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Research Article

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Decreased expression of miR-132 in CRC tissues and its inhibitory function on tumor progression

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Abstract: Objectives Colorectal cancer (CRC) is among the most common types of malignancies in the worldwide, and microRNAs (miRNAs) emerge as key regulators in carcinogenesis and tumor progression. Here we intended to address the expression and function of miR-132 in CRC cells. Methods Paired CRC tissues and several established cell lines were firstly collected. We performed qPCR to detect the expression of miR-132 in these tissues and cell lines. Cell proliferation and apoptosis were respectively monitored by CCK-8 assay and Annexin-V/PI staining followed by flow cytometry, after miR-132 was transiently overexpressed in RKO cells. Afterwards, Luciferase reporter assays were performed to examine the targeting of YAP1 by miR-132. Finally, qPCR and western blotting were also carried out to validate this targeting. Results MiR-132 was significantly decreased in CRC and its overexpression in RKO cells exerted tumor suppressing effects, including cell growth arrest and apoptosis promotion. Additionally, we proved that miR-132 could negatively regulate the expression of YAP1. Conclusion Our findings suggested that miR-132 was downregulated in CRC, and played as a tumor suppressor to inhibit cell proliferation and induce apoptosis. And these anti-tumor activities might be related with the targeting of YAP1 by miR-132.

Keywords: CRC, miR-132, proliferation, apoptosis, YAP1

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1 Introduction

Cancer is the leading cause of death in the developed countries over the past decades. Among the existing malignancies, colorectal cancer (CRC) ranks as the third most common type of human cancer in the worldwide, with over 1.2 million new cases each year [1,2]. Despite the progress of understanding the precise molecular mechanisms and treatment advances for CRC in surgical resection, radiotherapy and chemotherapy, the overall survival and prognosis of CRC remained unsatisfactory and has not been improved dramatically [3]. There are only a few genes validated as biomarkers or factors for clinical diagnosis. Therefore, future studies focusing on identifying novel biological events corresponding for CRC carcinogenesis and progression are of great significance, and new therapeutic targets for the treatment of this disease are urgently needed.

According to the conclusion of the human genome-sequencing project, more than 90% of the genomic DNA yields a complex network of transcripts consisting of tens of thousands of protein-coding and non-coding RNAs (ncRNAs) [4]. This finding indicates that ncRNAs exist as non-redundant RNAs that could function as important regulators in eukaryotic organisms. Those shorter than 200 nt in length are usually referred to as short/small ncRNAs, for instance, the microRNAs (miRNAs), which are approximately 21 nucleotides long and have been intensively studied in recent years [5]. Particularly, miRNAs have been shown to participate in the regulation of almost every cellular process investigated so far. By base pairing to specific targeting mRNAs, miRNAs mediate translational repression or mRNA degradation of these targets [5]. During the oncogenesis and metastasis processes of CRC development, miRNAs are also key factors to govern the progression of this disease [6-8]. Additionally, miRNA-based therapeutic potential for CRC in both animal models and clinical trials is being evaluated right now, and it might provide more useful hints for CRC prevention and therapy [9].

In a recent study using high-throughput qPCR method, the expression profile of some cancer-related genes and their regulatory miRNAs were identified in CRC patients, and miR-132 was found to be significantly deregulated in CRC [10]. Another previous study had reported that miR-132 could inhibit CRC cell invasion and epithelial-mesenchymal transition (EMT) *via* targeting ZEB2, an EMT regulator [11]. Nevertheless, the expression and inhibitory roles of miR-132 in CRC cells were far from been clearly understood.

2 Materials and methods

2.1 Patients and tissue samples

Surgical specimens of tumor tissues and paired non-tumoral tissues were obtained from 40 patients with CRC who underwent surgery at the 1st affiliated hospital of Henan university between 2014 and 2015. After collection, all samples were immediately frozen in liquid nitrogen and stored at -80°C until use. Written informed consent was obtained from each patient, and research protocols were approved by the 1st Affiliated Hospital of Henan University.

2.2 Cell culture

The human CRC cell lines Caco-2, HCT116, RKO, SW480, SW620 and the normal colon epithelium cell line CCD841 were purchased from the Cell Bank of Chinese Academy of Sciences (CBCAS) and cultured in DMEM or 1640 medium supplemented with 10% FBS. Cells were cultured at 37°C in a humidified incubator containing 5% CO_2.

2.3 MiRNA mimics and transfections

Oligonucleotides has-miR-132 mimics and scramble controls were purchased from GenePharma (Shanghai, China). Cells were transfected using Lipofectamine2000 reagent purchased from Invitrogen according to the manufacturer's guidelines.

2.4 Luciferase Reporter Assay

3'UTR of YAP1 was cloned into pmirGLO plasmid (Promega). Cells were seeded at a proper density and co-transfected with 0.05 mg of the reporter plasmid. *Renilla* luciferase was used as an internal control. Cell lysates were prepared 24 h after transfection, and the reporter activity was measured using the Promega Dual-luciferase reporter assay system.

2.5 RNA extraction and qPCR

Total RNA was extracted with Trizol Reagent (Invitrogen) according to the manufacturer's instructions. The concentration and purity of all RNA samples were detected by a NanoDrop. Special primers were used for the miRNA RT reaction, and U6B was used as an endogenous control of miRNA. The oligo$(dT)_n$ primer was used for the RT reaction of cDNAs, and GAPDH was used as an endogenous control of coding genes. The qPCR assay was performed using the Master SYBR Green Mix from TOYOBO. Each sample was examined in triplicate and analyzed by the 2^{-DDCT} method.

2.6 Western blotting

Cells were harvested by RIPA buffer on ice for 30 min and centrifuged at 12 000 rpm for 15 min at 4°C. And then the supernatant was collected. Cell lysates containing equal amount of protein were fractionated by 10% SDS-PAGE. Primary antibodies against YAP1 or ACTIN (Cell Signaling Technology) were used at a 1:1000 or 1:5000 dilution. ACTIN was used as an internal control.

2.7 Cell apoptosis assay

In order to detect the early and late apoptosis of RKO cells, flow cytometric analysis was applied with Annexin V-FITC/PI Apoptosis Detection Kit (BD) according to the protocol. Cells were discriminated into viable cells, dead cells, early apoptotic cells, and apoptotic cells. The ratio of early, late and total apoptotic cells were compared between the control group and test group.

2.8 CCK-8 Assay

The RKO cells were transfected with miR-132 mimics or scramble controls. After 24 h, cells were trypsined and seeded into 96-well plates at a density of 2×10^3 cells in each well. For the next 4 days, the plate was incubated at 37°C with 5% CO_2. CCK-8 assay was performed using CCK-8 Kit as per the protocol on each day. Absorbance at 450 nm (OD450) was detected by using a microplate reader.

2.9 Statistical Analysis

All statistical analyses were carried out by SPSS v18.0 software. The data were expressed as the mean ± SD (standard deviation) from at least three independent experiments. The differences between groups were analyzed using a double-sided Student's t-test, and the statistical significance was determined by a P-value of less than 0.05.

3 Results

3.1 Decreased expression of miR-132 in paired CRC tissues and cell lines

To study the expression of miR-132 in CRC, we collected 40 pairs of CRC tissues matched with adjacent normal colorectal tissues from our hospital. Results from qPCR shown in **Figure 1A** confirmed that miR-132 was indeed significantly down-regulated in CRC tissues ($P < 0.05$), as reported by Zheng's study [11]. We next detected the level of miR-132 among several CRC cell lines, and compared these with that of the normal CCD841 colon epithelial cells. As shown in **Figure 1B**, the expression of miR-132 was also decreased in most of these cell lines compared with the normal CCD841 cells. Among these established cell lines, miR-132 showed the middle expression level in RKO cells, which enticed us to further explore the roles of miR-132 in RKO cells.

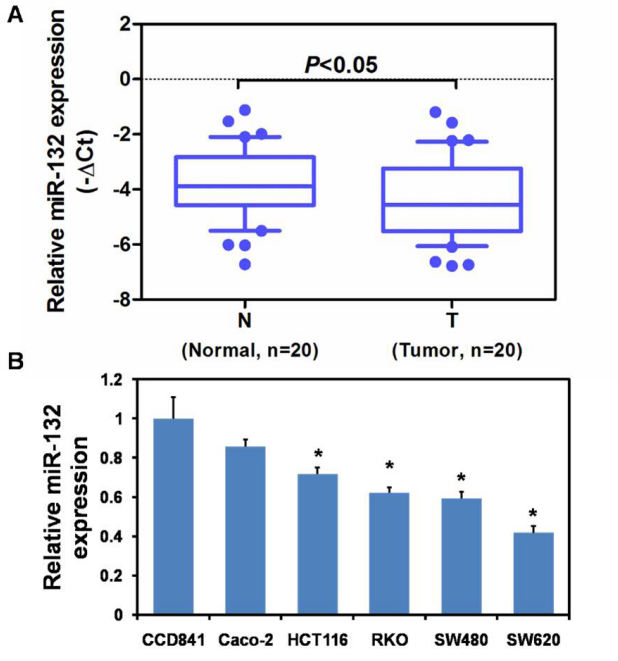

Figure 1. miR-132 in decreased in paired CRC tissues and cell lines. (A) Relative expression level of miR-132 in human CRC tumor tissues (n = 40) with paired non-tumor tissues (n = 40). **(B)** Relative expression level of miR-132 in human colorectal cancer cell lines and normal colon epithelium cell line CCD841 (*: P < 0.05).

3.1.1 MiR-132 inhibited cell proliferation and promoted apoptosis of CRC cells

After confirming the decreased level of miR-132 in CRC tissues and cell lines, we mainly focused on the roles of miR-132 in RKO cell proliferation and apoptosis, because Zhang et al. had already reported that miR-132 could impact on CRC cell invasion and metastasis [11]. MiR-132 was forced expressed in RKO cells (**Figure 2A**) and 48 h later, CCK-8 assay was performed to evaluate the cell proliferation rate by miR-132 overexpression compared with the scramble sequences transfection group. The results showed that RKO cells exhibited impaired proliferation after miR-132 was overexpressed (**Figure 2B**). Meanwhile, Annexin-V/PI staining followed by flow cytometry was also applied to analyze the early, late and total cell apoptosis rates of miR-132-overexpressing RKO cells. As shown in **Figure 2C**, miR-132 could also increase the Annexin-V or PI positive-cell percentage, in other words, promote early, late and total cell apoptosis.

3.2 YAP1 was one of the targets of miR-132 in CRC cells

From the above findings of our study, we concluded that miR-132 was an important tumor suppressing miRNA that not only impaired CRC cell metastasis but also impaired cell growth and promoted apoptosis. The mechanism responsible for metastasis inhibition was explained by its targeting of ZEB2 [11], whereas the possible reasons corresponding to the growth inhibition and apoptosis induction by miR-132 in CRC remain unknown. Since the Hippo signaling pathway is involved in restraining cell proliferation and promoting apoptosis, and YAP1 is a downstream nuclear effector of the Hippo signaling pathway, we set to investigate the expression of YAP1 after miR-132 overexpression in RKO cells. Surprisingly, we found that miR-132 overexpression could down-regulate the mRNA level of YAP1 as assessed by qPCR shown in **Figure 3A**. We then performed luciferase reporter assay to access the effect of miR-132 mimics on the YAP1 3'-UTR transcription. The result shown in **Figure 3B**

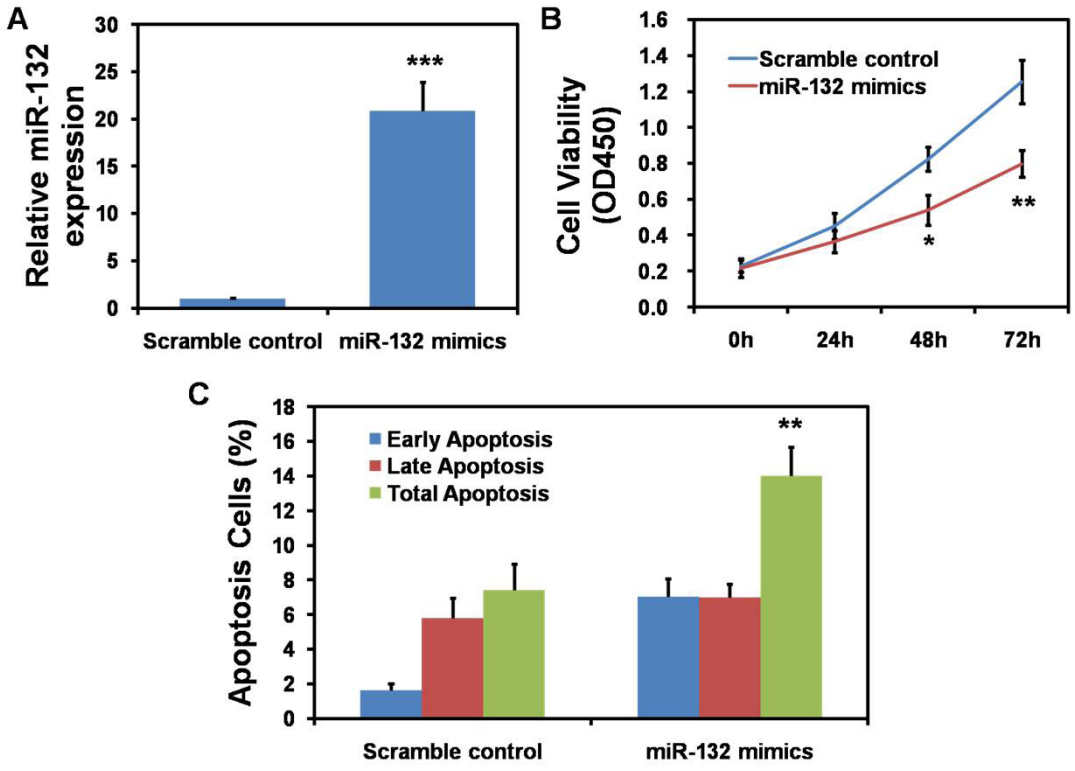

Figure 2. The inhibitory effects of miR-132 in RKO cells. (A) Transient transfection of miR-132 mimics or scramble control in human colorectal cancer cell line RKO. qPCR was performed to test the effect of overexpression (***: P < 0.001). **(B)** CCK-8 assay of RKO cells transfected with miR-132 mimics or scramble control for four days (*: P < 0.05, **: P < 0.01). **(C)** Effects of miR-132 on apoptosis of RKO cells as measured by Annexin-V/PI staining flow cytometric analysis. Early, late and total apoptotic cell death was quantified by software. Results were presented as mean ± SD (**: P < 0.01, n = 3).

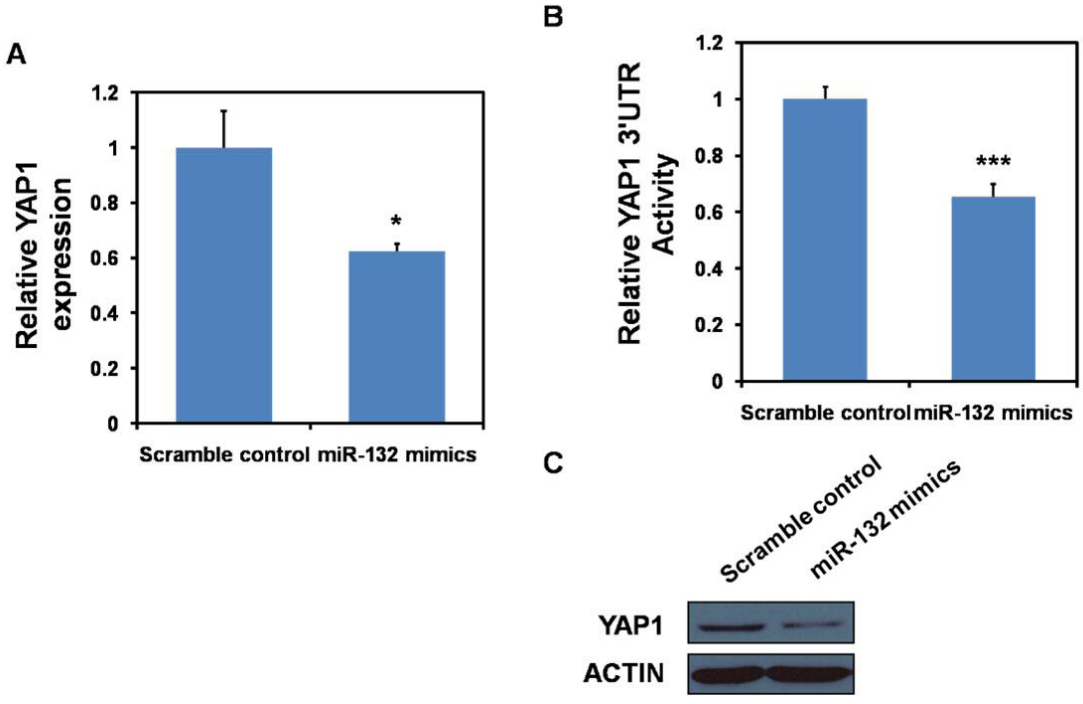

Figure 3. YAP1 is a potential target of miR-132 in colorectal cancer cells. **(A)** Ectopic expression of miR-132 reduces the YAP1 mRNA levels in RKO cells (*: P < 0.05). **(B)** Relative YAP1 3'-UTR activity in indicated cells reflected by luciferase reporter assay. (***: P < 0.001). **(C)** Ectopic expression of miR-132 decreases the YAP1 protein levels in RKO cells. ACTIN was used as internal control.

illustrated that miR-132 could negatively regulate the YAP1 3'-UTR activity. Importantly, western blotting results in **Figure 3C** further validated this targeting. These results indicated that the inhibitory functions of miR-132 on RKO cell proliferation and apoptisis promotion might be related with the targeting of YAP1, which needed further investigation.

4 Discussion

CRC is one of the most frequently seen malignancies in the world, and efforts to decode this disease and develop novel therapeutic strategies are always on the way to improve the cure rate. In the miRNA area, more and more studies consider miRNAs as vital modulators of CRC progression. Many miRNAs have been identified that are able to regulate known genes that are involved in the pathology of tumorigenesis and metastasis of CRC. For example, miR-21and miR-192 could induce CRC cell resistance to 5-fluorouracil (5-FU), which would shed light on the effectiveness of chemotherapy for patients [12,13].

MiR-132 was considered as a tumor suppressing miRNA in a series of human cancers: In osteosarcoma, miR-132 significantly suppressed in vitro cell proliferation and in vivo tumor growth via targeting the 3'-UTR of cyclin E1 (CCNE1) gene [14]. In ductal carcinoma in situ (DCIS) breast cancer, the most common type of non-invasive breast cancer, miR-132 was frequently down-regulated and suppressed cell proliferation and colony formation [15]. Zhang et al. also demonstrated a critical role of miR-132 in prohibiting cell proliferation, invasion, migration and metastasis in breast cancer through direct suppression of HN1 [16]. In hepatic carcinomas, Lei and his colleagues reported that miR-132 could inhibit the growth of hepatoma cells by targeting the YAP1 gene and reducing its expression level [17]. A recent report also found that miR-132 could regulate apoptosis in non-small cell lung cancer independent of acetylcholinesterase [18].

In the present study, we first confirmed that miR-132 was significantly down-regulated in CRC tissues as well as cell lines by using qPCR assays. To investigate the effects of miR-132 on cell proliferation and apoptosis, ectopic expression of miR-132 was achieved by transfection of mimics of miR-132 in RKO cells, matched with scramble sequences transfection. CCK-8 and flow cytometry assays were respectively carried out to evaluate the growth and apoptosis rates of RKO cells after miR-132 was overexpressed. We showed that forced expression of miR-132 inhibited cell proliferation and promoted apoptosis in CRC cells. These results suggested that miR-132 exerted tumor suppressing functions in CRC cells besides influencing invasion and metastasis as reported by Zheng's study[11]. We further validated that miR-132 could directly target the YAP1, a downstream nuclear effector of the Hippo signaling pathway which plays a role in the development and progression of multiple cancers as a transcriptional regulator of this signaling pathway. Although the exact mechanisms of miR-132 in CRC cells remained largely unclear, we speculated that miR-132 may regulate cell proliferation and apoptosis through its effects on YAP1 expression or other different target genes. However, further study is needed to investigate the underlying mechanisms of inhibitory functions of miR-132 in CRC. Furthermore, miR-132 may also represent a potential therapeutic target in CRC.

Conflict of interest: Authors declare nothing to disclose.

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