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Original Article

MicroRNA-505 inhibits proliferation and promotes apoptosis of colorectal cancer cells

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Abstract: MicroRNAs (miRs) have been demonstrated to play an important role in various cancers, but there has been no study on the role of miR-505 in colorectal cancer (CRC) and the mechanism of how miR-505 is regulated remains uncertain. In this study, we report that the expression of miR-505 can be strongly inhibited by some pro-tumor cytokines such as TNF-α, IL-1β, IL-17A and IL-6. In addition, NF-κB activation negatively regulates the expression levels of miR-505. The over-expression of miR505 can suppress proliferation and promote apoptosis of HCT116 and HT29 cells in vitro. In conclusion, our findings indicate that miR-505 might be a promising therapeutic target in patients with colorectal cancer.

Keywords: miR-505, CRC, cytokine, NF-κB, proliferation, apoptosis

Introduction

Colorectal cancer (CRC) has been reported to be the third most common malignant neoplasms [1]. Emerging evidences have suggested that the combination of genetic factors, epigenetic changes, intestinal inflammation, alteration of intestinal microbiota and deregulated immune response contributes to the pathogenesis of CRC [2-4]. However, the exact mechanisms underlying this disease still remain largely unknown.

Hyperactivation of cell proliferation and survival signaling is one of the hallmarks of cancer as well as suppressed cell apoptosis. Several pro-apoptotic gene mutations have been noted [5]. The first identified tumor suppressor gene associated with apoptosis is p53, and p53 mutations have been found in a number of human tumors and usually correlates poor prognosis [6]. Bax, as a classic pro-apoptotic molecule, has been observed to be inactivated in some types of colorectal tumors [7].

MicroRNAs (miRs) are a group of single-strain noncoding RNAs (about 18-25 nt), which have been demonstrated to play an important part in great amounts of biological processes. Recent findings have shown that miRs are able to influence cell development, differentiation, growth, and metabolism as well as the regulation of inflammation and tumorigenesis through direct binding to the 3'untranslational region (3'-UTR) of target mRNAs. In regard of the role of miRs in tumorigenesis, many studies have indicated that miRs could play as either tumor suppressors or oncogenes depending on the mRNAs they target. miR-150 has been identified as a potential biomarker in diagnosing and determining prognosis in CRC, due to its role in down-regulating tumor cell proliferation, migration and invasion through directly targeting c-Myb [8]. Studies using miR-21-knockout mice found reduced expression levels of STAT3 and Bcl-2 activation, leading to increased apoptotic activities of tumour cells in CAC [9].

In this study, we demonstrated that miR-505 expression was greatly decreased in patients with CRC and murine colitis-associated cancer (CAC) model, and revealed the mechanism of how miR-505 was down-regulated and how NF-κB activation affected this alteration. In vitro experiments showed miR-505 could inhibit tumorigenesis through suppressing cancer

cell proliferation and enhancing the apoptosis. Our findings indicate that miR-505 might be a novel therapeutic target for CRC.

Methods

Patients

CRC tissues, adjacent normal mucosal tissues and serum samples were obtained from patients with sporadic CRC. All the individuals enrolled in our study were from the Shanghai Tenth People's Hospital of Tongji University. No patient received radiotherapy or chemotherapy before surgery. The study was approved by the Institutional Review Board for Clinical Research of the Shanghai Tenth People's Hospital of Tongji University. Written informed consent was also obtained from all subjects before the study protocol.

Animal models

To establish the CAC model, two groups of 8- to 12-week old C57BL/6 wild type (WT) mice (n = 10) were enrolled. Briefly, one group of WT mice were received with one single intraperitoneal injection of AOM (Sigma-Aldrich; 12 mg/kg) and 7 days later, these mice were treated with 3 cycles consisting 7 days of 2% DSS (36000-50000 Da; MP Biomedicals) in drinking water, followed by 14 days of drinking water feeding. The other group of WT mice served as controls. On day 81 after AOM intervention, two groups of mice were sacrificed, and colon tissues were collected for further analysis. DSS-induced colitis model was established through administrating WT mice with 2.5% DSS in drinking water for 7 days, followed by 3 days of drinking water feeding. On day 10, colon tissues were collected for further analysis. Our study was performed in accordance with the recommendations in the guidelines for Animal Care and Use Committee of the Shanghai Tenth People's Hospital of Tongji University.

Cell lines

All the human colon cancer cell lines were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China).

RNA isolation and analysis of miR expression

Total RNA of cells or tissues was extracted by Trizol reagent (Invitrogen, Carlsbad, CA, USA). The analysis of miR expression was performed using mirVanaqRT-PCR miRNA Detection Kit (AM1558, Ambion). qRT-PCR was performed using the SYBR Premix Ex Taq mix (TaKaRa, Dalian, China). U6 small nuclear RNA was used as internal control.

Cell transfection

Plasmids encoding pre-miR-505, control pre-miR (pre-miR-ctrl), anti-miR-505 or control anti-miR (anti-miR-ctrl) were obtained from Biotend (Shanghai, China). HCT-116 or HT29 cells were transfected with indicated plasmids using Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Western blot analysis

The HCT-116 or HT-29 cells were plated into 10 cm plates and transfected with pre-miR-505 along with negative pre-miR-ctrl. After 48 h, cells were washed with PBS and harvested in radioimmunoprecipitation assay buffer (Beyotime Biotechnology) containing protease inhibitors, namely phenylmethylsulfonyl fluoride, phosphatase inhibitors and cocktail (Roche Applied Science). The cell lysates were incubated on ice for 8 min, then were collected and centrifuged at 12000 rpm for 10 min at 4°C. The supernatants were collected and mixed with 5 × loading buffer, denatured by 100°C for 10 min. The samples were separated by 12.5% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes (Millipore, Billercia, MA, USA) in the transfer buffer containing Tris (Sangon biotech, Shanghai, China), glycine (Sangon Biotech, Shanghai, China), and methanol (20%). The membranes were incubated in 5% non-fat milk for 30 min and probed with primary antibodiesβ-Actin (Sigma, USA), BCL-2 (Abcam, UK), Bax (Abcam, UK) in PBST overnight at 4°C. The membranes were then washed and incubated with secondary antibody for 30 min at 37°C. After washing thrice with PBST for 30 min, proteins were visualized using with an Odyssey LI-CDR scanner (BD biosciences, USA), and the gray value was measured using Image J software (National Institutes of Health (NIH), Bethesda, MD, USA).

Proliferation and colony formation assay

Cell proliferation was determined by performing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphen-

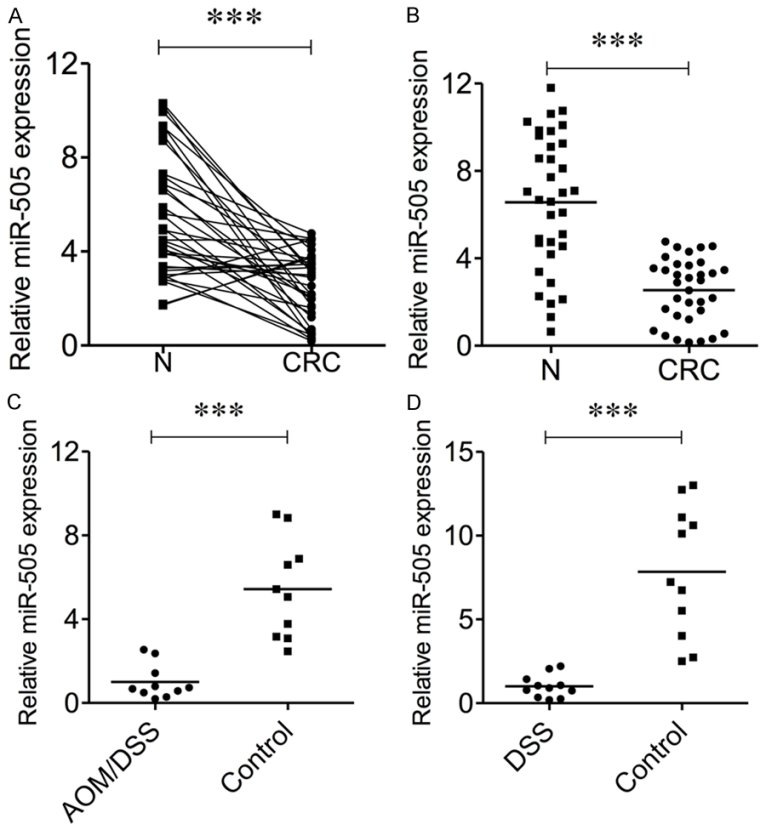

Figure 1. miR-505 is significantly decreased in CRC. (A) The expression of miR-505 was evaluated by qRT-PCR in human CRC tissues and normal mucosa tissues, (B) and in serum samples from CRC patients and healthy donors. (C) miR-505 expression in the colons resected frommice with AOM/DSS-induced CRC and control mice, and (D) in mice with DSS-induced acute colitisand control mice. Data are presented as mean ± SD of three independent experiments. ***P < 0.0001.

yltetrazolium bromide (MTT) assay as described elsewhere [10]. In order to investigate the colony formation ability, cells were seeded in 6-cm plates (2000 cells/dish). Ten days later, we fixed the colonies in methanol, and stained them with 0.1% crystal violet (Sigma, St Louis, MO, USA) and counted [10].

Assessment of apoptosis

To investigate apoptosis of HCT-116 cells transfection with pre-miR-505 or pre-miR-ctrl, we used an Annexin V-FITC Apoptosis Detection Kit II (BD Biosciences, San Diego, CA, USA). Briefly, transfected HCT-116 cells were trypsinized, washed and counted, and then immediately resuspended in Binding Buffer at a concentration of 1×10^6 cells/ml. Next, we stained these cells with FITC-conjugated Annexin V and PI for 15 min at room temperature. After adding Binding Buffer, flow cytometry data were acquired on a BD FACSCanto II and analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Statistical analysis

Data are expressed as the mean ± standard deviation. A Student t-test (two-tailed) was used to compare two groups (a $P < 0.05$ was considered significant) unless indicated otherwise (χ^2 test).

Results

miR-505 is markedly down-regulated in human and mouse CRC

To determine the expression of miR-505 in the cancer tissues, we collected cancer tissue samples and normal intestinal mucosa from 34 patients with CRC, and analyzed miR-505 expression using qRT-PCR. As shown in **Figure 1A**, significantly down-regulated expression of miR-505 was observed in the CRC tissues, compared to that in the normal mucosa tissues. Next, we examined the levels of miR-505 in the sera of CRC patients and healthy donors, and found that miR-505 was highly reduced in the sera of patients with CRC in comparison to that in healthy donors (**Figure 1B**). To further confirm the altered miR-505 expression, we detected the levels of miR-505 in AOM/DSS-induced CAC. After AOM/DSS treatment, colonic miR-505 expression was greatly decreased (**Figure 1C**). Moreover, we examined the expression levels of colonic miR-505 in mice with DSS-induced acute colitis. As expected, miR-505 was also significantly decreased in DSS mice (**Figure 1D**). Collectively, these data indicates that miR-505 might be intimately involved in the pathogenesis of sporadic CRC and inflammation-induced cancer.

TNF-, IL-1β, IL-17A and IL-6 strongly inhibit miR-505 expression

Since various studies have shown altered pattern of cytokines in the cancer tissues and

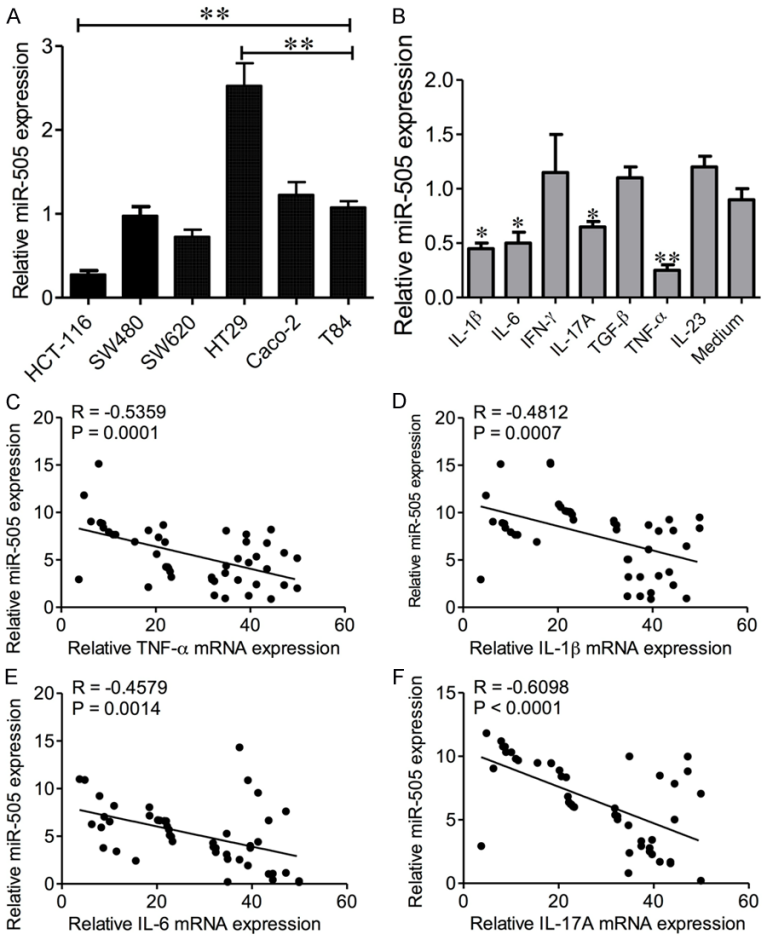

Figure 2. CRC related cytokines promote miR-505 expression. A. miR-505 expression in 6 human colon cancer cell lines. B. The expression of miR-505 was determined by qRT-PCR in HT29 cells with IL-1α, IL-6, IFN-γ, IL-17A, TGF-α, TNF-β, and IL-23. *P < 0.01, and **P < 0.001 versus cells cultured in medium alone. C-F. The correlation between miR-505 expression and TNF-α, IL-1α, IL-6, and IL-17A mRNA levels in the tumor tissues. R and *P* values are shown as indicated. Data are presented as mean ± SD of three independent experiments.

sera, [9, 11-13] we sought to determine whether the decreased expression of miR-505 was associated with these cytokines. To this end, we first measured the expression of miR-505 in 6 CRC cell lines, and we found the relatively lower expression of miR-505 in HCT-116 cells and higher miR-505 expression in HT29 cells (**Figure 2A**). We then stimulated human CRC cell line HT29 cells with IL-1, IL-6, IFN-γ, IL-17A, TGF-β, TNF-α, and IL-23 for 48 hours, and analyzed miR-505 expression by qRT-PCR. Interestingly, TNF-IL-1, IL-6, and IL-17A showed strong ability to suppress miR-505 expression (**Figure 2B**). Furthermore, we analyzed the mRNA levels of TNF-IL-1, IL-6, and IL-17A in CRC samples, and miR-505 expression was found to be negatively correlated with the mRNA levels of these four cytokines in CRC samples (**Figure 2C-F**). Taken together, miR-505 expression is markedly inhibited by TNF-IL-1, IL-6, and IL-17A, which were well established to play important roles in tumorigenesis, indicating miR-505 might exert anti-tumor function in the development of CRC.

miR-505 expression is inhibited by NF-κB activation

NF-κB is one of the most essential mediators in both inflammation and tumorigenesis, and animal models of CAC have emphasized the key roles of this master transcription factors in inflammation and cancer progression [14-17]. Two pathways have been implicated in NF-κB activation, including RelA/p50 and RelB/p52. RelA/p50 functions in the canonical NF-κB pathway, while RelB/p52 is involved in the noncanonical NF-κB pathway [18, 19]. In addition, many miRs have been pronounced to regulate or be regulated via NF-κB activity [20-22]. Here, we sought to examine whether miR-505 expression was mediated by NF-κB. To this end, we transfected HT29 cells with RelA/p50 and RelB/p52 expressing plasmid and cultured these cells for 48 hours. First, we examined the mRNA expression of RelA and RelB, which was strongly up-regulated (**Figure 3A**, **3E**). In this case, miR-505 was found to be significantly inhibited by NF-κB activity (**Figure 3B**, **3F**). To further confirm the role of NF-κB to suppress miR-505 expression, we depleted RelA or RelB by transfecting HCT-116 with siRNA. As outlined in **Figure 3C**, **3G**, siRNA strongly inhibited RelA or RelB expression, which resulted insignificantly up-regulated miR-505 expression (**Figure 3D**, **3H**). These findings collectively re-

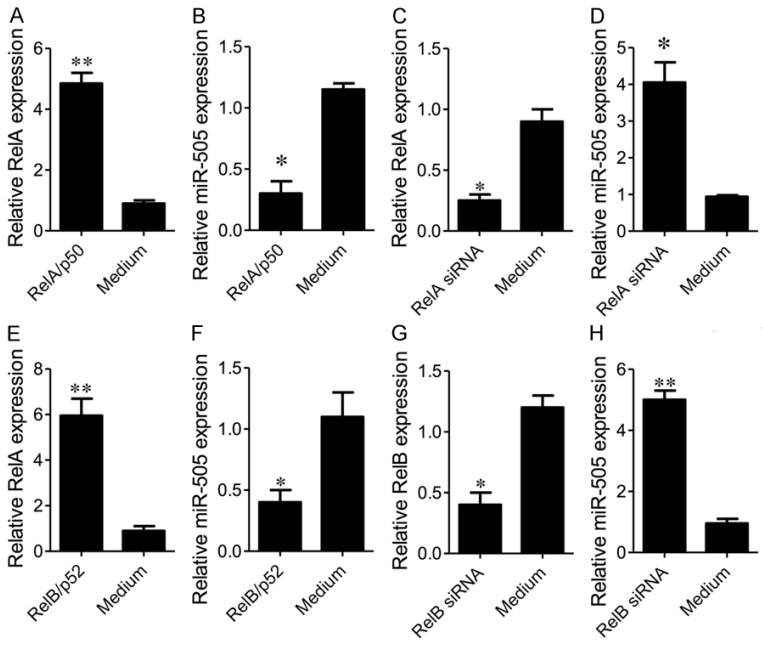

Figure 3. NF-κB inhibits miR-505 expression. A, B. The relative expression of RelA and miR-505 in HT29 cells transfected with RelA/p50. C, D. RelA and miR-505 expression in HT29 cells transfected with RelA siRNA. E, F. The relative expression of RelB and miR-505 in HT29 cells transfected with RelB/p52. G, H. RelB and miR-505 expression in HT29 cells transfected with RelB siRNA. Data are presented as mean ± SD of three independent experiments. *P < 0.05, **P < 0.001 versus cells cultured in medium alone.

veal that NF-κB activation negatively regulates the expression levels of miR-505.

miR-505 suppresses cell proliferation

Since dysregulated cell proliferation is one of the hallmarks of cancers, and the data presented above have indicated that miR-505 might play a role in regulation of colon cancer, we next sought to explore whether miR-505 could influence tumor cell proliferation to regulate the tumorigenesis. To this end, we transfected pre-miR-505 or pre-miR-ctrl into HCT-116 cells which express relatively lower levels of miR-505, and cultured them in DMEM containing 10% FBS for 6 days (**Figure 4A**). HCT-116 cell proliferation was assessed by MTT and colony-formation assays, and forced expression of miR-505 dramatically suppressed the growth rate of HCT-116 cells, while transfection with pre-miR-ctrl didn't affect the proliferation (**Figure 4C**, **4D**). Next, we introduced anti-miR-505 or anti-miR-ctrl into HT29 cells, which express relatively high levels of miR-505 (**Figure 4B**). MTT and colony-formation assays were also performed to examine the proliferation of cells, and we found that inhibition of miR-505 significantly promoted the proliferation of HT29 cells (**Figure 4E**, **4F**). Taken together, miR-505 suppresses cell proliferation, by which it functions as an inhibitor of colorectal cancer.

miR-505 promotes apoptosis of cancer cells

A number of investigators have documented that insufficient amount of apoptosis results in uncontrolled cell proliferation, which contributes to cancer progression. Given the findings above, we asked whether miR-505 could induce apoptosis in intestinal epithelial cells. To elucidate the effects of miR-505 on cancer cell apoptosis, we transfected pre-miR-505 to HCT-116 cells, which led to greatly increased expression of miR-505 in HCT-116 cells in comparison to those transfectants with control pre-miR-ctrl (**Figure 5A**), and measured the rate of apoptosis using flow cytometry. As described in **Figure 5B**, **5C**, flow cytometric analysis revealed that enforced expression of miR-505 induced significantly up-regulated apoptotic activity in HCT-116 cells, when compared to those transfected with pre-miR-ctrl. We also detected the protein expression of Bcl-2 and Bax in these transfected cells, and found that the expression of Bcl-2 that has been identified to act against apoptosis was markedly inhibited, while Bax pronounced to enhance apoptosis was strongly increased by enforced expression of miR-505 (**Figure 5D**). Taken together, our data suggest that miR-505 might contribute to tumorigenesis through promoting cancer cell apoptosis.

Discussion

In the past few years, there has been a large number of researches suggesting that miRs play a crucial role in the regulation of immune responses and tumorigenesis through mediating many cellular processes, such as cell prolif-

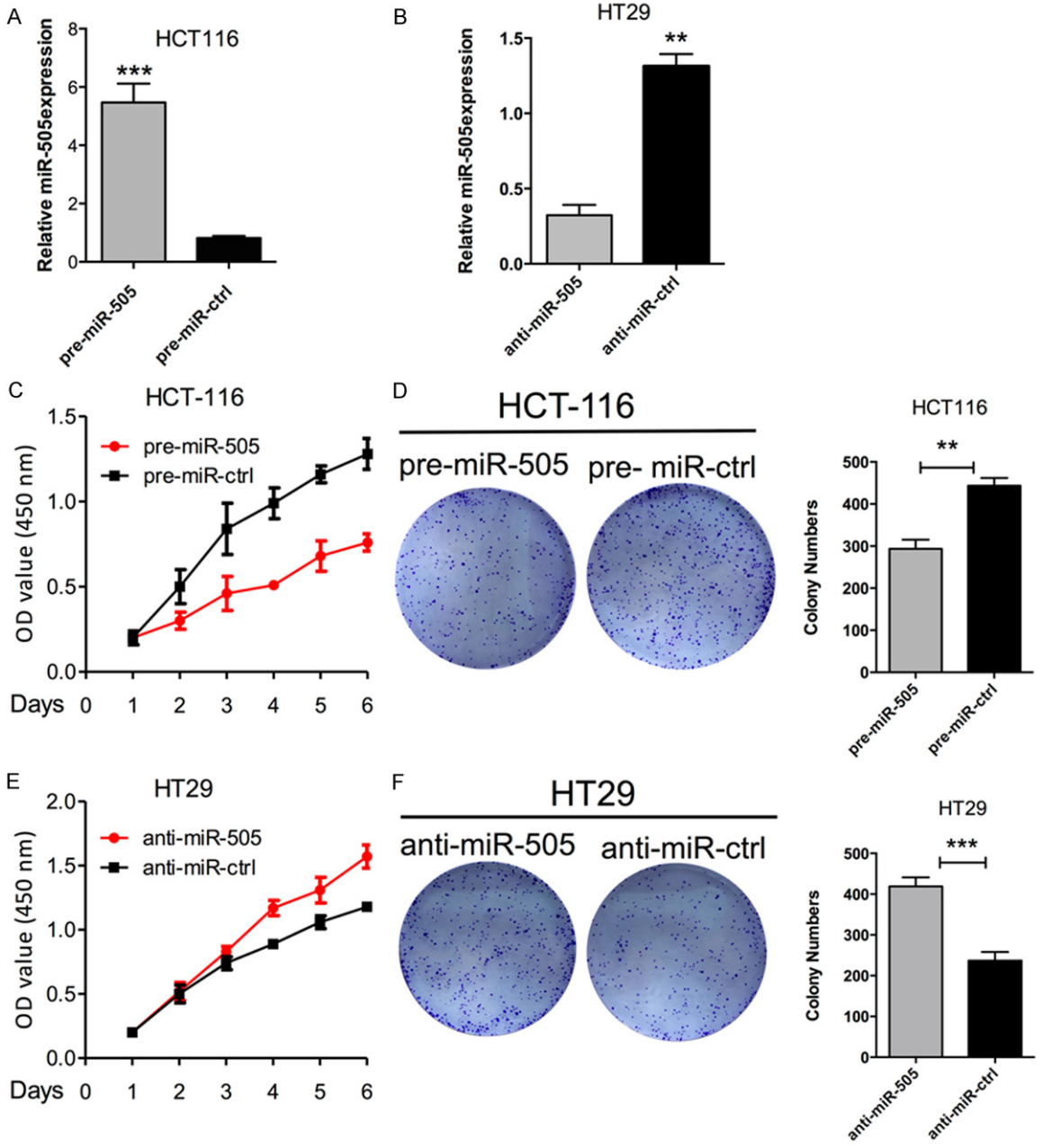

Figure 4. Ectopic miR-505 expression inhibits cell proliferation. (A) Ectopic expression of miR-505 in HCT-116 cells was validated by real-time PCR. (B) The inhibition of miR-505 expression in HT29 cells by anti-miR-505 cells was also confirmed using real-time PCR. (C) The proliferation of HCT-116 cells transfected with pre-miR-505 or pre-miR-ctrl was measured by MTT assay, and (D) representative results of colony formation of these HCT-116 cells; the numbers of colonies containing > 50 cells were scored (left panel) and colony numbers were shown in bar chart (right panel). (E) The proliferation of HT29 cells transfected with anti-miR-505 or anti-miR-ctrl was measured by MTT assay, and (F) representative results of colony formation of these HCT-116 cells; the numbers of colonies containing > 50 cells were scored (left panel) and colony numbers were shown in bar chart (right panel). Data are presented as mean ± SD of three independent experiments. **P < 0.01.

eration, differentiation, apoptosis and invasion [23, 24]. In the current study, we found, for the first time, that miR-505 expression was strongly suppressed in colorectal cancer tissues obtained from CRC patients, which was confirmed by detecting decreased expression of miR-505 in tumors from mice with AOM/DSS-induced CAC. In addition to the abnormal expression of miR-505 in colorectal cancer tissues, we also revealed for the first time that the

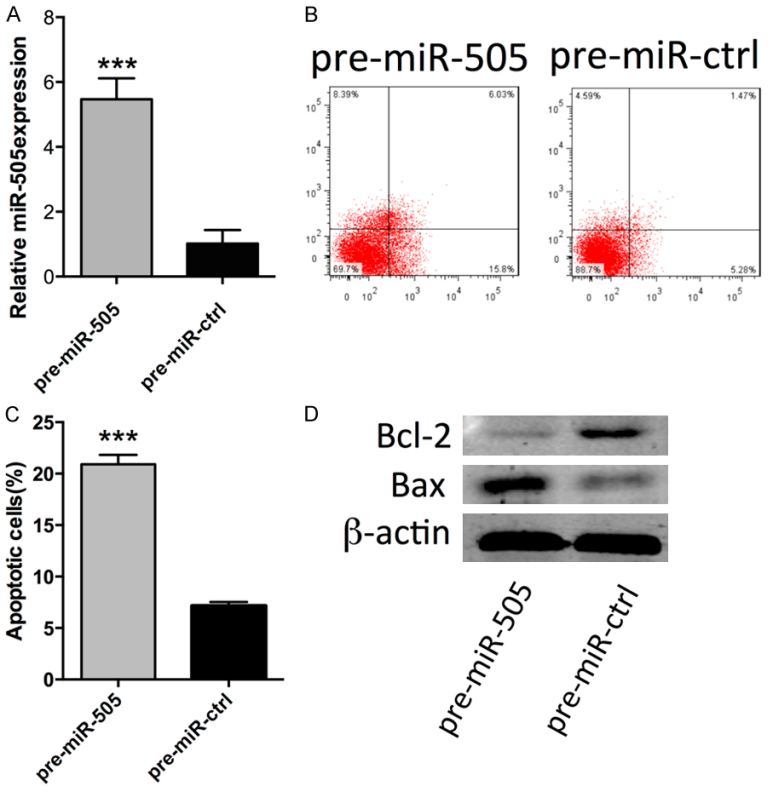

Figure 5. miR-505 promotes apoptosis of cancer cells. (A) Ectopic expression of miR-505 was evaluated in HCT-116 cells transfected with pre-miR-505, and in those transfected with pre-miR-ctrl. (B) The apoptosis of HCT-116 cells was measured by flow cytometry, and (C) the percentage of apoptotic cells was shown in bar chart. (D) The protein expression of Bcl-2 and Bax were detected in transfected HCT-116 cells via Western blot. b-actin was used as the internal references. Data are presented as mean ± SD of three independent experiments. ***P < 0.0001.

colonic expression of miR-505 was significantly reduced in mice with DSS-induced colitis. Given the relationship between colitis and colorectal cancer, these data strongly suggest the decreased expression of miR-505 in CRC.

Considering the fact that cytokines play an important role in the regulation of tumorigenesis and the altered pattern of cytokine expression has been implicated to contribute to tumor growth, differentiation and invasion, we investigated whether the abnormal pattern of miR-505 was associated with cytokine dysregulation. The down-regulated serum and mucosa expression of TNF-α has been noted by many researches, and markedly higher TNF-α level was found in tumour-node-metastasis stage IV in comparison to earlier stages of CRC and healthy controls. Additionally, significantly higher survival rate of CRC patients with low serum levels of TNF-α has been found, when compared to that of patients with high TNF-α serum level [25, 26]. IL-17A is produced by Th17 cells, a $CD4^+$ subtype distinct from Th1 and Th2 cells. This cytokine was primarily known to exhibit crucial pro-inflammatory abilities to protect the host against extracellular pathogens. In the field of cancer research, IL-17A has been found to act as an essential player in CAC [27]. The presence of $IL\text{-}17A^+$ cells have been demonstrated T in several both human colorectal cancers and murine models. Furthermore, IL-17A has been proposed to serve as a new prognostic indicator in CRC patients [28]. IL-6 is one of the most important pro-inflammatory cytokines in intestinal inflammation. Apart from the function to expand T cells and induce Th17 cells, IL-6 together with TGF-α has been shown to play a role in AOM/DSS induced murine CAC model. In vivo studies have demonstrated that high IL-6 serum level is positively associated with larger tumor size and liver metastasis, confirming the role of IL-6 in colorectal cancer [29]. A recent study has shown that neutrophils are able to secret IL-1β, which subsequently stimulate macrophage to produce IL-6, resulting promoting the CAC progression. Other labs have also revealed that IL-1β is capable of eliciting IL-17 expression, leading to promoting colon carcinogenesis [30]. Our data showed these four cytokine strongly inhibited the expression of miR-505 in HCT-116 cells, suggesting that miR-505 might play a protective role in colorectal cancer.

NF-κB signaling is a crucial pathway involved in multistage of colorectal cancer development, [31, 32]. This pathway have been implicated to be involved the growth, angiogenesis and metastasis of CRC. In the present study, we found that miR-505 expression in HT29 was

highly suppressed when RelA/p50 or RelB/p52 was overexpressed, and the inhibitory effect could be reversed when RelA/p50 or RelB/p52 was silenced. These data further indicated that miR-505 might exert an essential anti-tumor function.

In conclusion, we showed that the expression of miR-505 was significantly decreased in patients with CRC and murine CAC model, and NF-κB activation as well as pro-tumor cytokines such as IL-17A strongly inhibited miR-505 expression. More importantly, miR-505 was identified to be able to promote the apoptosis and suppress the proliferation of tumor cells, which suggests that miR-505 might be a promising colorectal cancer therapeutic target.

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Disclosure of conflict of interest

None.

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