MicroRNA-139-5p regulates chronic inflammation by suppressing nuclear factor-κB activity to inhibit cell proliferation and invasion in colorectal cancer

MINGMING ZHU, WEN ZHANG, JUN MA, YOU GUO DAI, QI ZHANG, QIN LIU, BURONG YANG and GANG LI
Department of Abdominal Tumor Surgery, Third Affiliated Hospital of Kunming Medical University, Kunming, Yunnan 650118, P.R. China

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Abstract. The inflammatory microenvironment, which mediates the initiation and malignant development of tumors, has been reported to be associated with microRNA (miRNA) dysregulation. In the present study, the expression of miR-139-5p was analyzed in colorectal cancer (CRC) cell lines SW480, HT29, HCT-8, LoVo and HCT116, aiming to investigate the function and mechanism of miR-139-5p in the regulation of the malignant phenotypes of CRC. miR-139-5p expression was found to be considerably downregulated in CRC cell lines compared with the human normal colon mucosal epithelial cell line NCM460. Subsequently, it was demonstrated that overexpression of miR-139-5p in colon cancer cell lines significantly suppressed the cell proliferation in vitro and in vivo. In addition, overexpression of miR-139-5p further inhibited the invasion ability of colon cancer cells in vitro, concomitantly with downregulation of key invasion-associated proteins, including matrix metalloproteinase 9 (MMP9) and MMP7. Furthermore, it was demonstrated that overexpression of miR-139-5p decreased the expression levels of inflammatory cytokines, including interleukin-1β (IL-1β), IL-6 and tumor necrosis factor-α (TNF-α), by suppressing nuclear factor (NF)-κB activity. Therefore, these findings collectively indicated that miR-139-5p regulated chronic inflammation by suppressing NF-κB activity in order to inhibit cell proliferation and invasion in CRC, thereby indicating a novel molecular mechanism in CRC therapy.

Introduction

Colorectal cancer (CRC) is the third most common malignancy worldwide and considered to be a major cause of cancer-associated mortality (1,2). Despite the rapid development of diagnostic and treatment methods, the 5-year survival rate of CRC remains poor, mostly due to recurrence and metastasis. A number of genetic factors and factors that affect the tumor microenvironment have been determined as key risk factors for CRC, including gene mutations, poor dietary habits, obesity and chronic intestinal inflammation. However, the exact mechanisms leading to the initiation and development of colon cancer remain unclear, and considerable scientific interest has been focused on the molecular pathogenesis of CRC.

Emerging evidence has indicated that inflammation is associated with tumor initiation and development, notably CRC (3-6). In addition, epidemiological studies have demonstrated that the development of CRC is closely associated with chronic inflammation (7). The inflammatory microenvironment facilitates tumorigenesis by a series of dynamic and reciprocal interactions between inflammatory and tumor cells (8). In this regulatory process, nuclear factor-κB (NF-κB) is regarded as a critical regulator that can promote the initiation and amplification of inflammation (9). The activation of NF-κB induces p65/p50 secretion into the nucleus, which regulates the expression of target genes (10), such as the inflammation-associated factors interleukin-1β (IL-1β), IL-6 and tumor necrosis factor-α (TNF-α). Consequently, NF-κB activation may link inflammation to tumor initiation and promotion by regulating the target genes of NF-κB.

MicroRNAs (miRNAs) are a class of small non-coding RNAs, approximately 17-24 nucleotides in length, which are involved in several pathological and physiological processes by regulating target gene expression. Recently, increasing evidence has confirmed that miRNAs may serve vital roles in regulating pro-inflammatory factor-induced initiation and progression of cancer (11-13). Accumulating evidence has indicated that reduced expression of miR-139-5p is a common characteristic of several types of cancer, including CRC (14-16). It was also reported that miR-139-5p was associated with abnormal inflammation in the animal models of
dextran sulfate salt-induced colon cancer and experimental colitis (17). However, the association between miR-139-5p and inflammation in CRC has not yet been fully clarified.

In the present study, it was demonstrated that overexpression of miR-139-5p was able to inhibit CRC cell proliferation and invasion. Furthermore, it was also observed that the mRNA and protein levels of the inflammatory cytokines IL-1β, IL-6 and TNF-α were significantly decreased by reduced NF-κB activity as a result of miR-139-5p overexpression. The results indicated that miR-139-5p regulated chronic inflammation by suppressing NF-κB activity in order to inhibit cell proliferation and invasion in CRC. These findings undoubtedly illustrated a novel molecular mechanism that may be applied in CRC therapeutics.

Materials and methods

Cell culture. The human CRC cell lines SW480, HT29, HCT-8, LoVo and HCT116 were purchased from the American Type Culture Collection (Manassas, VA, USA), whereas the human normal colon mucosal epithelial cell line NCM460 was obtained from INCELL Corporation LLC (San Antonio, TX, USA). The cell culture media of McCoy’s 5A, RPMI-1640, F12-K, Leibovitz’s L-15 and DMEM were purchased from GIBCO (Thermo Fisher Scientific, Inc.). HCT116 and HT29 cells were cultured in McCoy’s 5A medium. HCT-8, LoVo, SW480 and NCM460 cells were maintained in RPMI-1640, F12-K, Leibovitz’s L-15 and DMEM, respectively. All cell culture media aforementioned were supplemented with 10% fetal bovine serum (GIBCO; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and streptomycin. The cells were incubated under 5% CO₂ at 37°C.

miRNA transfection. Human hsa-miR-139-5p mimics (micrON™ hsa-miR-139-5p mimics; cat. no. miR10000250-1-5) and miRNA negative control (NC) mimics (micrON™ mimics Negative Control #24; cat. no. miR01201-1-5) were synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The cells were respectively seeded in 24-well plates at a density of 5x10⁴ cells/well and then transfected with 60 nM miR-139-5p mimics or NC mimics at 60% confluence using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturer’s protocol. The cells transfected with the miR-139-5p or NC mimics were harvested at 48 h post-transfection.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the cell pellets using the TRizol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturer’s protocol and subsequently quantified using NanoDrop™ 2000 machine (Thermo Fisher Scientific, Inc.). The total protein was extracted from the cell pellets using the TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturer’s protocol. For miRNA detection, the TaqMan MicroRNA assay kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used according to the protocol supplied by the manufacturer. For mRNA detection, the synthesis of cDNA was performed using the PrimerScript™ RT Reagent kit (Takara Bio Inc.) and qPCR was conducted by the FastStart Universal SYBR Green Master kit (Roche Diagnostics), according to the manufacturer’s protocol. The mRNA expression levels of IL-6, IL-1β and TNF-α were normalized to that of GAPDH, which was used as an internal control. The primer sequences were listed in Table I. The thermal cycling conditions for PCR were: Initial denaturation at 95°C for 15 min, followed by 35 cycles of denaturing at 95°C for 15 sec, annealing at 55°C for 30 sec and elongation at 72°C for 30 sec. The results of the detection were calculated using the 2^ΔΔCt analysis method (18).

Cell proliferation assay. The effect of miR-139-5p on cell proliferation was evaluated using the Cell Counting Kit-8 (CCK-8; Sangon Biotech Co., Ltd.). Briefly, cells transfected with the miR-139-5p or NC mimics were plated into the 96-well plates (2x10³ cells/well), and 10 μl CCK-8 solution was added to each well following culturing for 24, 48 and 72 h. The plate was then incubated at 37°C for 4 h, and cell proliferation was evaluated by measuring the absorbance at 450 nm using Multiskan MS (Thermo Fisher Scientific, Inc.). A total of three wells were used in each group, and the experiments were repeated three times independently. The results are expressed as the mean values ± standard deviation (SD).

Colony formation assay. The cells were seeded in a 24-well plate (5x10³ cells/well) and transfected with miR-139-5p or NC mimics. After 48 h of incubation, the cells were collected and seeded (1,000-1,500 cells/well) into a fresh 6-well plate for 10 days. The surviving colonies were counted following fixation with methanol/acetone (1:1) and were finally stained with 0.5% methylene blue (MedChemExpress). The experiments were repeated three times.

Cell invasion assay. Cell invasion was examined by a Matrigel invasion assay (BD Biosciences). In brief, the cells were transfected with the miR-139-5p or NC mimics for 48 h, and then harvested and suspended in serum-free medium. A total of 1x10⁵ cells were diluted in 500 μl serum-free medium and added to the upper chamber of the Transwell apparatus (Corning Inc.) that was precoated with 1 mg/ml Matrigel (BD Biosciences) for 2 h at 37°C Subsequently, 0.6 ml serum-free media with 10 ng/ml hepatocyte growth factor was added to the lower chamber. After 48 h of incubation, the cells that invaded through the Matrigel membrane were fixed with 4% paraformaldehyde at room temperature for 30 min, stained with 0.5% crystal violet at room temperature for 30 min and counted in five high-power fields (magnification, x200). A total of three independent experiments were conducted.

Western blot analysis. The total protein was extracted from the cells using radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology) with proteinase inhibitor cocktail (Roche Diagnostics). Subsequently, the protein concentration was quantified using the BCA Protein Assay Reagent kit (Thermo Fisher Scientific, Inc.) and ~30 μg total protein was then separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (EMD Millipore; Merck KGaA). Non-specific binding was blocked by incubating the membranes in 5% skim milk for 1 h at room temperature. Next, the membranes were incubated overnight at 4°C with rabbit anti-matrix metalloproteinase 9 (MMP9, cat. no. 13667), rabbit anti-MMP7...
The experimental protocol involving animals was to evaluate the effects of miR‑139‑5p in regulating tumor xenograft mouse model.

The bands were analyzed using an ImageJ Software (version placed together in a cassette and developed at -80˚C for 72 h. The membranes were wrapped in plastic film were placed in the middle of two X‑ray films, Fisher Scientific, Inc.). The membranes were then wrapped in plastic film were placed in the middle of two X‑ray films, Fisher Scientific, Inc.) was used for detection of DNA binding, EMSA kits (Thermo Fisher Scientific, Inc.) according to the protocols supplied the manufacturer. A total of three independent experiments were performed in triplicate.

Electrophoretic mobility shift assay (EMSA). The cells were transfected with miR-139-5p or NC mimics, and then seeded in 24-well plates (5x10^4 cells/well). After 48 h of incubation, the cell culture supernatant was collected from the 24-well plates, and the content of the inflammatory cytokines, including IL-1α, β, and TNF-α, was measured by ELISA kits (Sangon Biotech Co., Ltd.) according to the protocols supplied the manufacturer. A total of three independent experiments were performed in triplicate.

**Table I. Primer sequences used for reverse transcription‑quantitative PCR.**

| Gene name | Forward | Reverse |
|-----------|---------|---------|
| miR-139-5p | ACATCCAGCTGGGTCCTACAGTCACCTG | CTCAGCTGGTGTCGTCGA |
| U6 | CTCGTTTCGCCAGACA | AACGCTTTCAAGTTTGCGGT |
| IL-6 | ACTCACCTCTCAGAAAGAATTG | CCATCTTGGAAGGTTCAGGTG |
| IL-1β | TGGGATGATAGGGAGAG | TTTTGTGGATGTCGCCAGAG |
| TNF-α | CTTCTTCTTAATCGACCCCTCTG | CAGGACCTGGGAAGTAGATGAG |
| GAPDH | GAGGCGGAGATCCCTCCAAAT | GGCTGTGGTCATACCTTCTG |

**Results**

miR-139-5p is downregulated in human CRC cell lines. Previous studies have reported that the expression levels of miR-139-5p are downregulated in CRC tissues and are associated with poor disease prognosis (15,21). Although miR-139-5p shares the same precursor with miR-139-3p, miR-139-3p expression has been reported to be undetectable in CRC cells, the overexpression of which exhibited no significant effects on the malignant behavior of CRC cells (15). Therefore, in the present study, the function of miR-139-5p was examined. The data revealed significant downregulation of miR-139-5p in five human CRC cell lines, including SW480, HT29, HCT-8, LoVo and HCT116 cells, compared with its expression in the normal human colon mucosal epithelial cell line NCM460 (Fig. 1A). This observation suggests that miR-139-5p may serve an important role in CRC tumorigenesis. In accordance to these findings,
The reduction in the number of invading cells caused by the miR-139-5p mimic-transfected groups (Fig. 3A). and LoVo cells were significantly decreased compared with those in the miR-139-5p mimic-transfected HCT116 and LoVo cells following transfection with miR-139-5p mimics (Fig. 3B). Taken collectively, these results strongly indicated that miR-139-5p overexpression inhibited cell invasion.

miR-139-5p inhibits CRC cell proliferation and tumor growth. To investigate the effects of miR-139-5p on the proliferation and invasion of CRC cells, miR-139-5p mimics were transfected into CRC cells (HCT116 and LoVo), and RT-qPCR was used to determine the expression levels of miR-139-5p. The results indicated an increased expression of miR-139-5p in HCT116 and LoVo cells following transfection with miR-139-5p mimics, whereas NC mimics had no marked effect compared with the blank group (Fig. 1B). It was observed that ectopic expression of miR-139-5p significantly inhibited the proliferation of HCT116 and LoVo cells when compared with the blank group (Fig. 2A and B). The inhibitory effects of miR-139-5p on colon cancer cell proliferation were further confirmed by the colony formation assay. The results indicated that overexpression of miR-139-5p markedly suppressed the colony formation ability in miR-139-5p mimic-transfected HCT116 and LoVo cells compared with that noted in the blank or NC mimics groups (Fig. 2C). Subsequently, the inhibitory effects of miR-139-5p on tumor growth were further confirmed in vivo by conducting a xenograft tumor growth assay. The subcutaneous tumor growth curve is shown in Fig. 2D and indicates that the tumor growth rate of HCT116 cells transfected with miR-139-5p mimics was significantly lower compared with that of the blank and NC mimics cells on day 28. These results provided further evidence that miR-139-5p serves a tumor suppressive role in colon cancer.

miR-139-5p suppresses CRC cell invasion. Following the initial results of miR-139-5p on colon cancer cell growth, the current study further investigated whether this miRNA affects the invasiveness of colon cancer cells in vitro. The number of invading cells in the miR-139-5p mimic-transfected HCT116 and LoVo cells were significantly decreased compared with those in the blank or NC mimic-transfected groups (Fig. 3A). The reduction in the number of invading cells caused by the overexpression of miR-139-5p further confirmed the effect of this miRNA in suppressing cell invasion.

The MMP enzymes comprise a family of extracellular proteinases enzymes that regulate basic cellular processes, such as cell invasion. Two main members, namely MMP9 and MMP7, have been demonstrated to play significant roles in cell invasion (23,24). Therefore, the levels of MMP9 and MMP7 were measured by western blot assays in the current study. The data demonstrated that overexpression of miR-139-5p evidently inhibited the protein expression levels of MMP7 and MMP9 (Fig. 3B). Taken collectively, these results strongly indicated that miR-139-5p overexpression inhibited cell invasion.

miR-139-5p modulates chronic inflammation by suppressing NF-κB activity. The inflammatory response contributes to malignant tumor progression in the tumor microenvironment (25,26). Changes in the inflammatory microenvironment are frequently accompanied by molecular alternations in tumor tissues, and miRNAs are usually considered as potential mediators of these processes (27). In the present study, overexpression of miR-139-5p significantly decreased the mRNA expression levels of the inflammatory cytokines IL-1β, IL-6 and TNF-α in HCT116 and LoVo cells transfected with miR-139-5p mimics (Fig. 4A). Furthermore, transfection of the cells with miR-139-5p mimics significantly decreased the secretion of IL-1β, IL-6 and TNF-α in the culture supernatant of HCT116 and LoVo cells (Fig. 4B). It is well known that IL-1β, IL-6 and TNF-α are target genes of NF-κB, and its activation has been demonstrated to boost the production of these inflammatory cytokines (28). The results of the present study indicated that activation of NF-κB was apparent in HCT116 cells, whereas it was considerably suppressed by overexpression of miR-139-5p in these cells (Fig. 4C and D). Taken collectively, the data suggested that miR-139-5p was able to decrease the production of IL-1β, IL-6 and TNF-α by suppressing NF-κB activation.

Discussion

Unstable amplification of inflammatory cytokines secreted from the tumor microenvironment is a significant factor that maintains the malignant behavior of tumors, and is further...
associated with microRNA dysregulation. Accumulating evidence has indicated that miR-139-5p is downregulated in inflammatory bowel disease-associated neoplastic transformation, in primary CRC and in metastatic sites (29-31). In addition, it has been reported that miR-139-5p is downregulated in CRC tissues and is associated with poor disease prognosis (15,21). A previous study has also demonstrated that deletion of miR-139-5p promotes intestinal inflammation and CRC through activating the NF-κB signaling pathway in vivo (17).

It was further suggested that miR-139-5p may be involved in the initiation and development of CRC by regulating inflammation. In the present study, the results demonstrated that the expression levels of miR-139-5p in CRC cells were significantly downregulated, and that the overexpression of miR-139-5p decreased the levels of the inflammatory cytokines IL-1β, IL-6, and TNF-α by suppressing NF-κB activity. These molecular events led to the inhibition of CRC cell proliferation and invasion.

Figure 2. miR-139-5p suppressed colon cancer cell growth in vitro and in vivo. (A) HCT116 and (B) LoVo cell proliferative activity was evaluated by a Cell Counting Kit-8 assay at 24, 48 and 72 h after transfection. (C) A colony formation assay was performed to detect the proliferative activity of HCT116 and LoVo colon cancer cells following transfection with miR-139-5p or NC mimics. (D) HCT116 cells were transfected with miR-139-5p or NC mimics, and then injected into the left flank of BALB/c nude mice. The tumor growth was measured every 4 days and a tumor growth curve was constructed. The data are presented as the mean ± standard deviation (n=3/group). *P<0.05 and **P<0.01 vs. blank or NC group. miR, microRNA; NC, negative control.
miR-139-5p was previously identified as a tumor suppressor in endometrial cancer (32), hepatocellular carcinoma (33) and adult acute myeloid leukemia (34). Furthermore, the aberrant expression of miR-139-5p is a characteristic feature in the development of CRC. For instance, Zhang et al (21) reported that the expression levels of miR-139-5p in CRC tissues were significantly downregulated when compared with those noted in the adjacent normal tissues. Similar findings were also reported by Song et al (15). Based on these findings, the present study aimed to detect the expression levels of miR-139-5p in CRC cells and found that they were significantly downregulated in the CRC cell lines compared with those noted in the human normal colon mucosal epithelial cell line NCM460. These results indicated that miR-139-5p may serve as a tumor suppressor in CRC.

Aberrant miRNA expression levels are associated with the malignant progression of cancer. Shi and Guo (35) indicated that the overexpression of miR-139-5p suppressed osteosarcoma cell growth, migration and invasion by reducing DNA methyltransferase-1, or vice versa. In addition, a study by Maoa et al (36) reported that loss of miR-139-5p promoted colitis-associated tumorigenesis in a transgenic murine model of colorectal carcinoma by activating the PI3K/AKT/Wnt
signaling. In esophageal squamous cell carcinoma, overexpression of miR-139-5p inhibited cell proliferation, migration and invasion, and induced apoptosis and cell cycle arrest (37). Therefore, in the present study, the biological function of miR-139-5p in CRC cells was further investigated. The results demonstrated that overexpression of miR-139-5p markedly suppressed cell proliferation in vitro and xenograft tumor growth in vivo, which were in agreement with the findings of the aforementioned studies. Furthermore, the data indicated that overexpression of miR-139-5p exhibited significant inhibition of the invasion ability of CRC cells by downregulating the protein expression levels of MMP9 and MMP7.

Figure 4. miR-139-5p decreased the production of IL-1β, IL-6 and TNF-α by suppressing NF-κB activation. (A) The mRNA expression levels of IL-1β, IL-6 and TNF-α were determined using reverse transcription-quantitative polymerase chain reaction in HCT116 and LoVo cells following transfection with miR-139-5p or NC mimics. (B) The protein levels of IL-1β, IL-6 and TNF-α in the culture supernatant of HCT116 and LoVo cells were detected by ELISA following transfection with miR-139-5p or NC mimics. (C) The activation of NF-κB was assessed by EMSA in HCT116 cells following transfection with miR-139-5p or NC mimics. (D) NF-κB activation was quantified according to the EMSA density. *P<0.05 and **P<0.01 vs. blank or NC group. miR, microRNA; IL, interleukin; TNF-α, tumor necrosis factor α; NC, negative control; NF, nuclear factor; EMSA, electrophoretic mobility shift assay.
Chronic inflammation contributes to cancer development and can cause predisposition to carcinogenesis. It has been reported that ~20% of all cancer types are associated with chronic infections (38). In a previous study by Zou et al (17), the interaction of intestinal inflammation and colitis-associated CRC was explored, revealing that miR-139-5p knockout mice were highly susceptible to colitis and colon cancer, accompanied by increased production of inflammatory cytokines and activation of the NF-κB signaling pathway. To further identify the types of inflammatory cytokines regulated by miR-139-5p that are involved in CRC, the levels of inflammatory cytokines, such as IL-1β, IL-6 and TNF-α, were evaluated in miR-139-5p overexpressing CRC cells in the current study. The results indicated that overexpression of miR-139-5p markedly decreased the secretion of cytokines IL-1β, IL-6 and TNF-α in the culture supernatant of CRC cells. Concomitantly, IL-1β, IL-6 and TNF-α mRNA levels were also decreased. Therefore, the results demonstrated that inhibition of cell proliferation and invasion by miR-139-5p were associated with the induction of inflammation in CRC.

The NF-κB pathway is a complex signaling pathway that regulates oncogenesis to promote the initiation and development of cancer (39). The activation of the NF-κB pathway has been reported to boost the production of pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6 (28). Further experiments conducted in the current study confirmed our hypothesis, suggesting that overexpression of miR-139-5p markedly suppressed the activation of NF-κB. It has been reported that the activation of NF-κB pathway mediates the proliferation and metastasis of CRC cells (40), stimulates angiogenesis in CRC cells (41), and promotes CRC progression (42), while inhibition of the NF-κB pathway suppresses CRC growth and metastasis (43). As a result, in the present study, it can be concluded that miR-139-5p mediated the downregulation of TNF-α, IL-1β and IL-6 by suppressing the activation of NF-κB, which may be responsible for the suppressed proliferation of CRC cells and stunted growth of CRC tumors.

In conclusion, the present study demonstrated that miR-139-5p was significantly downregulated in CRC cells, and that overexpression of miR-139-5p inhibited cell proliferation and invasion by decreasing the secretion of inflammation-associated cytokines. Furthermore, downregulation of the inflammation-associated cytokines mediated by miR-139-5p was associated with the suppression of NF-κB activity. These results illustrated a novel molecular mechanism that can be used for the development of CRC therapeutics.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
GL and BRV initiated and designed the present study, YGD, QL and QZ analyzed and interpreted the results. MMZ, WZ and JM performed the various experiments. MMZ wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The experimental protocol involving animals was approved by the Animal Ethics Committee of the Kunming Medical University (Kunming, China).

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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