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MiR-874 inhibits cell growth and induces apoptosis by targeting STAT3 in human colorectal cancer cells

B. ZHAO, A.-S. DONG

Department of Nuclear Medicine, Changhai Hospital, Second Military Medical University, Shanghai, China

Abstract. – OBJECTIVE: MicroRNA-874 (miR-874) has previously been identified as a tumor suppressor in several cancers. However, its role in colorectal cancer (CRC) has not been studied. In the present study, we aimed to investigate its potential roles in regulating cell growth and apoptosis in human CRC cells.

PATIENTS AND METHODS: MiR-874 expression was detected by real-time PCR analysis. Cell viability was detected by CCK-8 assay. Protein expression level was detected by Western blot, and luciferase activity assay was used to validate the interaction between mir-874 and STAT3 mRNA 3'UTR.

RESULTS: We found that miR-874 was significantly downregulated in CRC tissues. Gain and loss of function of miR-874 proved that miR-874 could inhibit cell growth and induce apoptosis in CRC cells. Luciferase reporter assay and Western blot analysis showed that miR-874 repressed STAT3 expression by targeting its mRNA 3'UTR. Silencing STAT3 recapitulated the phenotype of miR-874 overexpression. Moreover, the inverse correlation between miR-874 expression and STAT3 expression was validated in CRC specimens.

CONCLUSIONS: These data demonstrate that miR-874 functions as a tumor suppressor by repression of STAT3, suggesting its potential therapeutic value in CRC treatment.

Key Words:
MicroRNA-874, STAT3, Proliferation, Apoptosis, Colorectal cancer (CRC).

Introduction

Colorectal cancer (CRC) is one of the most prevalent cancers and accounts for a substantial number of cancer-related deaths worldwide[1,2]. Although the detailed chemotherapeutic treatment regimen for CRC has been standardized, the treatment efficacy of these strategies is still unsatisfactory. Considerable laboratory studies have been conducted in order to develop better therapeutic methods. Despite the large amount of works that devoted to uncover the molecular mechanisms underlying the development and progression of CRC, the pathogenesis of this disease is still elusive.

MicroRNAs are single strand RNAs which are about 22nt long. Basically, microRNAs function as negative gene regulators by post-transcriptionally modification of the 3'UTR of the targeted mRNAs based on base pair matches[3]. The discovery of microRNAs has expanded our horizon to tackle problems in cancer treatment, and a large amount of microRNAs have been demonstrated to be critical molecules in regulating proliferation, apoptosis and cancer cell stemness. Several microRNAs have been shown to play important roles in promoting or suppressing the oncogenic potential of CRC cells[4,5]. MiR-874 has been previously identified as a tumor suppressor in malignancies including gastric cancer, breast cancer and head and neck squamous cell carcinoma[6-9]. However, it remains unclear whether it plays a role in colorectal cancer.

The transcriptional factor STAT3 (signal transducer and activator of transcription 3) is required for the expression of several oncogenes such as Bcl-xL, Mcl-1 and cyclin D1[10-13]. When phosphorylated, STAT3 is able to translocate into the nucleus to activate certain transcriptional programs. Aberrant STATs activation has been often observed in leukemia[14], and it has also been shown that STAT3 promotes the proliferation of colorectal cancer cells and modulates the microenvironment to promote the epithelial-mesenchymal transition (EMT) processes, which is critical for CRC metastasis[15]. STAT3 signaling is activated by upstream inflammation related cytokines. Given that inflammatory colon diseases have been well es-

Corresponding Author: Bo Zhao, MD; e-mail: zhaobo5896@163.com

tablished as important risk factors of CRC, STAT3 may play major roles in controlling the tumorigenesis and progression of CRC.

In the present study, we first reported the significant downregulation of miR-874 in CRC tissues compared with the adjacent normal tissues. We identified that miR-874 functions as a tumor suppressor by inhibiting cell growth and inducing apoptosis. Importantly, we demonstrated that these effects were mediated by its direct target STAT3. These findings suggest the tumor suppressive role of miR-874 in colorectal cancer for the first time and may expand our understandings of the role of microRNA and STAT3 signaling in the pathogenesis of CRC.

Patients and Methods

Human Tissue Specimens

19 human tissue samples were collected from Changhai Hospital under the regulation of the Ethics Committee. Informed consent was obtained from each patient. The tumor tissues and the adjacent normal tissues were collected during surgeries; the tissues were quickly frozen sectioned and diagnosed by experienced pathologists. After the diagnosis has been made, the tissues were snap-frozen in liquid nitrogen and transferred to a -80°C freezer.

Cell Lines and Transfection

CRC cell line SW480 and HCT116 and HEK293 cell line were all purchased from American Type Culture Collection (ATCC). Cells were cultured in DMEM containing 10% FBS. All cells were cultured in CO_2 (5%) incubator at 37°C with a humid atmosphere. To deliver miR-874 or si-STAT3 into cells, Lipofectamine 2000 (Carlsbad, CA, USA) was used. Cells were cultured in antibiotic-free reduced serum medium for 24h before transfection. The final concentration of the nucleotide is 100 nmol/L. The medium was replaced with complete medium 6h after transfection. Subsequent experiments were conducted 72h after transfection. MiR-874 and its inhibitor were purchased from RiboBio biotechnology (Guangzhou, China). The siRNAs for STAT3 were designed and synthesized by Genepharma (Shanghai, China).

Western Blot

Protein of cultured cells was extracted using RIPA buffer (Thermo Scientific, Waltham, MA, USA), briefly, after the cells have been lysed by RIPA buffer, the mixed contents were super sonicated and centrifuged. The supernatant was collected for Western blot. The proteins were denatured with loading buffer at 100°C for 3 min and subjected to 12% SDS polyacrylamide gels. The proteins were blotted onto a PVDF membrane. The membranes were blocked by 5% skimmed milk at room temperature for 1h. Primary antibodies were diluted in PBS, and membranes were incubated with primary antibodies overnight at 4°C. Secondary antibodies were incubated on the following day. The protein bands were revealed by chemiluminescence detection system (EMD Millipore Co., Billerica, MA, USA). All the antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and were used at the recommended dilutions.

Cell Viability Assay

Cell viability was determined by a CCK-8 kit (Vazyme, Nanjing, China). Cells were grown in a 96-well plates, the CCK-8 reagent was added to culture medium 72h after transfection, after a 3h-incubation, the absorbance value was detected.

Real-time PCR

To detect the levels of miR-874, we isolated cell RNA using a miRcute miRNA isolation kit (Tiangen, Beijing, China). We purchased the qRT-PCR primer set for miR-874 from RiboBio. The RNA samples were reverse transcribed by a first strand cDNA synthesis kit from Promega (Madison, WI, USA). The PCR amplification was deployed on a Bio-Rad CFX96 real-time PCR detection system.

Luciferase Reporter Assay

Luciferase reporter assay was used to identify the relationship between miR-874 and STAT3. The wild type (WT) 3'UTR and the mutant (Mut) 3'UTR were synthesized by Sangon Biotechnology (Shanghai, China). The 3'UTR sequence containing the potential binding site was then cloned into the 3'UTR of pMIRGLO luciferase reporter. MiR-874 was transfected into HEK293 cells along with either WT 3'UTR or Mut 3'UTR. Dual luciferase activity assay was conducted 36h after transfection using the corresponding detection kit (Promega, Madison, WI, USA).

Statistical Analysis

Data were shown as means ± SEM, the comparisons of miR-874 and STAT3 expression in

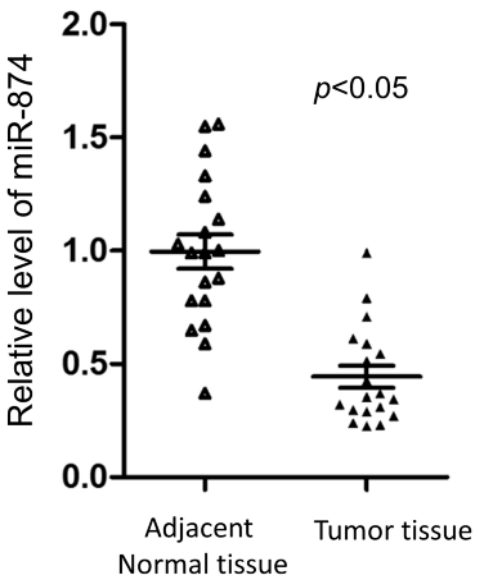

Figure 1. Downregulation of miR-874 in CRC tissues. The tissue samples were collected from surgeries, paired adjacent normal tissues and tumor tissues were subjected to real-time PCR analysis. $p<0.05$, n=19.

clinical tissue samples were performed by paired *t*-test. The data of the *in vitro* studies were analyzed by unpaired *t*-test. Spearman correlation coefficient was used to analyze the relationship between STAT3 expression and miR-874 expression in clinical tumor samples. A two tailed $p < 0.05$ was considered statistically significant.

Results

MiR-874 is Downregulated in Human CRC Tissues

To determine the potential role of miR-874 in CRC, miR-874 expression levels were analyzed by Real-time PCR. We collected 19 paired samples of tumor tissue and the adjacent normal tissue. As displayed in Figure 1, we found that the miR-874 expression was significantly lower in CRC tissues.

Overexpression of miR-874 Inhibits Cell Growth and Induces Apoptosis in CRC Cells

To further investigate whether the aberrant expressed miR-874 level exhibited biological functions. We first overexpressed miR-874 in CRC cell lines SW480 and HCT116. The gain of function of miR-874 was confirmed by Real-time PCR analysis (Figure 2A). By CCK-8 assay, we found that the cell viability was significantly decreased after miR-874 over expression in both SW480 and HCT116 cell lines (Figure 2B). Western blot analysis showed that the molecular marker of apoptosis, cleaved-caspase-3, was significantly induced by miR-874. By contrast, cyclin D1, a cell cycle regulator that is required for cell proliferation, was found significantly upregulated in both cell lines (Figure 3C and D).

Inhibition of miR-874 Promotes the Growth of CRC Cells

We then transfected cells with the antisense nucleotide against miR-874 to inhibit its endogenous expression (Figure 3A), as shown in Figure 3B, cell viability was significantly decreased after miR-874 knockdown. Western blot analysis revealed that cyclin D1 expression was increased upon miR-874 suppression (Figure 3C and D). Together with the data of gain of function experiments, these findings suggest that miR-874 plays a tumor-suppressive role by inhibiting the proliferation and apoptosis of CRC cells.

STAT3 is Targeted by miR-874

To investigate the exact molecular mechanism of miR-874 in CRC, we searched the miRanda database and found that miR-874 can potentially target STAT3 (Figure 4A). Western blot and luciferase gene reporter assay were conducted to validate our hypothesis. As shown in Figure 4B, overexpression of miR-874 in SW480 cells caused a remarkable reduction of STAT3 expression. Forced expression of miR-874 inhibited luciferase activity in wild-type 3'UTR, but it did not influence the luciferase activity of reporter containing mutant STAT3 3'UTR. These data indicates that STAT3 is targeted by miR-874.

The expression of STAT3 in clinical samples was also analyzed; we observed that STAT3 was significantly higher in CRC tissues (Figure 4D), and linear correlation analysis revealed that miR-874 and STAT3 were inversely correlated (Figure 4E). These findings confirmed the interaction between miR-874 and STAT3 in the clinical setting.

Inhibition of STAT3 Recapitulated the Effect of miR-874

Finally, we inhibited STAT3 expression by transfection of specific siRNAs. Silencing STAT3 showed consistent results with miR-874

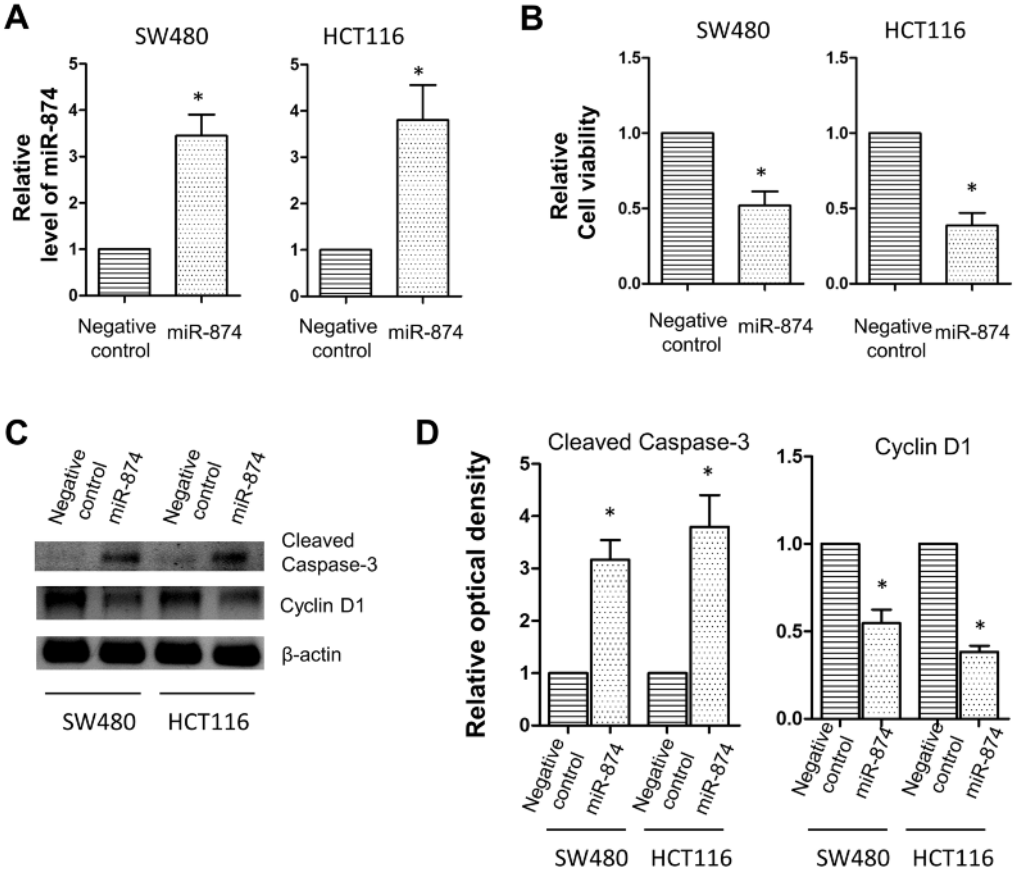

Figure 2. Overexpression of miR-874 inhibits cell growth and induces apoptosis in CRC cell lines. SW480 and HCT 116 cells were transfected with the miR-874; 72h after transfection, cells were subjected to real-time PCR, CCK-8 assay or Western blot. ***(A)*** The relative expression of miR-874 after transfection. ***(B)*** The relative cell viability after transfection. ***(C)*** Detection of cleaved caspase-3 and cyclin D1 after transfection of miR-874 and negative control in CRC cell lines. ***(D)*** The histogram of protein expression after transfection of negative control and miR-874. *$p < 0.05$ vs. negative control, n = 3.

overexpression: it resulted in decreased cell viability (Figure 5A), decreased cyclin D1 expression as well as induction of the cleavage of caspase-3 (Figure 5B and C). These results suggested that STAT3 inhibition recapitulated the cell phenotype of miR-874 overexpression and indicated that STAT3 is a functional target of miR-874.

Discussion

Posttranscriptional regulation of gene expression is of obvious importance for the oncogenic behaviors of tumor cells. MicroRNAs, which have been identified decades ago, are broadly implicated in the process of carcinogenesis[5]. In the current study, we showed that miR-874 acted as a tumor suppressor by repression of the transcriptional factor STAT3. We demonstrated for the first time that miR-874 was downregulated in CRC clinical samples. Supplementing miR-874 exhibited decreased growth rate and increased apoptosis, and inhibition of miR-874 had an opposite effect. Importantly, we demonstrated a novel mechanism, which revealed that STAT3 was directly targeted by miR-874. Finally, we confirmed that STAT3 was a functional target of miR-874 by RNA silencing experiment: knockdown of STAT3 totally recapitulated the growth inhibition and pro-apoptotic phenotype of miR-874 overexpression. Therefore, we propose the anti-tumor role of miR-874 for the first time in the system of CRC, and our study highlights the potential application of miR-874 for CRC treatment.

Accumulating studies have demonstrated the important roles of noncoding RNAs in the pathogenesis of CRC[4]. Although recent findings

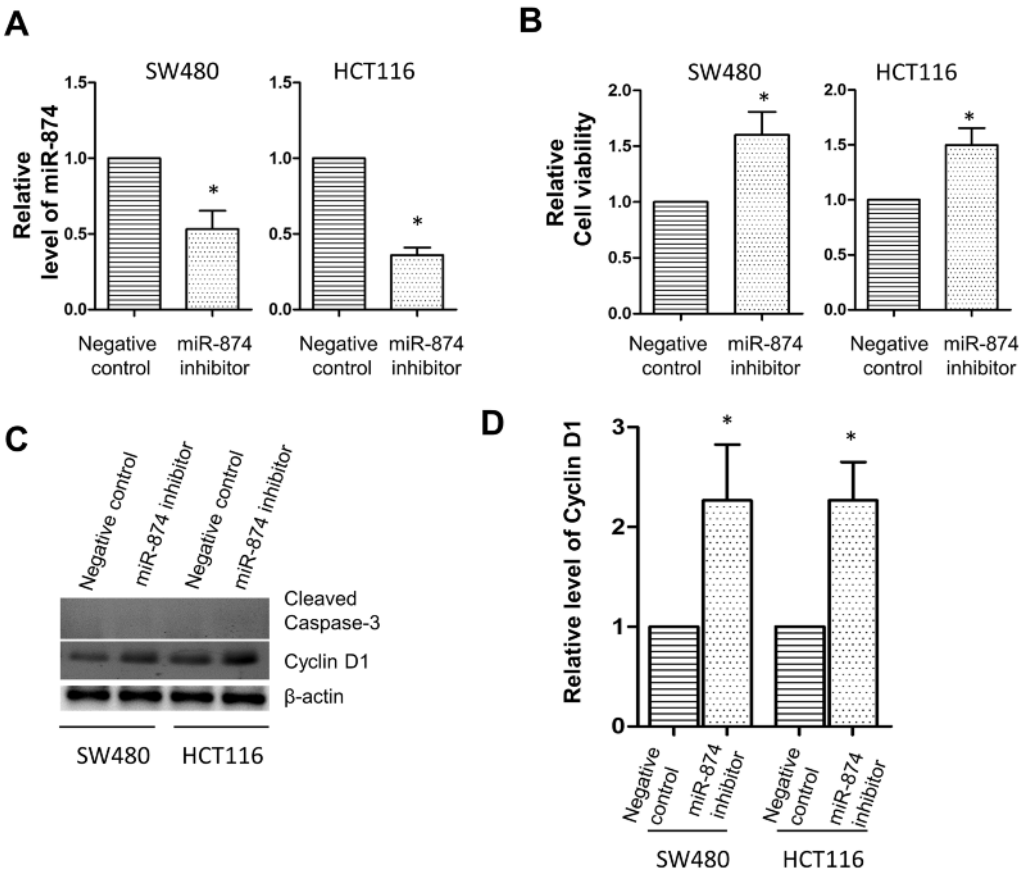

Figure 3. Overexpression of miR-874 inhibits cell growth and induces apoptosis in CRC cell lines. SW480 and HCT 116 cells were transfected with the miR-874; 72h after transfection, cells were subjected to real-time PCR, CCK-8 assay or Western blot. ***(A)*** The relative expression of miR-874 after transfection. ***(B)*** The relative cell viability after transfection. ***(C)*** Detection of cleaved caspase-3 and cyclin D1 after transfection of miR-874 and negative control in CRC cell lines. ***(D)*** The histogram of protein expression after transfection of negative control and miR-874. $^{*}p < 0.05$ vs. negative control, n = 3.

demonstrated novel classes of non-coding RNAs such as long non-coding RNAs (LncRNAs) and Piwi-Interacting RNAs in CRC (PiRNAs) are crucial in regulating the CRC carcinogenesis, the functions of a substantial number of microRNAs remain not understood. Recent reports on miR-874 have suggested its tumor suppressive role in cancers occurring in breast, stomach and maxillary sinus[7,8,16]. However, there has been no study working on its role in CRC. As deregulation of microRNA profile has been commonly involved in tumor formation and metastasis, we tested the expression of miR-874 in paired tumor samples and adjacent normal samples. We reported for the first time that miR-874 was downregulated in CRC tissues. This results encouraged us to hypothesize that miR-874 might repress the proliferative activity of cancer cells. *In vitro* analysis of cell viability and cell apoptosis marker cleaved caspase-3 supported our hypothesis. Moreover, the anti-growth phenotype after miR-874 transfection was inconsistent with other studies in gastric cancer and breast cancer[6-8].

Identifying the mechanism by which miR-874 regulates apoptosis and proliferation is one of the key issues to harness the antitumor activity of this microRNA in the treatment of CRC. By the prediction of bioinformatics tools, STAT3 was assumed as one of the candidate genes that are regulated by miR-874. Forced expression of miR-874 indeed suppressed the endogenous expression level of STAT3, and luciferase activity assay confirmed the direct interaction between STAT3 mRNA 3'UTR and miR-874. Thus, we identified a novel targeting relationship between STAT3 and miR-874. Notably, consistent with the *in vitro* experiments, we also observed a strong inverse correlation between miR-874 and

STAT3 in tumor samples. STAT3 is believed to be the critical linker between inflammation signaling and cancer. Given that inflammatory gut diseases such as inflammatory bowel disease, ulcerative colitis and Crohn's disease are believed to increase the risk of CRC[17-19], it is conceivable that inflammatory signaling plays critical roles in gut carcinogenesis. STAT3 receives the signals from the JAK kinase, which is mainly activated by the cytokine IL6. Remarkably, there are also reports showing the hyperactivation of STAT3 in CRC. STAT3 probably acts as a main nodal point to regulate cell proliferation. It has been demonstrated by many studies that oncogenes such as Survivin, Cyclin D1 and Mcl-1 are transcriptionally regulated by STAT3[13,20-22]. In the

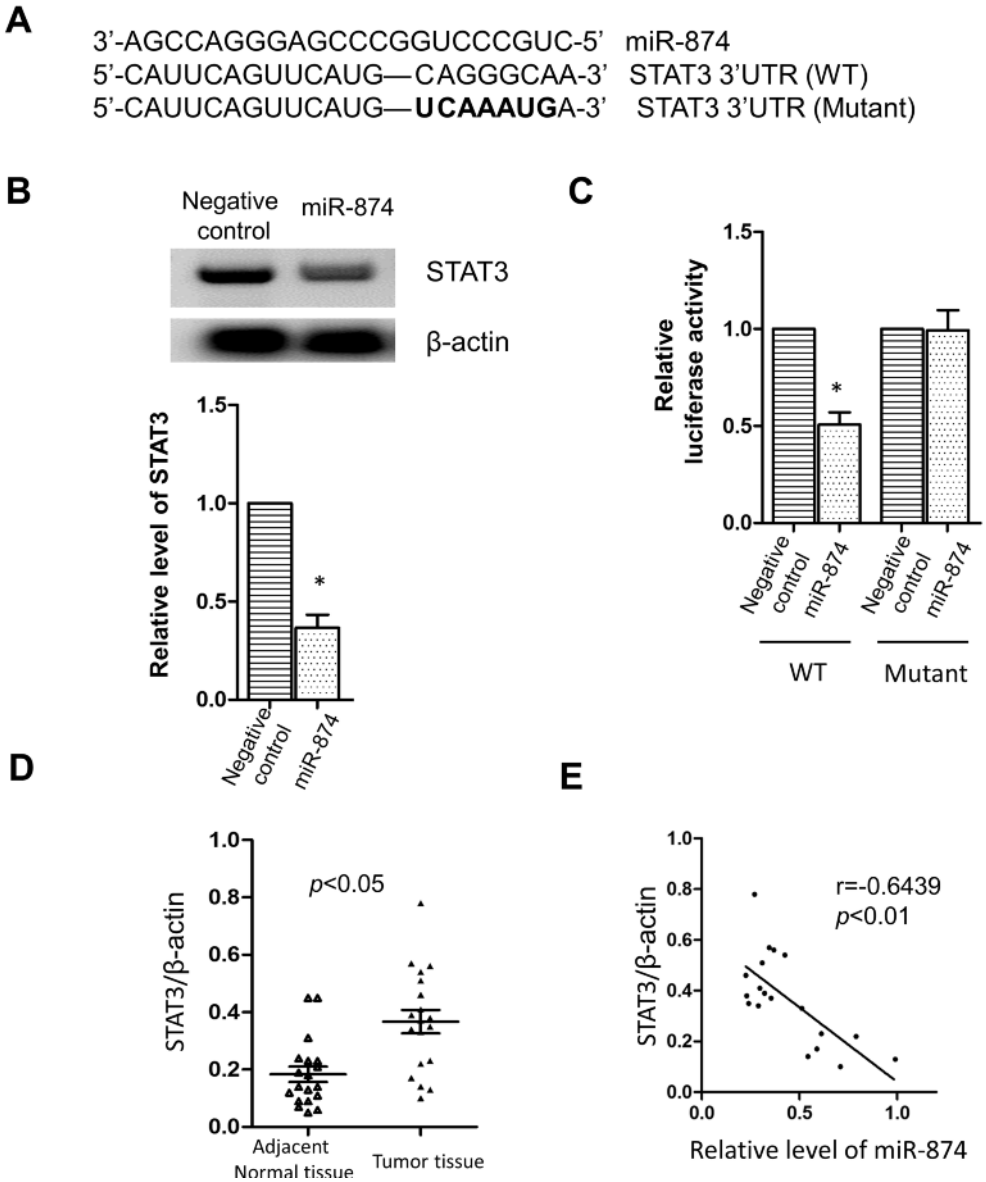

Figure 4. MiR-874 targets STAT3. ***(A)*** The alignment of the base pair match between miR-874 and STAT3 3'UTR, the boldface base sequence represented the mutant sequence. ***(B)*** Western blot showing STAT3 expression was downregulated by miR-874 in SW480 cells. ***(C)*** MiR-874 inhibited the luciferase activity in wild-type STAT3 (WT) 3'UTR, and it failed to do so in mutant (Mut) STAT3 3'UTR. ***(D)*** Western blot analysis showing upregulated STAT3 expression in CRC tumor tissues compared with its adjacent normal tissues. ***(E)*** Spearman correlation analysis showing the negative linear correlation between STAT3 expression and miR-874 expression in tumor samples. $^{*}p < 0.05$ *vs.* negative control, n = 3.

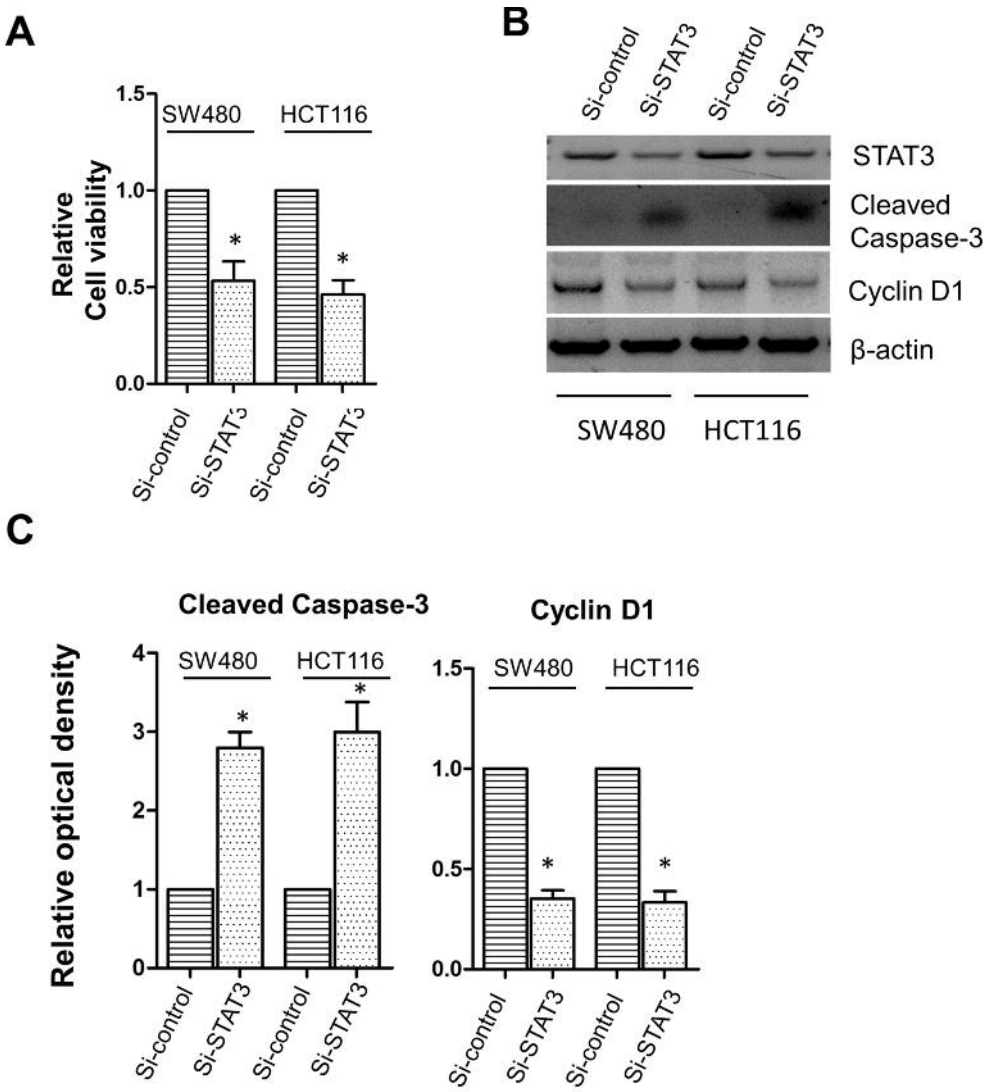

Figure 5. Inhibition of STAT3 recapitulated the effect of miR-874. SW480 and HCT 116 cells were transfected with specific siRNAs against STAT3; 72h after transfection, cell viability and protein expression were determined by CCK-8 assay and Western blot. ***(A)*** Knockdown of STAT3 inhibited cell viability in SW480 and HCT116 cells. ***(B)*** The expression of STAT3, cyclin D1 and cleaved caspase-3 after transfection of STAT3siRNAs (si-STAT3) and the control siRNAs (si-control). ***(C)*** The histogram of protein expression level of cyclin D1 and cleaved caspase-3. $*p < 0.05$ *vs*. si-control, n = 3.

present work, we found that the expression of STAT3 was positively correlated with Cyclin D1 in both loss and gain of function of miR-874, which is consistent with these previous demonstrations. Furthermore, increased apoptosis was observed, as evidenced by an increased cleavage of caspase-3 after miR-874 or STAT3 siRNA transfection. So we can conclude that miR-874/STAT3 interaction influenced both the apoptotic machinery and the proliferative program. Notably, using HUVEC as a cellular model, Zhang et al reported that STAT3/VEGF signaling acts downstream of miR-874 to promote the angiogenesis of gastric cancers[6]. This work also implied the potential role of miR-874 in the regulation of the microenvironment of CRC.

Since a microRNA can have multiple targeted mRNAs, we cannot eliminate other possible mechanisms. Several other genes have been previously identified as targets of miR-874 including aquaporin-3 in gastric cancer[8], CDK9 in breast cancer[7], HDAC1 in head and neck squamous cell carcinoma[9] and PPP1CA in maxillary sinus squamous cell carcinoma[16]. All of the cur-

rently identified targets have oncogenic potential, suggesting that the antitumor role of miR-874 is universal in different systems. Wang et al[23] showed that, in cardiomyocytes, miR-874 is involved in necrosis. In the present work, we only detected the parameters of apoptosis, whether necrosis is implicated in the antitumor role of miR-874 is going to be studied in the future.

Conclusions

We present evidence that miR-874 plays a crucial antitumor role in CRC. We show that downregulation of miR-874 in CRC correlates with increased expression of its target gene STAT3 both in CRC cells and in clinical samples, which further triggers growth inhibition and proapoptotic effects. Elucidation of the biological function of miR-874 in CRC not only deepened our understanding on the pathogenesis of cancers but also provided a new therapeutic approach for the clinical treatment of CRC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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