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CircRNA circ_0026344 as a prognostic biomarker suppresses colorectal cancer progression via microRNA-21 and microRNA-31

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ABSTRACT

Recently, circular RNA (circRNA) is identified as a novel class of noncoding RNA with important roles in human diseases, such as cancer. However, how circRNA participates in colorectal cancer (CRC) remains unclear. In this study, we aimed to illustrate the role of circ_0026344 in CRC progression. We showed that circ_0026344 expression was downregulated in CRC tissues compared to adjacent normal tissues. Moreover, the level of circ_0026344 was inversely correlated with CRC advance and lymphoid node metastasis. Additionally, circ_0026344 low expression predicted poor prognosis in CRC patients. We identified circ_0026344 as a miRNA sponge for microRNA-21 (miR-21) and microRNA-31 (miR-31) whose expression levels were elevated in CRC tissues. And the level of circ_0026344 was reversely correlated with both miR-21 and miR-31 levels in CRC tissues. Functionally, we found that ectopic expression of circ_0026344 decreased the growth and invasion of CRC cells while promoting apoptosis *in vitro*. The xenograft experiment indicated that circ_0026344 overexpress led to CRC growth inhibition *in vivo*. Rescue assays further demonstrated that circ_0026344 exerted biological functions by sponging miR-21 and miR-31 in CRC. In conclusion, this study revealed that circ_0026344/miR-21/miR-31 regulatory signaling was implicated in CRC progression.

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1. Introduction

Colorectal cancer (CRC) is one of the most frequent cancers worldwide among both females and males [1]. The development of CRC is a multi-step processes involving various factors such as genetic mutation [2]. The CRC incidence is still increasing every year while the five-year overall survival rate remains low [3]. Especially, cancer advance and metastasis contribute to the death in CRC patients [4]. Thus, it is important to understand the molecular mechanism underlying CRC progression, which is critical for the development of therapeutic strategies.

Circular RNAs (circRNAs) belong to the family of noncoding RNAs, which attracted a great of attention recently due to their vital biological functions [2]. CircRNAs are characterized by a covalently closed loop without either a 5′ cap or 3' polyadenylate tail [5]. In the past years, a large amount of circRNAs have been identified in human genomic transcripts as the advancement of sequencing technology. Functional studies indicate that circRNAs are widely implicated in many human diseases, such as cancer [5]. For instance, Zhang et al. indicated that circSMAD2 underexpression in hepatocellular carcinoma tissues regulates cell migration, invasion, and epithelial-mesenchymal transition (EMT) [6]. Liu et al. showed that hsa_circ_0008039 sponging miR-432-5p and upregulating E2F3 expression felicitates breast cancer progression [7]. CircRNA_0084043 modulates miR-153-3p/Snail signaling to enhance malignant melanoma progression [8]. Additionally, a few of important circRNAs have been identified in CRC, such as circHIPK3 [9] and hsa_circ_0007534 [10]. Thus, above evidences support the significance of investigating circRNA function in cancer.

The function of circ_0026344 remains unknown until now. In this study, we aimed to explore the role and mechanism of circ_0026344 in CRC progression. Our data showed that circ_0026344 expression was downregulated and reversely correlated with CRC progression. *In vitro* and *in vivo* experiments showed that circ_0026344 inhibited the proliferation and invasion of CRC cells while promoting apoptosis through acting as a miRNA sponge for miR-21 and miR-31. Taken together, our study revealed a novel regulatory mechanism and suggested that circ_0026344/miR-21/

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miR-31 signaling might be a potential therapeutic target for CRC prevention.

2. Materials and methods

2.1. Patient tissues

32 pairs of human CRC tissues and adjacent normal tissues were obtained from Xuzhou Central Hospital. CRC tissues and normal tissues were validated by pathologists. All tissues were frozen and stored at liquid nitrogen until use immediately after surgery. This study was approved by the Ethics Committees of Xuzhou Central Hospital. Written informed consent was obtained from each patient.

2.2. Cell culture and transfection

Human CRC cell lines (HCT116, SW480, SW620 and HT29), normal colon cell lines (NCM460 and FHC) and 293 T cells were from the type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and cultured according to the standard guidelines.

For circ_0026344 overexpression, circRNA sequence was cloned into PLCDH-cir vector (Ribobio, Guangzhou, China) for producing lentivirus. Si-circ_0026344 (5′-GTTAAATCCTGAGTCCTCTCA-3′), miR-21 mimics, miR-31 mimics and corresponding controls were purchased from GenePharma (Shanghai, China) and transfected into SW480 and HT29 cells using Lipofectamine™2000 according to the standard protocol.

2.3. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from CRC tissues or cell lines using Trizol reagent (Invitrogen). 1 μg total RNA was used as temple for cDNA synthesis. qRT-PCR was performed using the SYBR Premix Ex Taq (Takara) for circ_0026344 detection and the Taqman Universal Master Mix II (ABI) for miRNA analysis on an ABI 7300 system (ABI). Gene expression was normalized to U6 and calculated according to $2^{-\Delta\Delta Ct}$ method. Primer sequence information was available if requested.

2.4. Transwell assay

To analyze CRC cell invasion, 2×10^5 cells in 200 μl culture medium were seeded in the upper chamber (8 μm pore size and precoated with Matrigel) of the 24-well plate. The lower chamber was added with 600 μl medium containing 10% FBS. After incubation for 24 h, the medium was removed and cells in the upper chamber were scraped. Then cells were fixed with methanol for 2 h and stained with 5% crystal violet, followed by photographed using an inverted microscope.

2.5. Cell counting Kit-8 (CCK8) assay

Cell proliferation was measured using CCK8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) assay according to the standard instruction. In brief, 1×10^3 CRC cells were seeded in a 96-well plate. After cultured for 24, 48, 72 or 96 h, 10 μl CCK-8 solution was added and incubated for 2 h at 37 °C. Then absorbance was measured at 450 nm.

2.6. Xenograft experiment

For analysis of tumor growth *in vivo*, the xenograft experiment was performed according to a previous study [2]. In brief, 2×10^6 treated SW480 cells were subcutaneously injected into the flank of nude mice. Tumor volume was measured every 7 days and tumor weight was determined on day 28. The animal experiments were approved by Animal Ethics Committee of Xuzhou Central Hospital.

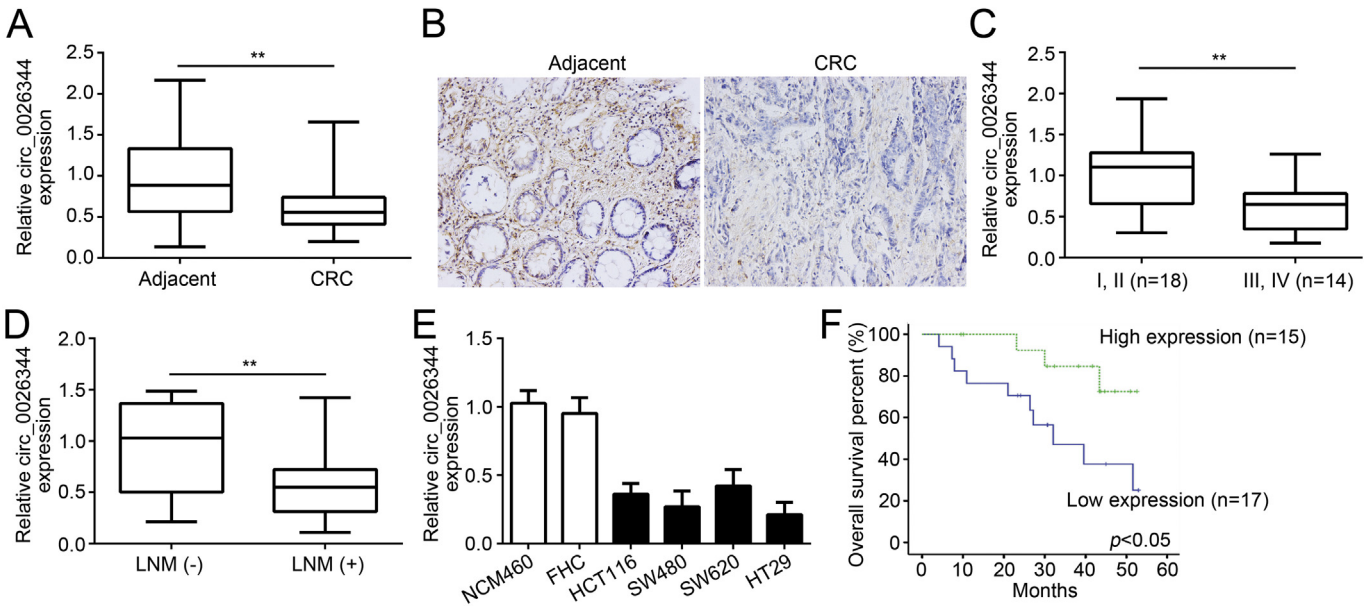

Fig. 1. Circ_0026344 was inversely associated with CRC progression. (A) qRT-PCR results indicated that circ_0026344 expression was downregulated in CRC tissues (n = 32) compared with adjacent normal tissues (n = 32). (B) *In situ* hybridization (ISH) analysis showed that lower expression of circ_0026344 was observed in CRC tissues but not in matched adjacent normal tissues. (C) Circ_0026344 expression was lower in CRC tissues of stage III-IV than that in stage I-II tissues. (D) Circ_0026344 expression was lower in lymphoid node metastatic CRC tissues than that in non-metastatic tissues. (E) Circ_0026344 expression was lower in CRC cell lines (HCT116, SW480, SW620 and HT29) than in normal colon epithelial cell lines (FHC and NCM460). (F) Lower expression of circ_0026344 predicted poorer prognosis in CRC patients. Kaplan-Meier curve was performed to analyze overall survival. $^{**}p < 0.01$.

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