MiR-423-3p Enhances Cell Growth Through Inhibition of p21Cip1/Waf1 in Colorectal Cancer

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Abstract
Background/Aims: Colorectal cancer (CRC) is one of the leading causes of cancer-related deaths globally, with many oncogenes and tumor suppressors involved. The miRNAs are small non-coding RNAs known to play a vital role in the pathogenesis of CRC. The miR-423-3p was reported to act as an oncogene; however, its role in CRC growth remains unknown. Methods: qPCR assay was used to detect miR-423-3p expression in CRC specimens. Cell proliferation assay and transwell assay were conducted to evaluate CRC cell proliferation and migration. Luciferase reporter assay was to identify the target gene of miR-423-3p. And tumorigenesis model was established to test the role of miR-423-3p in CRC development in vivo. Results: Here, we showed that miR-423-3p was significantly up regulated in CRC tissues and cells compared with normal tissues and cells. Overexpression of miR-423-3p promoted CRC cell proliferation via enhancing the G1/S transition phase of the cell cycle, while inhibition of miR-423-3p repressed cell growth. Further studies showed that p21Cip1/Waf1 mediated the function of miR-423-3p, and overexpression of p21Cip1/Waf1 reversed the augmented effect of miR-423-3p on cell proliferation. Importantly, all these data were validated in the tumorigenesis assay in vivo. Conclusions: In conclusion, our findings demonstrated a critical impact of miR-423-3p on CRC growth.

Introduction
The miRNAs are a class of endogenous small non-coding RNAs with 19–23 nucleotides that negatively modulate gene expression by binding to the 3′ untranslated regions (UTR) of the target messenger RNAs (mRNAs). Binding causes a block of protein translation or mRNA degradation, depending on the level of complementarity [1, 2]. Partial complementarity

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between the miRNA and the 3′-UTR of the target mRNA transcript resulted in inhibition of translation, while perfect complementarity led to degradation of the mRNA [3, 4]. It has been reported that there are about 1000 miRNAs that regulate about 60% of the protein-coding genes in human cells. Therefore, miRNAs constitute one of the largest gene regulation families [5]. Increasing evidence shows that miRNAs control various cellular processes, such as cell proliferation, differentiation, apoptosis, and migration [6, 7]. Abnormal expression of miRNAs may contribute to the occurrence of a wide range of human diseases. Especially, deregulation of miRNAs has been observed in some types of tumors. The miRNAs act as oncogenes or tumor suppressor genes during tumor development and progression [8].

Colorectal cancer (CRC) is the third most common cancer worldwide, accounting for about 10% of all cancer cases [9]. There were 1.4 million new cases and 694,000 deaths from the disease in 2012 [10]. Distant metastasis and recurrence are major causes of death due to CRC. Especially, some cases of CRC are detected at later stages, making it difficult to cure. Occurrence of CRC is a multistep process involving both non-genetic and genetic factors. Several signaling pathways involved in the occurrence and metastasis of CRC, including PI3K-AKT signaling and RAS signaling pathways, which have been tested as therapeutic drug targets [11-14]. Recent studies have shown that many small-molecules such as YLT205 [15] and EMD638683 [16] inhibited CRC development. However, the mortality of CRC was still high. Therefore, it is urgent to develop new targets for CRC intervention. miRNAs were reported to be involved in CRC occurrence and development. For example, miR-31 and miR-21 regulated CRC tumorigenesis by modulating cell proliferation, and miR-221, miR-222 and miR-30a inhibited CRC migration and invasion by suppressing angiogenesis [17, 18]. Importantly, many miRNAs such as miR-29a, miR-92a, and miR-17-3p have been identified as diagnostic markers [17]. Some miRNA-CHIP microarray assay studies revealed aberrant miRNA expression in CRC, with elevation of a set of miRNAs in CRC compared with tumor adjacent samples, including miR-203, miR-224, and miR-423-3p [19]. It was recently reported that miR-423-3p was an oncogene, especially in hepatocellular carcinoma (HCC) [20]. However, the critical role of miR-423-3p in CRC remains unknown.

In the current study, we showed that miR-423-3p controlled CRC development. MiR-423-3p was up regulated in CRC tissues and CRC cells. Overexpression of miR-423-3p significantly promoted cell proliferation by enhancing the G1/S transition of the cell cycle. Further studies determined that p21Cip1/Waf1 was the direct target of miR-423-3p, and that ectopic expression of p21Cip1/Waf1 totally rescued the effect of miR-423-3p on CRC growth. Importantly, a CRC xenograft model in nude mice also showed miR-423-3p augmentation of CRC development by inhibiting p21Cip1/Waf1 expression.

### Materials and Methods

#### Human clinical specimens

Thirty CRC and thirty normal tissues adjacent to tumors were obtained from patients who underwent surgery with curative intent at the first affiliated hospital of Bengbu medical college. All participants gave informed written consent before participating in this study. This study was approved by the ethics committees of Bengbu medical college and was in accordance with the principle of the Helsinki Declaration II. All tissues were immediately dissected, placed on ice, snap-frozen in liquid nitrogen, and stored at −80°C until processing.

#### Cell culture and cell transfection

The normal colonic mucosal epithelial cell line HIEC, and CRC cell lines HT29, HCT116, and Caco-2 were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) in a humidified cell incubator with an atmosphere of 5% CO₂ at 37°C.

The miR-423-3p mimic (Invitrogen Life Technologies, Grand Island, NY, USA) was transfected into CRC cells for miR-423-3p overexpression, and two sets of miR-423-3p inhibitor anti-miR-423-3p
oligonucleotides (Invitrogen) were used for inhibition of miR-423-3p. For cell transfection assays, the synthetic oligonucleotides were transfected into cells using a Lipofectamine RNAiMAX kit (Invitrogen) at about 50% cell confluency, according to the product manual. The media was changed 24 h post-transfection and the indicated cells were subjected to further investigations.

**Generation of recombinant lentivirus**

The human miR-423-3p precursor and human p21Cip1/Waf1 coding sequences were cloned into the lentivirus pCDH vector to generate stably-transfected CRC cell lines. Virus was produced in 293T cells cotransfected with lentiviral vector pCDH and packaging plasmid. Cells were incubated overnight at 37°C and 5% CO₂. The supernatant was collected 48 h post-transfection. The virus stock solution was then used to infect CRC cells.

**Quantitative polymerase chain reaction (qPCR)**

Total RNA was extracted from cultured cells or CRC tissues using TRIzol® buffer (Invitrogen) according to the manufacturer's instructions. Total miRNA was reverse transcribed using the TaqMan® miRNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). The qPCR was carried out using the appropriate TaqMan miRNA assay (Applied Biosystems) and a Prism 7900 instrument (Applied Biosystems), with U6 as an endogenous control. Total mRNA was reverse transcribed using PrimeScript reverse transcriptase and random primers (TakaRa Bio, Otsu, Japan), followed by qPCR with SYBR Green (Applied Biosystems), with β-actin as an endogenous control. The qPCR analyses were performed in triplicate and repeated at least three times. Results are presented as means and standard deviations (SD) from three independent experiments.

**Western blotting**

Tissues and cells were harvested and lysed with sodium dodecyl sulfate (SDS) lysis buffer. Equal amounts of protein were separated by electrophoresis in SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). After blocking in 5% nonfat milk or bovine serum albumin, the membrane was immunoblotted with primary antibodies and visualized with horseradish peroxidase-coupled secondary antibodies. The primary antibody against p21Cip1/Waf1 was from Cell Signaling Technology (Danvers, MA, USA); antibody against β-actin was from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA).

**Cell proliferation assays**

Cell viability was evaluated by the Cell Counting Kit-8 (CCK-8; Dojindo Corporation, Kumamoto, Japan) following the manufacturer's instructions. CRC cells were plated into 96-well plates at an initial density of 5000 cells per well. The kit reagents were added to each well at 0, 1, 2, 3, or 4 days after plating, and all plates were scanned by a microplate reader at 450 nm after further 2 h incubation (Thermo Fisher Scientific, Sunnyvale, CA, USA). CCK8 assays were performed in triplicate and repeated at least three times. Results are presented as means and SD from three independent experiments.

**Propidium iodide (PI) staining and FACS analyses**

CRC cells were harvested and fixed in ice-cold 70% ethanol overnight. After washing with phosphate-buffered saline (PBS), the fixed cells were stained with a freshly prepared PBS solution containing PI (Sigma-Aldrich), RNaseA, ethylene diamine, and Triton X-100 for 30 min at 37°C in the absence of light. For each sample, at least 20000 cells were analyzed, and DNA content was determined by FACS cytometry (Beckman Coulter Epics Altra, Miami, FL, USA).

**Cell migration assays**

CRC cell migration was analyzed using a Transwell® system (Corning, Corning, NY, USA). Cells were plated at a density of 10000 cells into the upper chamber with serum-free medium. Medium containing 10% FBS was added to the lower chamber. Cells were allowed to migrate for 12 h at 37°C. Nonmigrating cells on the upper surface of the membrane were gently removed. The remaining cells were fixed, stained, and analyzed by inverted microscopy. The migrating cells were counted in five to six randomly-selected areas to evaluate the migration activity of the indicated cells. Results are presented as means and SD from three independent experiments.
Luciferase reporter assay

The wild-type 3′-UTR of p21Cip1/Waf1 containing the predicted binding site for miR-423-3p was cloned into the psiCHECK2 vector, as well as the binding site1-deleted mutant, the site2-deleted mutant, and the doubly-deleted mutant. For the luciferase reporter assay, CRC cells were seeded in 24-well plates and cotransfected with miR-423-3p mimic, luciferase reporter construct, and the Renilla luciferase reporter construct. At 36 h after transfection, luciferase activity was detected using a dual-luciferase reporter assay system (Promega, Madison, WI, USA) and normalized to Renilla enzyme activity. Data were normalized to the luciferase activity of cells transfected with miR-control. Results are presented as means and SD from three independent experiments.

Tumorigenesis in nude mice

Three types of CRC cells (1 × 10⁶) stably expressing miR-423-3p or p21Cip1/Waf1 were collected and inoculated subcutaneously into the right flank regions of 4-week-old male BALB/c nude mice (Institute of Zoology, Chinese Academy of Sciences, Shanghai, China). Mice were sacrificed after 1 month and the tumor growth rate and rate of inhibition were calculated. Three independent experiments were performed for each experimental group.

Statistical analyses

SPSS 19.0 software was used for statistical analyses (SPSS, Chicago, IL, USA). All values were presented as mean ± SD of at least three separate experiments, using the independent t-test to carry out comparisons of two independent groups.

Results

MiR-423-3p expression was positively correlated with CRC occurrence

In several types of cancers, including HCC, miR-423-3p was reported as an oncogene [20]. We therefore determined whether miR-423-3p expression was altered during CRC development. To detect miR-423-3p expression, qPCR analyses were conducted, finding out that miR-423-3p was significantly upregulated in CRC tissues compared with that in normal tissues (Fig. 1A). Consistently, miR-423-3p expression in CRC cells was much higher than that in HIEC cells (Fig. 1B). Therefore, miR-423-3p was increased during CRC occurrence.

![Fig. 1](image)

Up regulated expression of miR-423-3p in CRC tissues and cells. (A) qPCR analysis of miR-423-3p expression in the CRC tissues compared to tumor adjacent normal tissue samples. Data were normalized to the expression level in tumor adjacent normal tissue. (B) qPCR was used to measure relative expression of miR-423-3p in three CRC cell lines compared to HIEC cells. Data were normalized to the expression level in HIEC cells. All the data are presented as means and SD from three independent experiments. *p < 0.05.
MiR-423-3p regulated the proliferation and migration of CRC cells

In order to determine the role of miR-423-3p in CRC cell viability, the miR-423-3p mimic was transfected into CRC cells, including HT29, HCT116, and Caco-2 cells. CCK-8 assays were conducted to detect cell proliferation, demonstrating that expression of miR-423-3p significantly augmented the proliferation activity of all three CRC cell lines (Fig. 2A-C). We then determined if miR-423-3p had an effect on cell cycle progression of CRC cells. The cell population in G1 phase was reduced by miR-423-3p, whereas the cell number in S phase was increased in all three CRC cell lines (Fig. 2D-F). Thus, miR-423-3p promoted CRC cell growth by enhancing the G1/S transition. Additionally, transwell assays were performed, and demonstrated that miR-423-3p overexpression significantly promoted the number of migratory cells (Fig. 2G). To validate these results, two sets of anti-miR-423-3p inhibitors were transfected into CRC cells to suppress miR-423-3p. Consistently, inhibition of miR-423-3p repressed cell proliferation (Fig. 3A-C), cell cycle progression (Fig. 3D-F), and migration activity in CRC cells (Fig. 3G). These results indicated that miR-423-3p was a key mediator for CRC cell growth.

The p21Cip1/Waf1 was the direct target of miR-423-3p

It has been shown that miR-423-3p modulated HCC occurrence by inhibiting p21Cip1/Waf1. We therefore determined the effect of miR-423-3p on p21Cip1/Waf1 expression in CRC cells. There are two predicted binding sites for miR-423-3p in the 3′-UTR region of p21Cip1/Waf1. We constructed full-length fragments of the p21Cip1/Waf1 3′-UTR, including the wild type and the binding site deleted mutant, and inserted them immediately downstream of the luciferase reporter gene (Fig. 4A). The results of luciferase assays showed that the miR-423-3p mimic significantly down regulated the luciferase activity in the WT 3′-UTR group. The miR-423-3p mimic had little effect on the luciferase activity of the site1-deleted group,
while significantly inhibiting luciferase activity in the site2-deleted group (Fig. 4B). These data indicated that miR-423-3p inhibited the p21Cip1/Waf1 3′-UTR by direct binding to site1. Consistently, we observed an obvious decrease in p21Cip1/Waf1 protein level in CRC cells with miR-423-3p overexpression, whereas p21Cip1/Waf1 expression was significantly increased in all three CRC cell lines with miR-423-3p inhibition (Fig. 4C). More importantly, p21Cip1/Waf1 protein level was inversely correlated with miR-423-3p expression in CRC specimens (Fig. 4D), indicating that miR-423-3p regulated p21Cip1/Waf1 expression during CRC development. These data suggested that miR-423-3p directly regulated p21Cip1/Waf1 expression in CRC.

The p21Cip1/Waf1 reversed the effect of miR-423-3p on CRC cell growth

Because miR-423-3p regulated p21Cip1/Waf1 expression in CRC cells, we determined whether p21Cip1/Waf1 rescued augmented CRC cell growth caused by miR-423-3p. First, we detected p21Cip1/Waf1 expression in four randomly selected CRC specimens, finding out that p21Cip1/Waf1 protein level was much lower in CRC tissues than in normal tissues (Fig. 5A). We then generated three types of CRC cells, the control cell group, the miR-423-3p overexpression group, and the miR-423-3p and p21Cip1/Waf1 double overexpression group. CCK-8 assays showed that miR-423-3p overexpression significantly augmented proliferation activity in all three CRC cell lines, while p21Cip1/Waf1 overexpression inhibited cell proliferation activity and rescued the effect of miR-423-3p on cell growth (Fig. 5B).
Additionally, the transwell assay results indicated that ectopic expression of miR-423-3p significantly promoted CRC cell migration, whereas p21Cip1/Waf1 overexpression reversed these effects (Fig. 5C). These data collectively suggested that p21Cip1/Waf1 exhibited an inhibitory impact on CRC cell proliferation and migration, and rescued the effect of miR-423-3p.

The miR-423-3p promoted CRC development in vivo

The p21Cip1/Waf1-dependent effects of ectopic expression of miR-423-3p on tumor growth in vivo were determined. Three types of CRC cell lines stably overexpressing miR-423-3p, p21Cip1/Waf1, or both miR-423-3p and p21Cip1/Waf1, and control CRC cells were produced and injected subcutaneously into nude mice. The mice were sacrificed and photographed at 1 month post-implantation. The qPCR analyses were conducted on the tumors, demonstrating that miR-423-3p expression was significantly upregulated upon miR-423-3p overexpression (Fig. 6A), which was accompanied by downregulated expression of p21Cip1/Waf1 (Fig. 6B). Notably, tumor size in the miR-423-3p overexpression group was larger than in the control group. Overexpression of p21Cip1/Waf1 significantly decreased tumor size, and rescued the augmentation effect of miR-423-3p on tumor growth (Fig. 6C). These data suggested that miR-423-3p promoted CRC development in vivo.
Fig. 5. The p21Cip1/Waf1 mediated the effects of miR-423-3p on CRC growth. (A) Western blotting analysis of p21Cip1/Waf1 expression in randomly selected CRC and normal specimens. (B-D) The miR-423-3p and p21Cip1/Waf1 were ectopically expressed in CRC cells. CCK8 assays were conducted in the indicated cells. (E) MiR-423-3p and p21Cip1/Waf1 were ectopically expressed in CRC cells. Transwell assays were performed in the indicated cells. All data are presented as means and SD from three independent experiments. For miR-control vs. miR-423-3p: *, p < 0.05; for miR-423-3p vs. miR-423-3p + p21: #, p < 0.05.

Fig. 6. The miR-423-3p inhibited tumor growth in vivo. (A) CRC cells (1 × 10⁶) that stably expressed miR-423-3p or p21Cip1/Waf1 were collected and inoculated subcutaneously into right flank regions of 4-week-old male BALB/c nude mice. Xenograft tumors isolated from nude mice on day 30 in different groups. The qPCR analyses of the expression of miR-423-3p in different tumor groups. (B) Western blotting analysis of p21Cip1/Waf1 in different tumor groups. (C) The isolated tumors were photographed. All the data are presented as means and SD from three independent experiments. *, p < 0.05.
Discussion

MiR-423-3p was previously reported to be an oncogene, and aberrant expression of miR-423-3p was observed in several types of cancers, including HCC [20], endometrial cancer [21], and head and neck squamous cell carcinoma [22]. However, the role of miR-423-3p in CRC occurrence remains unknown. The current investigation determined that miR-423-3p was critical to CRC development. It promoted the proliferation activity of CRC cells, the underlying mechanism of which was that miR-423-3p augmented cell cycle G1/S transition via inhibition of p21Cip1/Waf1 expression. Importantly, the in vivo tumorigenesis assay validated that miR-423-3p facilitated CRC growth.

Additionally, we showed that miR-423-3p elevated CRC cell migration activity, which was essential for tumor metastasis. Approximately 50% of the patients diagnosed with CRC die as a result of complications related to distant metastasis [23]. Metastasis is a multistep process in which malignant cells disseminate from the primary tumor to colonize distant organs, followed by proliferation, induction of angiogenesis, and evasion of apoptotic cell death [24]. CRC metastasis involves epithelial-mesenchymal transition (EMT), whereby tumor cells become more invasive and metastatic. EMT is a biological process that drives polarized, immobile epithelial cells to undergo multiple biochemical changes to acquire a mesenchymal cell phenotype [25]. Therefore, we doubt whether miR-423-3p regulated the expression of EMT-related proteins, thereby promoting the migration of CRC cells, which was need clarification.

The inhibitor of cyclin-dependent kinase (CDK), p21Cip1/Waf1 functions as a downstream effector of tumor suppressors including p53 and TGFβ [26]. Because p21cip1/waf1 negatively regulated the progression of the cell cycle, its potential usefulness in gene therapy was proposed [27]. The p21Cip1/Waf1 was reported to be involved in suppression of several cancer types. For example, silencing of p21Cip1/Waf1 enhanced EMT, which resulted in increased breast tumor metastasis, while ectopic expression of p21Cip1/Waf1 repressed tumor metastasis [28]. Overexpression of p21cip1/waf1 caused a growth inhibition in CRC cells, suggesting that introduction of the p21cip1/waf1 gene into CRC cells may be useful for restriction of CRC [29, 30]. Our current study showed that p21cip1/waf1 was the direct target of miR-423-3p in inhibition of CRC occurrence. Two potential target sites of miR-423-3p were found in the p21Cip1/Waf1 3′-UTR. Deletion of site1, but not site2, rescued the impaired luciferase activity caused by miR-423-3p, suggesting that site1 was the effective target of miR-423-3p. The miR-423-3p mimic significantly elevated protein levels of p21cip1/waf1, while anti-miR-423-3p down regulated p21cip1/waf1. However, p21cip1/waf1 was not a unique target of miR-423-3p. There may be other proteins that mediated the effect of miR-423-3p on CRC proliferation. However, we found that p21cip1/waf1 overexpression reversed the impact of miR-423-3p, implying p21cip1/waf1 was the key mediator for miR-423-3p-driven cell growth.

In summary, we demonstrated for the first time that miR-423-3p had an important role in CRC occurrence through inhibiting the expression of p21cip1/waf1. Our findings provided new insight into CRC pathogenesis and suggested a therapeutic application for miR-423-3p in CRC intervention.

Disclosure Statement

No potential conflicts of interest relevant to this article were reported.

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