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miR-590-3p promotes colon cancer cell proliferation via Wnt/β-catenin signaling pathway by inhibiting WIF1 and DKK1

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Abstract. – OBJECTIVE: Colon cancer is one of the most common and deadly types of gastrointestinal tumor. Despite progressive treatments, the patient prognosis has not been improved effectively.

MATERIALS AND METHODS: Expression of miRNA and mRNA were tested by Realtime PCR. Cell cycle was detected by flow cytometry. Cell viability was evaluated by MTT assay. Cell spheroid formation was determined by colony assay. Wnt signaling pathway activity was evaluated by TOP/FOP ratio. Protein expression was tested using Western blot. β-catenin binding ability was detected by ChIP assay. miRNA target gene was confirmed by luciferase assay.

RESULTS: miR-590-3p was found to be overexpressed in both glioma tissues and cell lines. miR-590-3p is upregulated in colon cancer cells and tissues compared to non-tumorigenic colon cells and normal colon tissues. miR-590-3p positively regulated cell proliferation, spheroid formation, and cell cycle in LS174T cells. Conversely, inhibition of miR-590-3p reduced these effects. We confirmed that WIF1 and DKK1 are targets of miR-590-3p. Overexpression of miR-590-3p promoted TOP flash luciferase activity, enhanced nuclear β-catenin levels and increased target genes expression of Wnt signaling pathway. The results indicated that miR-590-3p activates the Wnt/β-catenin signaling pathway.

CONCLUSIONS: We demonstrate that miR-590-3p regulates colon cancer progression via WIF1 and DKK1, which suggests that miR-590-3p may be a promising candidate for therapeutic applications in colon cancer treatment.

Key Words:
miR-590-3p, Colon cancer, WIF1, DKK1, Wnt signaling pathway.

Introduction

Colon cancer is one of the most common types of cancer and the leading cause of cancer-associated mortality in Western countries[1-3]. In China, the incidence is lower while is a substantial burden[4]. The molecular mechanism underlying the development of cancer remains unclear. Therefore, limited therapeutic option is currently existed[5]. miRNAs are a family of small non-coding RNAs of 18-27 nucleotides in length[6]. Generally, miRNAs negatively regulate gene expression via binding to the 3'-untranslated region (3'-UTR) of their target double-stranded mRNA, resulting in degradation of the mRNA upon dicer complex[7]. Recently, miRNAs were proved to participate in various physiopathologic proceeding[8-10]. Abnormal expression of certain miRNAs has been found in various types of solid tumor, including colon cancer[11]. For instance, miRNA-21 has recently been found to be exhibited as a novel biomarker in colon cancer, with the potential to be used as a diagnostic and therapeutic target[12]. Also, several miRNAs have been confirmed to regulate colon cell growth, migration, and invasion, such as miR-32, -224, and -203[13,14].

The miRNA 590-3p (miR-590-3p) was previously found to act as an important tumorigenic factor for hepatocellular carcinoma[15]. This miRNA was also reported to play an inhibitory role in glioblastoma on cell migration, invasion, and epithelial-mesenchymal transition by targeting ZEB1 and ZEB2[16]. miR-590-3p was also indicated to be associated with the osteogenic differentiation of human mesenchymal stem cells by regulating *APC* gene[17]. In addition, it was suggested that miR-590-3p may regulate the p50 subunit of Nuclear Factor Kappa-B and provide a novel strategy for the treatment of myocarditis[18].

In the current study, we reported that miR-590-3p is up-regulated in colon cancer tissues and cell lines compared to normal colon tissue and normal human colon cell line. miR-590-3p significantly promoted colon cancer cell growth and

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proliferation *in vitro*. We further found that Wnt inhibitory factor 1 (WIF1) and Dickkopf-related protein 1 (DKK1) are direct targets of miR-590-3p. The results indicated that miR-590-3p may be a potential therapeutic target for colon cancer treatment in clinic.

Materials and Methods

Tissue Samples and RNA Extraction

A total of 10 colon cancer tissue samples were collected. Total RNA extraction was performed using the RNA extraction kit from Takara (TaKaRa Bio, Otsu, Shiga, Japan). This study was approved by the Institutional Ethics Board at Department of Gastrointestinal Surgery, the Third Affiliated Hospital of Guangzhou Medical University.

Plasmids, Virus Production and Infection of Target Cells

The different regions of the human miR-590-3p promoter were generated by PCR amplification from LS174T cells and cloned into the KpnI/HindIII sites of the pGL3-basic-luciferase reporter plasmid, respectively (Promega, Madison, WI, USA). The full length of WIF1-3'-UTR region and DKK1-3'-UTR were generated by PCR amplification from DNA of LS174T cells and subcloned into pEGFP-C3 and a modified pGL3-control-luciferase vector. To silence endogenous WIF1 or DKK1 expression, RNAi oligonucleotides were synthesized by Invitrogen (Carlsbad, CA, USA) and cloned into the pSuper-retro puro plasmid (Oligoengine, Seattle, WA, USA). The miR-590-3p mimic and miR-590-3p inhibitor were purchased from RiboBio (RiboBio Co., Ltd., Guangzhou, Guangdong, China). Transient transfection was performed using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

Cell Culture and Generation of Transfected Cell Lines

The CCD18Co, Lovo, HCT116, RKO, HT29, LS174T, and SW480 cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA).The cells were cultured in DMEM culture medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone). Cells were grown in a humidified atmosphere of 5% CO_2 at 37°C. The sequence of miR-590-3p mimics, miR-590-3p inhibitor, siRNA-WIF1, and siRNA-DKK1 were cloned into pMSCV-puromycin plasmid. Then, the plasmids were transfected into 293T cells that were used as virus-generating host cells by calcium phosphate precipitation. For transfection, the cells were incubated at 37°C for 6 h, the media were changed, and the cells were incubated overnight. To produce new viruses, the media were collected three times a day until 293T cells achieved total confluence. Media containing viruses were adopted to infect LS174T cells. Twenty-four hours after the addition of viruses, the infected cells were selected by adding puromycin to the growth medium. Stable cell lines were verified by qRT-PCR.

MTT Assay

The LS174T cells were seeded into a 96-well plate at 2×10^3cells/well for cultivation. Cells were then transfected with miR-590-3p mimics or miR-590-3p inhibitor as described above for 24 h. During the last 4 h of each day of culture, cells were treated with MTT (50 mg per well, Sigma-Aldrich, St. Louis, MO, USA). Dimethyl sulfoxide was used to dissolve the formazan, and the absorbance was measured at 450 nm using an ELISA plate reader (Bio-Tek Instruments, Winooski, VT, USA).

Spheroid Formation Assay

LS174T cells were seeded into 6-well Ultra Low Cluster plate (Corning, NY, USA) at 500 cells/well and were cultured in suspension in serum-free DMEM-F12 (BioWhittaker, Walkersville, MD, USA), supplemented with B27 (1:50, Invitrogen, Carlsbad, CA, USA), 20 ng/ml EGF (BD Biosciences, Franklin Lakes, NJ, USA), 0.4% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA), and 4 mg/ml insulin (Sigma-Aldrich, St. Louis, MO, USA) for 10-12 days. After 10-12 days, the number of LS174T cell spheres (tight, spherical, non-adherent masses > 50 μm in diameter) were counted, and images of the spheres were scored under an inverse microscope. Sphere formation efficiency = colonies/input cells × 100%.

Cell Cycle

LS174T cells were collected and washed with PBS for twice. The cells were added to 1 ml 70% precooled ethanol and incubated at 4°C overnight. Then, the cells were washed with PBS and treated with 100 mg/L RNase at 37°C for 30 min. After stained by 50 mg/L PI at 4°C avoid

of light for 30 min, the cells were detected by flow cytometry with the excitation wavelength at 488 nm. The primary result was analyzed by cell cycle matching software to record hypodiploid peak, namely sub-G1 phase, G0/G1 phase, S phase, and G2/M phase. All the experiments were repeated for three times.

Luciferase Assay

miRNAs and luciferase reporter plasmids were co-transfected into LS174T cells in 6-well plates and cultured for 48 h before the cells were collected and lysed for luminescence detection. The following procedure and detection were performed using a luciferase assay kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. Renilla luciferase was activated to emitting the primary luminescence, and firefly luminescence was selected for normalization. Each experiment was repeated in triplicate.

TOP/FOP Detection

TOPglow/FOPglow TCF reporter kit (Millipore, Billerica, MA, USA) was applied for detection of Wnt signaling pathway activity. LS174T cells were seeded in 6-well plates and transfected with TOPglow and FOPglow according to the manual. All transfections were performed in triplicates and repeated at least 3 times.

ChIP

ChIP assays were performed as described previously[19]. Briefly, LS174T cells were cross-linked for 10 minutes with 1% formaldehyde and quenched with 125 mM glycine. After nuclei were isolated by centrifugation, the pellet was re-suspended in lysis buffer containing 0.1% sodium dodecyl sulfate (SDS) and sonicated to achieve fragment sizes of 200 to 600 bp. The assay was conducted with ChIP-grade protein G magnetic beads using an antibody against β-catenin. IgG protein was used as the negative control. To validate the enrichment, qRT-PCR was performed with tiled primers.

Real-time PCR

TRIzol reagent (Invitrogen) was used to extract total RNA according to the manufacturer's instructions. RNA was eluted in 50 mL of RNase-free water (Promega, Madison, WI, USA) and stored at -70°C. To analyze gene expression, the qRT-PCR mixture system containing cDNA templates, primers, and SYBRGreen qPCR Master Mix were subjected to qRT-PCR according to standard methods. Fold changes in gene expression were calculated using $2^{-\Delta\Delta Ct}$ method with 18S rRNA used as an internal control.

Western Blot

LS174T cells were lysed with loading lysis buffer that was diluted with 5× loading lysis buffer (0.5 mol/LTris–HCl 2.5 mL, dithiothreitol 0.39 g, SDS 0.5 g, bromophenol blue 0.025 g, glycerin 2.5 mL). Equal amount of protein was transferred onto the polyvinylidene difluoride (PVDF) membrane. The membrane was then incubated with primary antibodies at 4°C for 1 h. Then the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature. The binding signals were visualized by enhanced chemiluminescence (ECL; Thermo Fisher Scientific, UK).

Statistical Analysis

Statistical analysis was performed with the SPSS software (version 16.0, SPSS Inc., Chicago, IL, USA). Means ± SD was calculated and two-tailed Student's *t*-test or one-way ANOVA was performed using the data analysis tools provided by the software. A p-value of < 0.05 was considered as significant difference.

Results

miR-590-3p was Upregulated in Colon Cancer Tissues and Cell Lines

To explore the expression of miR-590-3p in human colon cancer, 6 colon cancer cell lines were selected. It was found that miR-590-3p levels were increased compared with normal colon cell line CCD18Co ($p < 0.05$, Figure 1A). Next, 10 paired colon cancer and adjacent normal tissues were tested using Real-time PCR. It was revealed that miR-590-3p level in colon cancer tissues was markedly higher than that of adjacent tissues ($p < 0.05$, Figure 1B). Moreover, the expression level of miR-590-3p in colon cancer tissues was gradually increased following grade upregulation, which confirmed its overexpression in colon cancer ($p < 0.05$, Figure 1C).

miR-590-3p Facilitated LS174T Cell Growth and Proliferation

As miR-590-3p was upregulated in colon cancer, we proposed that miR-590-3p participated in promoting the development of colon cancer.

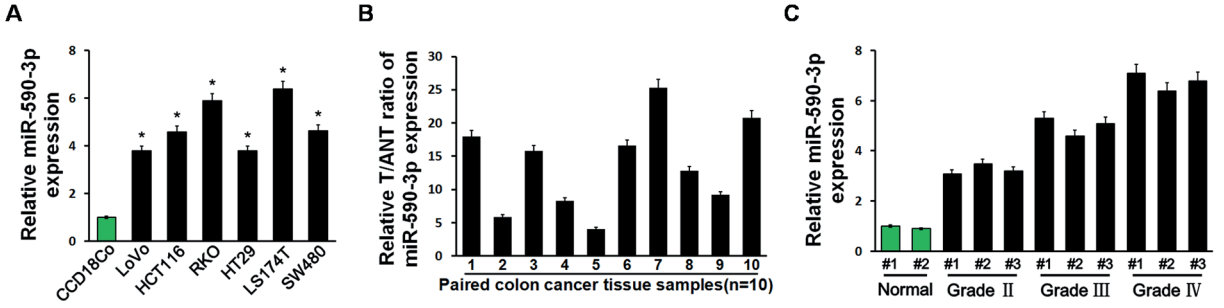

Figure 1. MiR-590-3p overexpressed in colon cancer cells and tissues. ***A,*** MiR-590-3p expression in colon cancer and normal colon cell lines. ***B,*** MiR-590-3p expression in paired colon cancer and adjacent normal tissues. ***C,*** MiR-590-3p expression in colon cancer tissues at different grades. *$p < 0.05$, compared with control.

miR-590-3p expression was regulated by miR-590-3p mimics or inhibitor transfection ($p < 0.05$, Figure 2A). The results of cell cycle detection revealed that miR-590-3p mimics elevated cell proportion in S phase and decreased proportion in G1/G0 phase, while miR-590-3p inhibitor exhibited the opposite effect of miR-590-3p mimics (Figure 2B). Moreover, miR-590-3p mimics promoted LS174T cell viability, whereas miR-590-3p inhibitor markedly declined LS174T cell viability (Figure 2C). At the same time, miR-590-3p mimics significantly facilitated the spheres formation compared with control group, while miR-590-3p inhibitor reduced the spheres formation (Figure 2D). It indicated that miR-590-3p may affect LS174T cell growth and proliferation.

miR-590-3p Activated Wnt Signaling Pathway

Previous studies demonstrated that Wnt signaling pathway is involved in colon cancer cell proliferation and stemness. Therefore we speculated that miR-590-3p may achieve its influence on co-

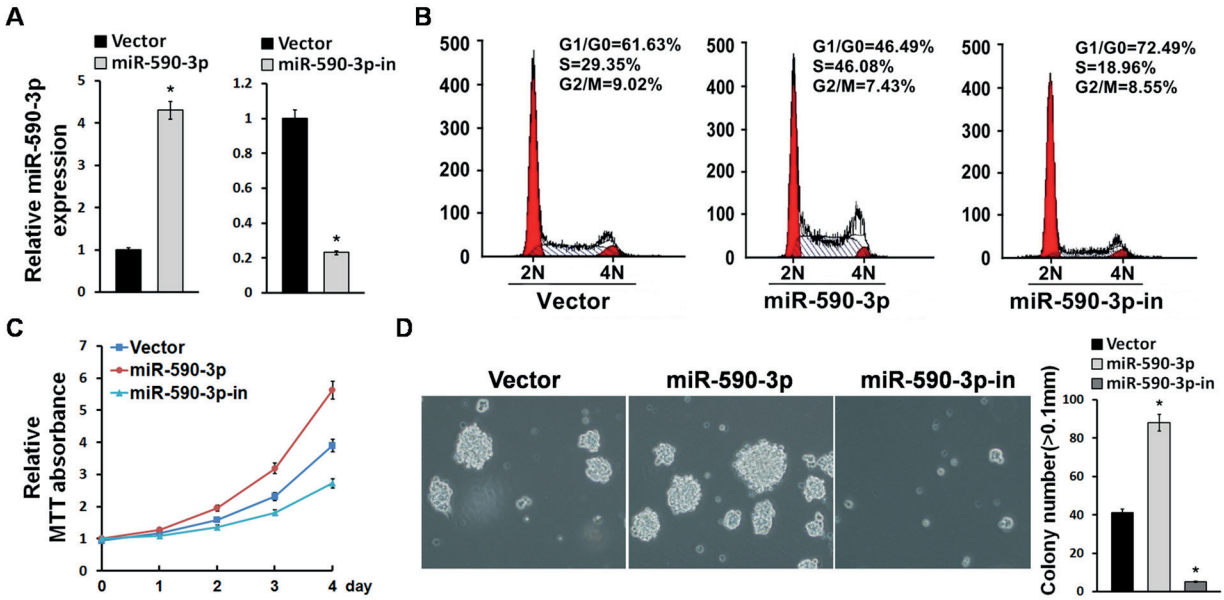

Figure 2. MiR-590-3p facilitated LS174T cell growth and proliferation. ***A,*** MiR-590-3p expression in LS174T cells after miR-590-3pmimics or inhibitor transfection. ***B,*** LS174T cell cycle detected by flow cytometry after miR-590-3p mimics or inhibitor transfection. ***C,*** LS174T cell viability detected by MTT assay after miR-590-3p mimics or inhibitor transfection. ***D,*** LS174T cell tumorigenesis ability tested by spheroid formation assay after miR-590-3p mimics or inhibitor transfection. *$p < 0.05$, compared with control.

lon cancer cells via Wnt signaling pathway. TOP/FOP ratio was significantly increased in LS174T cells after miR-590-3p overexpression, whereas it was declined after miR-590-3p inhibitor transfection ($p < 0.05$, Figure 3A). The influence of miR-590-3p on β-catenin nuclear translocation was tested. After extracted from cytoplasm and nucleus, the protein was detected by Western blot. It was shown that β-catenin level in nucleus was markedly increased in miR-590-3p mimics group ($p < 0.05$, Figure 3B). Furthermore, a variety of target genes were increased by miR-590-3p mimics and was downregulated by miR-590-3p inhibitor ($p < 0.05$, Figure 3C). Meanwhile, stemness-related genes, such as OCT4, SOX2, and TCF4, were found to be upregulated in LS174T cells after miR-590-3p transfection ($p < 0.05$, Figure 3D). In addition, ChIP assay was performed to evaluate activation of Wnt signaling pathway. It was found that miR-495-3p overexpression facilitated nuclear β-catenin binding with the promoter of p300 and Nanog, enhancing gene transcription and mediating colon cancer cell proliferation ($p < 0.05$, Figure 3E).

miR-590-3p Targeted WIF1 and DKK1 in Spheroid Formation

We searched the miRNA database and found that miR-590-3p may bind with WIF1 and DKK1 mRNA (Figure 4A). Luciferase assay demonstrated that miR-590-3p markedly reduced the luciferase activity of WIF1 3'-UTR and DKK1 3'-UTR, whereas exhibited no significant impact on WIF1 3'-UTR or DKK1 3'-UTR mutation (Figure 4B). On the other hand, the knockdown of WIF1 or DKK1 enhanced spheroid formation ability of LS174T cells which was decreased by miR-590-3p inhibitor ($p < 0.05$, Figure 4C).

Discussion

Identification of cancer-specific miRNAs is important to understand the role in tumorigenesis and exploring novel therapeutic targets. Previous studies have revealed that miR-590-3p plays a role in several types of physiopathologic process, including mesenchymal stem cells differentiation, mitochondrial dysfunction, and

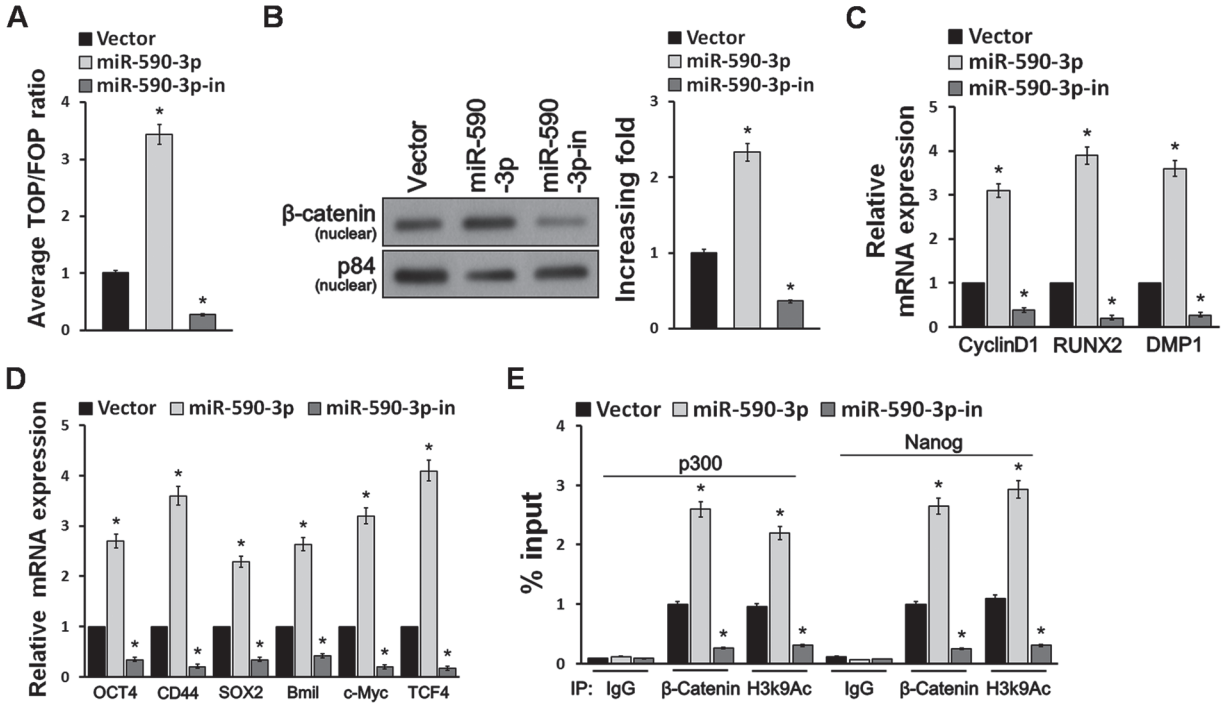

Figure 3. MiR-590-3p promoted Wnt signaling pathway. ***A,*** TOP/FOP ratio determination of Wnt signaling pathway activity in miR-590-3p mimics or inhibitor transfected LS174T cells. ***B,*** Western blot detection of β-catenin protein nuclear expression in miR-590-3p mimics or inhibitor transfected LS174T cells. ***C,*** Real time PCR detection of cyclin D1, RUNX2, and DMP1 mRNA expressions in miR-590-3p mimics or inhibitor transfected LS174T cells. ***D,*** Cell stemness associated mRNA expressions determined by real time PCR in miR-590-3p mimics or inhibitor transfected LS174T cells. ***E,*** ChIP detection of p300 and Nanog in miR-590-3p mimics or inhibitor transfected LS174T cells. *$p < 0.05$, compared with control.

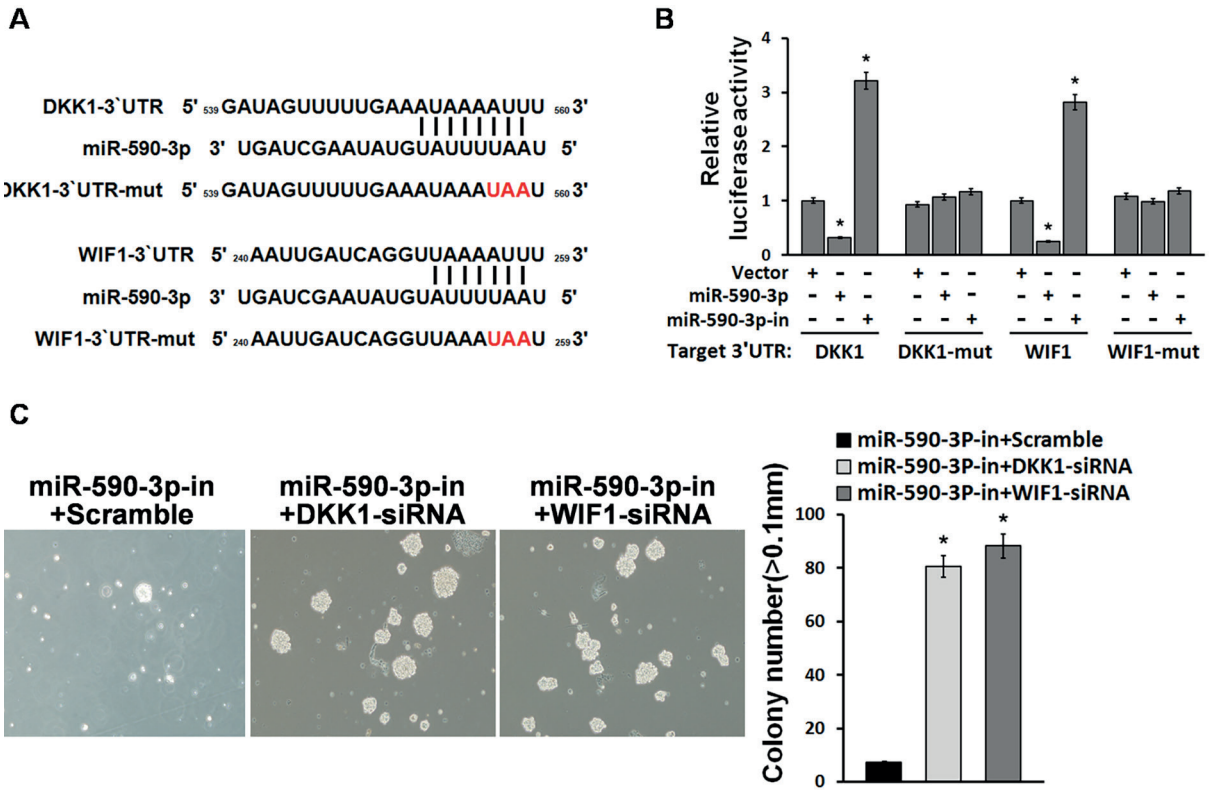

Figure 4. MiR-590-3p targeted WIF1 and DKK1 in spheroid formation. ***A,*** Predicted miR-590-3p mimics or inhibitor transfected LS174T cells target sequence in the 3' UTR of WIF1 and DKK1. ***B,*** Dual-luciferase reporter assay of the LS174T cells transfected with the WIF1-3' UTR or DKK1-3' UTR reporter and miR-590-3pmimics or inhibitor. ***C,*** HCT-116 cell tumorigenesis ability tested by spheroid formation assay after WIF1 or DKK1 knockdown together with miR-590-3p inhibitor transfection. *$p < 0.05$, compared with control.

hepatocellular carcinoma[15,17,20]. However, the function of miR-590-3p in colon cancer biology remains unclear.

By comparing colon cancer cell lines and normal colon cell, we found that miR-590-3p expression was significantly higher in colon cancer cells than in normal colon cell. Moreover, it was also revealed that miR-590-3p was upregulated in colon cancer tissues compared with adjacent normal tissue, and elevated following upgrading. Thus, we proposed that miR-590-3p may participate in tumorigenesis of human colon cancer. We further evaluated the effect of miR-590-3p overexpression and miR-590-3p inhibition on colon cancer cells using flow cytometry, MTT assay, and spheroid formation assay. The results showed that miR-590-3p overexpression promoted cell cycle, proliferation, and spheroid formation. Conversely, miR-590-3p inhibition led to cell cycle blockage, proliferation inhibition, and spheroid formation reduction. These results provided evidence to support the hypothesis that miR-590-3p regulates cell proliferation in colon cancer cells.

We further searched miRNA databases to identify potential targets of miR-590-3p, which we further tested using luciferase assay. The results indicated that miR-590-3p directly restrain the translation of WIF1 and DKK1 mRNA into mature protein. To investigate whether WIF1 and DKK1 regulate colon cancer cell tumorigenesis, WIF1 and DKK1 knockdown and spheroid formation assay were performed *in vitro*. Cell spheroid formation, which was decreased by miR-590-3p inhibition, was increased by WIF1 or DKK1 downregulation. WIF1 is a lipid-binding protein that binds to Wnt proteins and blocks them from triggering the Wnt/β-catenin pathway[21]. Currently, *WIF1* gene was demonstrated to be dysregulated in the peripheral blood of human colon cancer and could be used as a promising marker[22]. Consistent with the previous reports, we found

that WIF1 may be involved in colon cancer cell proliferation and tumorigenesis. DKK1 prevents β-catenin mediated signal transduction via binding to LRP and Kremen proteins, promoting cell differentiation and apoptosis. DKK1 was also found to be downregulated in colon cancer. And multiple molecules contributes to colorectal tumorigenesis via downregulating DKK1[23,24].

Wnt/β-catenin signaling has been proved to participate in the development and promotion of colon cancer[25] and is considered to be important in tumorigenesis[26]. This pathway affects a variety of biological processes, including cell cycle, proliferation, invasion, migration, apoptosis, and angiogenesis[27-29]. Abnormal expression of molecules within Wnt/β-catenin signaling plays important roles in colon cancer development. Wnt/β-catenin signaling can be inhibited by several factors that bind either to the Wnt ligand or the degrading complex, such as WIF1, Frizzled-related proteins (sFRPs), and DKK130. Our study found that WIF1 and DKK1 are negatively regulated by miR-590-3p in colon cancer tissues and cells, resulting in abnormal activation of Wnt signaling. Also, miR-590-3p overexpression facilitated the expression of stem cell-related markers, such as OCT4, SOX2, and TCF4.

Since we found that WIF1 and DKK1 are regulated by miR-590-3p and previous reports have shown that WIF1 and DKK1 are involved in Wnt/β-catenin signaling, we further investigated the effect of miR-590-3p expression on TOP/FOP ratio, nuclear translocation of β-catenin, and target gene activation. We found that miR-590-3p overexpression promoted β-catenin nuclear translocation, elevated TOP/FOP ratio, facilitated the binding of β-catenin with p300 and Nanog promoter. The results indicate miR-590-3p plays an important role in the regulation of Wnt/β-catenin signaling pathway. Multiple downstream genes are transcriptionally activated by Wnt/β-catenin signaling, such as cyclin D1, OCN, ALP, MMP-9, and RUNX2[31-34]. We also found that the expressions of cyclin D1, RUNX2, and DMP1 were enhanced by miR-590-3p overexpression and suppressed by miR-590-3pinhibition.

Conclusions

We found that miR-590-3p is upregulated in colon cancer. This oncogene is a negative modulator of the Wnt/β-catenin signaling regulator, WIF1, and DKK1. Aberrant overexpression of miR-590-3p in colon cancer induces Wnt/β-catenin signaling in colon cancer cells. This abnormal signaling further promotes spheroid formation and cell cycle, leading to colon cell proliferation and tumorigenesis. Our observations provide new insight into colon cancer pathogenesis and a potential choice for colon cancer therapy, as miR-590-3p activity affects central mechanisms in colon cancer progression.

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Conflict of Interest

The authors declare that do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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