried out to explore the correlation between circRNA-100876 and the prognosis of Cc patients by Kaplan-Meier method and Log-rank method. Subsequently, Chi-square test was used to investigate the clinical significance of circRNA-100876 in the clinicopathological parameters of Cc patients. Moreover, the expression of circRNA-100876 was inhibited by small interfering RNAs (siRNAs) in loss-of-function assay. Finally, the invasion ability of Cc cells was determined by transwell assay.

RESULTS: The results of this study manifested that circRNA-100876 was abnormally overexpressed in Cc tissues and cell lines, and the high expression of circRNA-100876 was clearly associated with the Clinical stage, T classification and Lymph node metastasis of Cc patients. Besides, Cc patients with high expression worsened overall survival. In addition, it was demonstrated that the inhibition of circRNA-100876 reduced the invasion ability of Cc cells.

CONCLUSIONS: Acting as a tumor promoter, circRNA-100876 might be regarded as a new potential biomarker for the diagnosis and therapy of Cc.

Key Words:
Colorectal cancer, CircRNA-100876, Clinicopathological parameters, Overall survival.

Introduction

Colorectal cancer (Cc) is one of the ubiquitous malignant tumors of the digestive system that seriously threatens human health, and its prevalence is rising constantly with the improvement of people's living standards and changes in eating habits[1,2]. Radiotherapy, chemotherapy and surgical treatment are the commonly used therapies for Cc at present[3,4], but the majority of Cc patients have been in the intermediate and advanced stage when definitely diagnosed as the incidence of Cc is an accumulative process involving multiple steps as well as mutation and deletion of many genes[5], in combination with its characteristics, such as insidious onset and inconspicuous early clinical symptoms[6]. Moreover, Cc is prone to metastasis, relapse and poor prognosis[7], so investigating the pathogenesis of Cc and seeking for efficacious therapeutic methods are the research hotspots at present.

As a novel category of non-coding ribonucleic acids (RNAs), circular RNAs (circRNAs) widely exist in the cytoplasm of eukaryocytes and have extremely favorable stability[8]. In fact, circRNAs possess various functions, including mediating splicing and transcription, binding to proteins and transporting RNAs[9,10]. According to the latest studies, circRNAs are well known for their critical determinants in diverse cancers biobehavior[11,12], and certain circRNAs may act as biomarkers for cancer diagnosis and prognosis and are tightly associated with the procession of cancers. Yao et al[12] revealed that circRNA-100876 played important role in the non-small cell lung cancer development and was clearly related with the prognosis of patients with non-small cell lung cancer. Jin et al[13] demonstrated that the inhibition of circRNA-100876 induced apoptosis and suppressed proliferation ability of osteosarcoma cancer cells. However, currently, no literature has illustrated the role of circRNA-100876 in regulating the Cc progression.

Therefore, the present study was carried out to investigate the potential role of circRNA-100876 in Cc development and to clarify the connections

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between circRNA-100876 and clinicopathological characteristics of Cc patients.

Patients and Methods

Patients and Tissues

A total of 124 fresh specimens of colorectal cancer tissues and their corresponding adjacent cancer tissue specimens were collected from patients definitely diagnosed with Cc by clinical pathologists for quantitative Real Time-PCR detection. Inclusion criteria were in accordance with the guideline proposed by the Union for International Cancer Control (UICC). The specimens used were from Putuo People's Hospital of Tongji University. All patients did not receive radiation or chemotherapy before surgery. This study was approved by the Putuo People's Hospital of Tongji University Ethics Committee and agreed by the patient.

Culture of Cells

Human Cc cell lines (HCT116, SW480, HT29 and SW620) and a human colon epithelial cell line (HCM460) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA), and were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% dual antibodies at 37°C under 5% CO_2. When the cell density reached about 90%, the cells were trypsinized and passaged at 1:3.

Cell Transfection

The cells were digested and seeded into a 6-well plate, followed by transfection when the density was 50-70%. Firstly, the siRNA and Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) were separately added into Eppendorf (EP, Hamburg, Germany) tubes containing serum-free DMEM and incubated at room temperature for 5 min. Then, siRNA and Lipofectamine 2000 were mixed gently and placed at room temperature for 15 min. Subsequently, the mixture was added into the medium for cell culture for 6 h. Finally, the medium was replaced with complete medium for further experiments.

Transwell Assay

After transfection for 48 h, the cells were digested, centrifuged, and resuspended in serum-free DMEM, followed by cell counting and adjustment of cell density to 1×10^5 cells/mL. Later, the transwell chambers were put into a 24-well plate, and 200 μL of cell suspension was added into the upper chamber while 500 μL of complete medium was added into the lower chamber. Next, the chambers were normally cultured in the incubator for 42 h, then, taken out and gently washed with phosphate-buffered saline (PBS) for three times. After the cells in the chambers were wiped out using cotton swabs, the chambers were fixed with 4% paraformaldehyde at room temperature for 15 min, air dried and stained with crystal violet for 5 min, followed by cleaning with PBS for three times. Finally, the transmembrane cells were observed under a microscope, photographed, and counted using software.

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

When the density reached 80-90%, the cells in the growth phase were collected and washed with PBS for 2-3 times after discarding the medium, and then, the waste liquid was absorbed. Later, 1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was added into each culture dish and placed on ice for 5 min. The concentration and purity of RNAs in each group of samples were determined using a NanoDrop 2000 nucleic acid detector. Subsequently, the RNAs were reversely transcribed into complementary deoxyribonucleic acids (cDNAs) by reference to the instructions of SYBR Premix Ex Taq™ II kit (TaKaRa, Otsu, Shiga, Japan), and proper quantities of cDNAs were taken for qRT-PCR under the following conditions: 95°C for 5 min, 95°C for 10 s, 59°C for 30 s and 72°C for 30 s, 40 cycles in total. The average value of 3 replicate wells was regarded as the Ct value for each group, with Actin as an internal reference. The primers used in this study were listed as follows: circRNA-100876, forward 5′-CTGGTGCAGTGGAAGCAGAG-3′ and reverse 5′-CGACCCTCCATTGCTCTTCT-3′. β-actin, forward 5′-CCACCATGTACCCAGGCATT-3′ and reverse 5′-ACTCCTGCTTGCTGATCCAC-3′. Finally, experimental results were subjected to data processing, histogram plotting, and result analysis and statistics by $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

All the data were analyzed by Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA). The differences between the two groups were analyzed using

the Student's *t*-test. The comparison among multiple groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). Kaplan-Meier method and Log rank analysis was used to analyze the overall survival of Cc patients. The association between circRNA-100876 and clinicopathological characteristics of Cc patients was performed by Chi-square test. The duplicated data were measured *via* repeated measures analysis of variance, and $p<0.05$ suggested statistically significant differences.

Results

CircRNA-100876 Was Upregulated In Cc Tissues and Cells

To demonstrate the effect of circRNA-100876 on Cc, qRT-PCR was firstly performed to analyze its expression status in collected tumor tissues and related adjacent normal tissues of Cc patients. The results in Figure 1A clearly showed that circRNA-100876 was notably upregulated in Cc tissues when compared to the adjacent non-cancerous tissues. Meanwhile, the circRNA-100876 expression was compared in four Cc cell lines (HCT116, HT29, SW480, and SW620) and a normal human colon epithelial cell line (HCM460). CircRNA-100876 was also observed to be highly expressed in Cc cells lines compared with that in the normal cell line (Figure 1B). Hence, the verified enhanced expression of circRNA-100876 both in Cc tissues and cell lines suggested that circRNA-100876 might be closely associated with the Cc progression.

CircRNA-100876 Was Closely Associated With Clinicopathological Characteristics of Cc Patients

The association between circRNA-100876 expression and the clinicopathological parameters of patients with Cc was further analyzed by the Chi-square test. As shown in Table I, evident associations of highly expressed circRNA-100876 with Clinical stage (p=0.013), T classification (p =0.031) and Lymph node metastasis (p =0.029) were detected, whereas no relationships with clinicopathological characteristics including age, gender, venous invasion and histological type were found.

High Expression of CircRNA-100876 Predicted Poor Overall Survive In Cc Patients

In addition, Kaplan-Meier method and Log rank analysis were performed to explore whether a connection exists between the altered expression of circRNA-100876 expression and the prognosis of Cc patients. As shown in Figure 2, Cc patients with high circRNA-100876 expression exhibited shorter overall survival compared with patients with low expression of circRNA-100876.

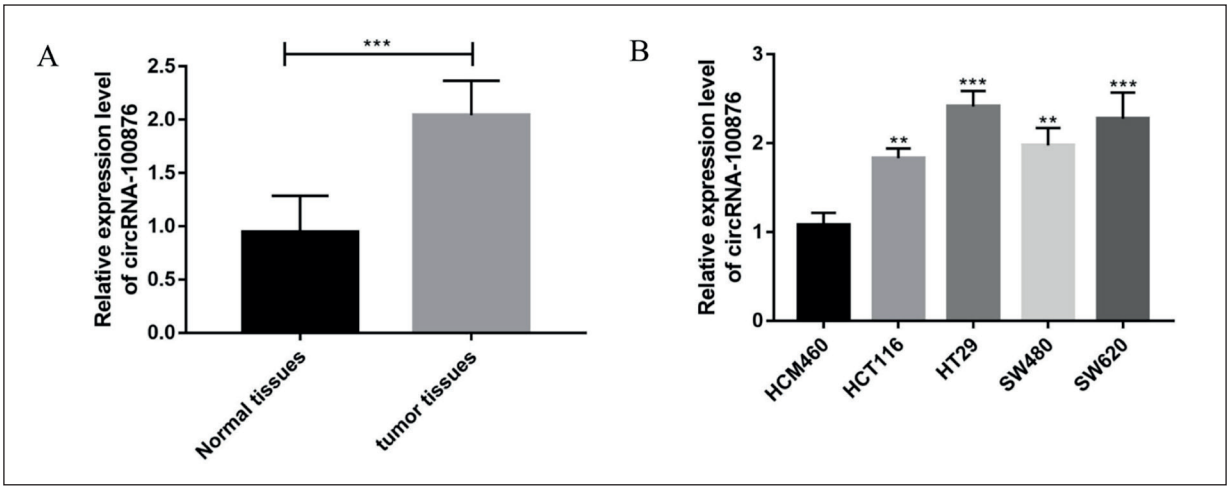

Figure 1. Expression of circRNA-100876 in Cc tissues and cells. **A,** CircRNA-100876 was significantly highly expressed in Cc tissues compared with that in adjacent normal tissues. **B,** CircRNA-100876 was significantly upregulated in HCT116, HT29, SW480 and SW620 cell lines in contrast to that in HCM460 cell line. (**p<0.01, ***p<0.001).

Table I. Correlations between circRNA-100876 expression and clinic-pathological characteristics of patients with colorectal cancer.

Characteristics	No.	Low expres-sion	High expres-sion	*p*-value
Age (years)				
< 50	48	26	22	0.265
≥ 50	76	49	27	
Gender				
Female	52	30	22	0.069
Male	72	29	43	
Clinical stage				
I-II	40	14	26	0.013*
III-IV	84	50	34	
T classification				
T1-T2	60	24	36	0.031*
T3-T4	64	39	25	
Lymph node metasta-sis				
Negative	74	32	42	0.029*
Positive	50	32	18	
Venous invasion				
Negative	51	27	24	0.857
Positive	73	40	33	
Histological type				
Differentiated	76	29	47	0.141
Undifferentiated	48	25	23	

Transfection of SiRNA Inhibited CircRNA-100876 Expression In Cc Cells

To better illustrate the roles of circRNA-100876 in mediating the Cc development, circRNA-100876 siRNA was transfected into HT29 and SW620 cell lines due to the abnormal upregulated expression of circRNA-100876 for loss-of-function assay. qRT-PCR results in Figure 3 showed that siRNA transfection successfully suppressed the circRNA-100876 expression both in HT29 and SW620 cell lines compared with that in untreated cells.

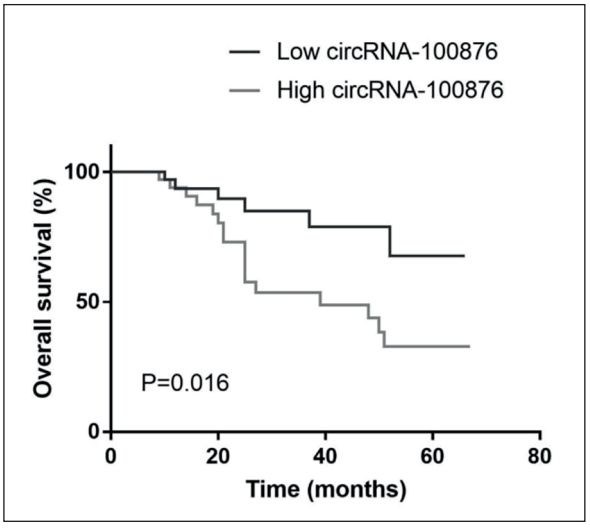

Figure 2. Association between the circRNA-100876 and survival of Cc patients. Patients with high expression of circRNA-100876 showed a poor overall survival than those with low expression of circRNA-100876.

Suppression of CircRNA-100876 Expression Impaired the Invasion Ability of Cc Cells

Considering the above significant correlation of circRNA-100876 upregulation with the Clinical stage, T classification, and lymph node metastasis of Cc patients, transwell assay was conducted to explore whether circRNA-100876 impacted the invasion ability of Cc cells. Results in Figure 4 revealed that number of migrated cells in siRNA group was notably decreased in contrast to NC group, suggesting that suppression of circRNA-100876 expression remarkably impaired their invasion ability.

Discussion

In spite of certain progress in the diagnosis and treatment of tumors so far, the fatality rate of Cc is on the rise since it can be easily ignored in the early stage[14,15]. Nowadays, the treatments with Cc mainly rely on surgery and chemotherapy[16], but the subsequent recurrence and metastasis still

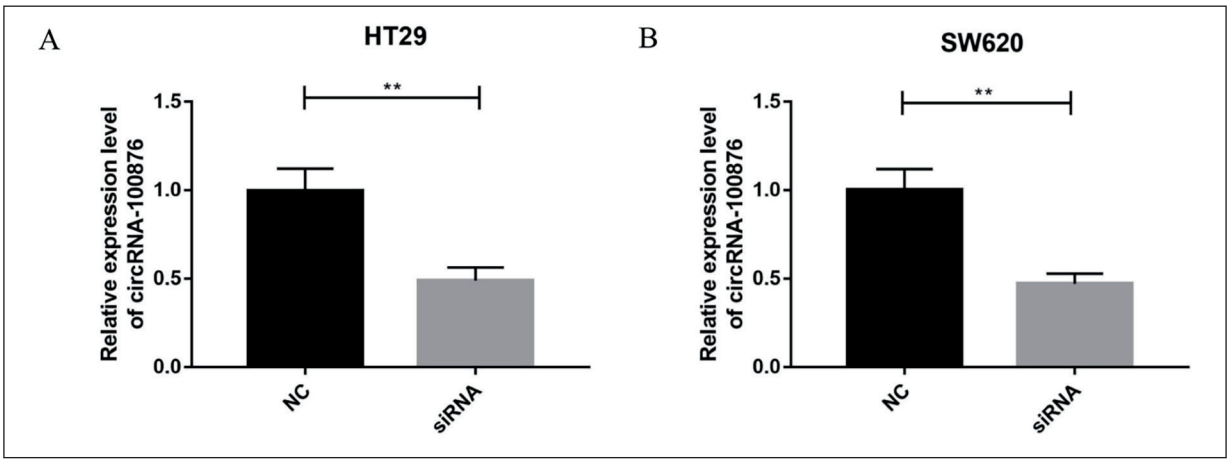

Figure 3. SiRNA inhibited the expression of circRNA-100876. The expression of circRNA-100876 was inhibited both in HT29 cell line (**A**) and SW620 cell line (**B**) after transfection of circRNA-100876 detected by RT-qPCR, respectively. (**p<0.01).

result in poor prognosis of the patients[17]. Presently, molecularly targeted therapy has stronger specificity and fewer side effects relative to traditional chemotherapy drugs, while there is a lack of effective molecular targeted drugs for Cc[18,19]. Therefore, it is essential to find new molecular targets to provide directions for the diagnosis and therapy strategies for Cc.

Defined as new non-coding RNAs, circRNAs are extensively distributed in eukaryotes and highly evolutionarily conserved, whose loop structures are stable and can be hardly decomposed[20]. Studies[21,22] have revealed that circRNAs have multiple potential biological functions, including miRNA sponging, transcription factor function and gene expression regulation, which

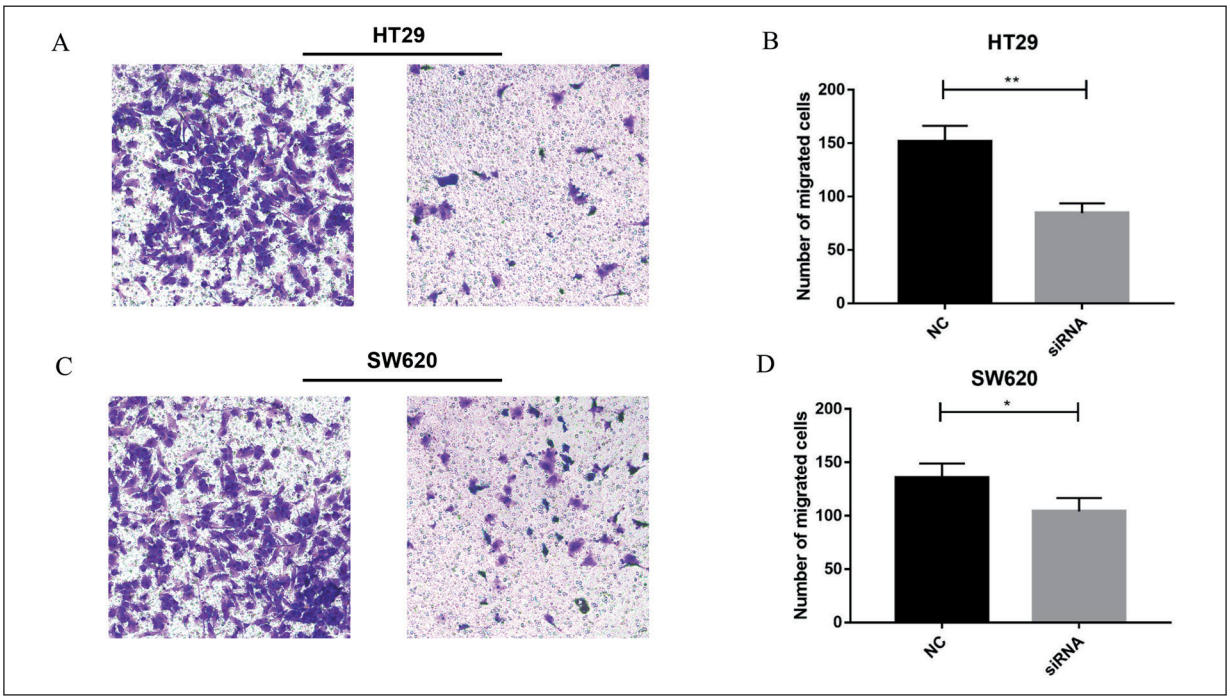

Figure 4. Inhibition of circRNA-100876 impaired the invasion ability of Cc cells. **A, B**, Down regulation of circRNA-100876 decreased the invasion ability of HT29 cells (magnification: 4×). **C, D,** The invasion ability of SW620 was reduced remarkably when the circRNA-100876 expression was inhibited (magnification: 4×). (*p<0.05, **p<0.01).

are important regulatory factors for the occurrence and development of tumors. Currently, large quantities of studies have demonstrated that circRNAs are critical players in the occurrence and progression of various cancers. Rong et al[23] demonstrated that circ-0066444 was significantly upregulated in gastric cancer tissues and could largely affect the proliferation ability and migration capability of gastric cancer cells. In addition, Yang et al[24] found that circ-ITCH was downregulated both in bladder cancer tissues and cell lines, respectively, and patients with low circ-ITCH expression had shorter overall survival. Besides, Pan et al[25] showed that circ-0006948 enhanced cancer progression and EMT in esophageal squamous cell carcinoma, as well as positively associated with lymphatic metastasis and poor prognosis in patients. Collectively, these data revealed the important role of circRNAs in regulating the progression of various tumors.

CircRNA-100876 is involved in the progression of breast cancer, non-small cell lung cancer and osteosarcoma cancer. In this article, to our current knowledge, it was the first time that an internal association between circRNA-100876 and colorectal cancer has been reported. Specifically, it was found that the expression level of circRNA-100876 was higher both in Cc tissues and cell lines, respectively. In addition, it was found that the abnormally high expression of circRNA-100876 was related to the Clinical stage, T classification and Lymph node metastasis of patients with Cc. Moreover, the overall survival in Cc patients with high expression of circRNA-100876 was even worse than those with lower expression of circRNA-100876. Collectively, these results implied a non-negligible effect of circRNA-100876 on colorectal cancer progression.

Combining the above results, in order to further verify the effect of circRNA-100876 on Cc cells, the loss-of-function assay was performed by siRNA transfection. After verifying the inhibitory effect by qRT-PCR, transwell assay was then conducted, and the results showed that the invasion ability of Cc cells with circRNA-100876 downregulation was significantly decreased compared with that of normal Cc cells, which better complemented the molecular role played by circRNA-100876 in the Cc development.

To the best of our knowledge, there is no literature that specifically studies the role of circRNA-100876 in the development of Cc. This paper first revealed that circRNA-100876 was not only associated with the pathological characteristics and prognosis of colorectal cancer patients, but also closely related to the invasion ability of Cc cells, which is of great significance for improving the systematic research of Cc progression.

Conclusions

Taken together, the data of this study indicated that circRNA-100876 was highly expressed in Cc tissues and cell lines, and was clearly correlated with the clinical stage, T classification and Lymph node metastasis of patients with Cc. In addition, it was demonstrated that high expression level of circRNA-100876 predicted poor overall survival of patients with Cc. Hence, this paper provided a strong foundation for application of circRNA-100876 as a new target for the diagnosis and therapy of Cc.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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