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Hsa_circ_0014717 is downregulated in colorectal cancer and inhibits tumor growth by promoting p16 expression

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ABSTRACT

Circular RNAs (circRNAs) represent a novel class of non-coding RNAs that may regulate gene expression in eukaryotes. Nevertheless, their potential functions in human malignancies are still largely unknown. The aim of this study was to explore the expression of hsa_circ_0014717 in colorectal cancer (CRC) and its role involving in tumor growth. We found that hsa_circ_0014717 was downregulated in CRC tissues and cell lines, and was significantly correlated with TNM stage, distal metastasis and poor overall survival of CRC patients as well. Moreover, our gain-of-function analysis further showed that hsa_circ_0014717 overexpression could significantly suppress CRC cell proliferation and colony formation, as well as induce cell cycle G0/G1 phase arrest *in vitro* and inhibit xenograft tumor growth *in vivo*. In addition, hsa_circ_0014717 could upregulate the expression of cell cycle inhibitory protein p16. Besides, p16 knockdown could reverse hsa_circ_0014717 mediated growth inhibition of CRC cells. Taken together, hsa_circ_0014717 acts as a potential tumor suppressor that inhibits CRC growth, partly at least, through upregulating p16 expression. Furthermore, our present study may provide a promising therapeutic target for CRC patients.

1. Introduction

Colorectal cancer (CRC) is one of the most frequent malignancies with approximately 1.2 million new cases each year, which ranks as the third in the cancer morbidity and the second leading cause of cancer-related death worldwide [1]. Apart from the developed countries, CRC is more and more prevalent in developing countries in recently years, especially in China. Colorectal canceration is a multi-step biological process, including polygenic alteration, and imbalance of cell proliferation, differentiation, and apoptosis. Despite much improvements have been achieved in diagnosis, treatment and understanding of the molecular mechanisms of CRC, the recurrence and metastasis of CRC are still closely associated with poor prognosis [2,3]. Therefore, it is emergently needed to deeply understand the molecular mechanisms in CRC progression and identify new therapeutic strategies as well as diagnostic and prognostic biomarkers.

Recently, circular RNAs (circRNAs) have been reported as new stars in noncoding RNA (ncRNA) world that are highly prevalent in the eukaryotic transcriptome. Unlike traditional linear RNAs, circRNAs have covalently linked ends formed by a junction of the 5' end and 3'end together via exon or intron circularization, which enables them to resist RNA exonucleases. Consequently, they are highly stable and conserved than linear RNAs, and often exhibit cell-, tissue-specific and developmental stage specific expression [4–6]. CircRNAs have been previously thought to be functionless owing to errors in splicing in the past few years. However, to date, a lot of circRNAs have been identified in a variety of cell lines and species. Moreover, emerging evidence has revealed that circRNAs are also involved in the onset and development of some human diseases, including a majority of malignancies [7,8]. Several deregulated circRNAs, such as homo sapiens circ_0020397 (hsa_circ_0020397), hsa_circ_ 0000069, and circ-BANP, etc., have been reported to contribute to CRC cell proliferation, metastasis and poor prognosis, suggesting their crucial roles in CRC [9–11]. Nevertheless, the association between circRNAs and CRC tumorigenesis remains largely unknown.

Hsa_circ_0014717 is one of circRNAs with 516 nt in spliced sequence length. Its gene is located at chr1:156290629-156304709, which is associated with gene symbol CCT3 (chaperonin containing TCP1 subunit 3). It has been reported that hsa_circ_0014717 is downregulated in gastric cancer, and is related to tumor stage, distal

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metastasis, tissue carcinoembryonic antigen and carbohydrate antigen 19-9 expression, showing potential clinical value for screening of high-risk gastric cancer patients [12]. However, the expression pattern and functional roles of hsa_circ_0014717 in CRC are still elusive. In the current study, we investigated the expression levels of hsa_circ_0014717 and its clinical performance in CRC. Then, functional studies about the effects of hsa_circ_0014717 on tumor cell growth were further evaluated *in vitro* and *in vivo*. Furthermore, the potential mechanism of hsa_circ_0014717 involving in tumor growth was also explored. The present study might offer a novel biomarker for CRC diagnosis and the feasibility of circRNA- oriented therapy for this deadly disease.

2. Materials and methods

2.1. Patients and sample collection

A total of 46 patients with primary CRC were recruited and the diagnosis was histopathologically confirmed. All patients were not underwent preoperative treatment, such as radiation or chemotherapy. Fresh cancer tissues and pair-matched adjacent normal tissues were obtained by surgical resection and immediately frozen and stored in liquid nitrogen until required. The detailed clinical characteristics of all the patients were summarized in Table 1. The follow-up data were obtained through outpatient visits at 1–3 month intervals or telephone calls and mail. The study was performed with the approval of the Ethics Committee of the Affiliated Hospital of Nantong University and written informed consent was obtained from all patients.

2.2. Cell culture, vector construction and transfection

Three human CRC cell lines HCT116, HT29 and SW480 were purchased from the Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences (Shanghai, China), and a normal colonic epithelial cell line FHC (ATCC, USA). All cells were cultured in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (Hyclone, USA) at 37 °C in humidified incubator containing 5% CO_2.

To construct the hsa_circ_0014717 overexpression vector, hsa_circ_0014717 genomic region with its flanking introns was amplified from the genomic DNA using PrimerSTAR Max DNA Polymerase Mix (Takara, Dalian, China) and was subcloned into a pcDNA3.0 vector, and an empty vector was used as a control. The short hairpin siRNA sequence targeting p16, GGAGCAGCATGGAGCCTTCGGaagcttCCGAAG GCTCCATGCTGCTCC, was used according to previous publication [13], which was synthesized and cloned into eukaryotic expression plasmid pGenesil-1 vector (Biosci, Hangzhou, China), and an empty vector was used as a control.

HCT116 and HT29 cells were seeded in 6-well plates 24 h prior to plasmids transfection or co-transfection with 70% confluence, and then cells were transfected with Lipofectamine 2000 (Invitrogen, USA) according to the manufacture instructions and Geneticin (G418, 500 μg/mL, 3 weeks) was used to screen stable transfection cells for further studies.

Table 1
Relationship of circ_0014717 expression with clinicopathological features in CRC patients.

Parameters	Cases	circ_0014717 expression		*P* value
	(n = 46)	Low (n = 23)	High (n = 23)	
Age (year)				0.556
< 60	23	10	13	
≥60	23	13	10	
Gender				0.353
Male	30	17	13	
Female	16	6	10	
Tumor size (cm)				0.139
< 5	22	8	14	
≥5	24	15	9	
Tumor location				0.554
Colon	25	14	11	
Rectum	21	9	12	
Tumor differentiation				0.130
Well/moderately	28	11	17	
Poorly	18	12	6	
TNM stage				0.013
I/II	17	4	13	
III/IV	29	19	10	
Distal metastasis				0.023
Negative	32	12	20	
Positive	14	11	3	

Fisher's exact test.

2.3. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA from the tissues and cultured cells was extracted using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The concentration and purity of RNA were measured by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). QRT-PCR assays were conducted with PrimeScript RT Master Mix (Takara) on the Applied Biosystems 7500 PCR System (ABI, USA). The results were normalized to the expression of glyceraldehyde-3- phosphate dehydrogenase (GAPDH) and calculated by using the equation $2^{-\Delta\Delta CT}$. All reactions were repeated at least triplicate. The specific primers sequences were listed in Table 2.

2.4. Cell counting kit 8 proliferation assay

Cells were seeded into the 96-well plates at a density of 5×10^3 cells per well the day before transfection. After transfection at 24, 48, and 72 h time point, cell growth viability was measured with Cell Counting Kit-8 (CCK-8; Beyotime, China) according to the manufacturer's instructions. The optical density was determined with the spectrophotometer (Universal Microplate Reader, Bio-Tek, USA) at a wavelength of 450 nm. Experiments were performed in triplicate.

2.5. Colony formation assay

About 800 cells treated with different transfected vectors were seeded in six-well plates and cultured in media with 10% fetal bovine serum. After 14 days, cells were fixed with methanol and stained with Giemsa dye. The number of colonies containing ≥ 10 cells was counted under a microscope. Experiments were repeated in triplicate.

2.6. Cell cycle detection

At 48 h after transfection, CRC cells were fixed in 70% ethanol at −20 °C for 24 h, and incubated at 37 °C with RNase A for 30 min.

Table 2
The primer sequences were used in this study.

Gene	Primer sequences
hsa_circ_0014717	forward 5'-TTGCCCTGGATGCTGTCAAG-3' reverse 5'-GGTCATCACAATGCCTCCCAT-3'
p15	forward 5'-GGTGAACCCACAACTTAGGC-3' reverse 5'-TTAGCATCTGTCGTCGCTTG-3'
p16	forward 5'-ACCGGAGGAAGAAAGAGGAG-3' reverse 5'-CGTAACTATTCGGTGCGTTG-3'
p21	forward 5'-GGCTCCTTCCCATCGCTGTCA-3' reverse 5'-GTCACCCTGCCCAACCTTAGA-3'
p27	forward 5'-ATGTCAAACGTGCGAGTGTCT-3' reverse 5'-TTACGTTTGACGTCTTCTGA-3'
GAPDH	forward 5'-CGCTCTCTGCTCCTCCTGTTC-3' reverse 5'-ATCCGTTGACTCCGACCTTCAC-3'