

Effects of miR-490-5p targeting CDK1 on proliferation and apoptosis of colon cancer cells *via* ERK signaling pathway

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Abstract. – **OBJECTIVE:** The aim of this study was to investigate the effects of micro ribonucleic acid (miR)-490-5p on the proliferation and apoptosis of colon cancer cells, and to explore its potential mechanism.

PATIENTS AND METHODS: The mRNA expression of miR-490-5p in 30 pairs of colon cancer tissues and adjacent normal tissues was detected *via* reverse transcription-polymerase chain reaction (RT-PCR). Human colon cancer SW480 cell lines were cultured *in vitro* and divided into Control group and miR-490-5p overexpression group (miR-490-5p mimic group). The nonsense sequence and miR-490-5p mimic were transfected using liposome transfection technique into colon cancer cells in control group and miR-490-5p mimic group, respectively. Cell proliferation and apoptosis in each group were then observed. At the same time, the effect of miR-490-5p on the growth of colon cancer *in vivo* was explored using subcutaneous tumorigenesis assay. The protein expressions of extracellular signal-regulated kinase (ERK)1/2 signaling pathway and cyclin-dependent kinase 1 (CDK1) were determined *via* Western blotting. Furthermore, immunohistochemical staining was performed to verify the protein expression of CDK1 *in vivo*.

RESULTS: The expression of miR-490-5p in colon cancer tissues was significantly lower than that in adjacent normal tissues ($p<0.05$). After transfection with miR-490-5p mimic *in vitro*, EdU staining and colony formation assay showed that the proliferation ability of SW480 cells was significantly weakened ($p<0.05$). Meanwhile, the number of colonies in miR-490-5p mimic group was markedly less than that in Control group ($p<0.05$). The results of Western blotting revealed that overexpression of miR-490-5p remarkably up-regulated the Bax/Bcl-2 and C-Caspase3/T-Caspase3 ratios in cancer cells ($p<0.05$). Subsequent results indicated that the subcutaneous tumorigenesis of colon cancer cells was markedly inhibited by overexpression of miR-490-5p ($p<0.05$). According to the results of Western blotting, the activation of ERK signaling pathway and the protein expression of CDK1 were significantly suppressed by overexpression of miR-490-

5p ($p<0.05$). *In vivo* experiments further revealed that the protein expression of CDK1 in colon cancer tissues increased significantly ($p<0.05$).

CONCLUSIONS: MiR-490-5p was found lowly expressed in colon cancer patients. In addition, overexpression of miR-490-5p inhibited the proliferation and promoted the apoptosis of colon cancer cells *via* down-regulating CDK1 both *in vitro* and *in vivo*.

Key Words:

MiR-490-5p, Colon cancer, Proliferation, Apoptosis, CDK1.

Introduction

As one of the most common malignant tumors in the digestive tract, colon cancer is characterized by high morbidity and mortality rates. In Western countries, the morbidity rate of colon cancer is among the top five. Meanwhile, it is the third major cause of cancer-related death in the Western population^{1,2}. The occurrence and development of colon cancer is a complex progressive process involving multi-factors, multi-stages, multi-mechanisms, multi-links and multi-gene changes. However, its early development is relatively slow, and the prognosis is good^{3,4}. Therefore, it is of great significance to explore the occurrence and development mechanisms of colon cancer for early diagnosis and treatment.

Micro ribonucleic acids (miRNAs) are a group of single-stranded non-coding RNAs existing in eukaryotes, with 20-24 nucleotides in length⁵. MiRNAs can regulate the expression of a variety of genes through targeted binding, thus playing important roles in cell proliferation, differentiation, invasion and apoptosis⁶. A large number of clinical and basic studies have confirmed that there are abnormal changes in miRNA expression

Table I. Primer sequences in RT-PCR.

Target gene	Primer sequences	
<i>U6</i>	Forward	5'-CTCGCTTCGGCAGCACA-3'
	Reverse	5'-AACGCTTCACGAATTTGCGT-3'
<i>MiR-490-5p</i>	Forward	5'-ACGTAGTCGGATGTTTCAGTTA-3'
	Reverse	5'-ACGTAGTCGTAGTCGTAGTTGC-3'

profile during tumorigenesis. This can further promote or inhibit the occurrence and development of malignant tumors *via* affecting proliferation, apoptosis, differentiation, invasion and endothelial-mesenchymal transition of cells^{7,8}. MiR-490-5p has been found to have many targets, so its regulatory mechanism in malignancies is complex. For example, miR-490-5p inhibits the proliferation, migration and invasion of liver cancer cells through directly regulating ROBO1⁹. In triple-negative breast cancer, miR-490-5p suppresses the proliferation and invasion of cells, whose mechanism may be related to its inhibition on ETV1¹⁰. Moreover, miR-490-5p inhibits the growth of renal cell carcinoma *via* targeting PIK-3CA¹¹. However, the effect of miR-490-5p on the proliferation and apoptosis of colon cancer cells *in vitro* has not been fully elucidated.

In the present study, the expression of miR-490-5p in 30 pairs of colon cancer tissues and adjacent normal tissues was first detected. The effects of miR-490-5p on the proliferation and apoptosis of human colon cancer SW480 cells were investigated. All our findings might help to provide references for further optimizing the therapeutic regimen of colon cancer patients in the future.

Patients and Methods

Tissue Specimens

Thirty pairs of colon cancer tissues and adjacent normal tissues were collected from colon cancer patients undergoing operation in Colorectal Surgery of our hospital from April 2017 to August 2019. The selection of patients was based on the guideline proposed by the Union for International Cancer Control (UICC). After the blood stains were washed away with normal saline, the specimens were cut into pieces and placed into Eppendorf (EP) tubes and stored in a refrigerator at -80°C for molecular biology experiments, or they were fixed in 10% neutral formalin without being cut into pieces for pathological experiments. This

study was approved by the Medical Ethics Committee of The Second Clinical Medical College of Jinan University. Informed consent was obtained from each subject before the study.

Culture and Transfection of Human Colon Cancer SW480 Cells

Human colon cancer SW480 cells were purchased from Shanghai Gaining Biological Co., Ltd. All cells were cultured in Dulbecco's Modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) in an incubator at 37°C. Cell passage was performed once every 2-3 d. For cell transfection, SW480 cells were first inoculated into 6-well plates and cultured for 36 h. When cell density reached 70-80%, the cells could be transfected. Nonsense sequence and mimic of miR-490-5p were transfected into SW480 cells according to the instructions of LipofectamineTM RNAi MAX (Invitrogen, Carlsbad, CA, USA).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA in colon cancer tissues and adjacent normal tissues was extracted using TRIzol method (Invitrogen, Carlsbad, CA, USA). The concentration and purity of RNA extracted were detected by an ultraviolet spectrophotometer, and qualified RNA with absorbance (A)₂₆₀/A₂₈₀ of 1.8-2.0 could be used. Subsequently, extracted messenger RNA (mRNA) was synthesized into complementary deoxyribonucleic acid (cDNA) through RT and stored in a refrigerator at -80°C. RT-PCR was performed under the system consisting of 2.5 µL of 10 × Buffer, 1 µL of cDNA, 0.5 µL of forward primers (20 µmol/L), 0.5 µL of reverse primers (20 µmol/L), 10 µL of LightCycler[®] 480 SYBR Green I Master (2×), and 5.5 µL of ddH₂O. The amplification system of RT-PCR was the same as above. Primer sequences used in this study were shown in Table I.

Colony Formation Assay

Cells in logarithmic growth phase were digested with 0.25% trypsin and prepared into sin-

gle cell suspension (the proportion of single cells >95%). Subsequently, the suspension was inoculated into 6-well plates at a density of about 500 cells/well. 2 mL of Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) was added into each well. The culture medium was replaced once every 2 d. After 10 d of culture, formed colonies were fixed with formaldehyde and stained with crystal violet. Finally, colonies were observed under a microscope, and the number of colonies was counted.

5-Ethynyl-2'-Deoxyuridine (EdU) Staining

At 48 h after transfection with miR-490-5p mimic, the cells were stained according to the instructions of Click-iT EdU staining kits (Invitrogen, Carlsbad, CA, USA). Next, the cells were photographed under a fluorescence microscope. Finally, EdU-positive cells were counted and quantified in 3 randomly-selected fields of view in each glass slide.

Western Blotting

First, the culture medium was discarded, and the cells were washed with PBS for 3 times. 1000 μ L of lysis buffer was added into every dish and fully vibrated for 10 min. Cells at the bottom of the dish were scraped off using a brush and placed into Eppendorf (EP) tubes, followed by lysis using an ultrasonic lysis instrument for no more than 15 s (1-2 s/time). After standing for 15 min, the cells were centrifuged at 12,000 rpm for 5 min. The supernatant was taken and placed into EP tubes. Protein concentration was detected *via* ultraviolet spectrometry, and all protein samples were adjusted to the same concentration. Next, protein samples were sub-packaged and stored in a refrigerator at -80°C for use. After separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), protein samples were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Then, the membranes were incubated with primary antibodies at 4°C overnight. On the next day, the membranes were incubated again with HRP conjugated goat anti-rabbit secondary antibody in the dark for 1 h at room temperature. Immuno-reactive protein bands were scanned and quantified using an Odyssey scanner (Seattle, WA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference.

Subcutaneous Tumorigenesis Assay in Nude Mice

A total of 20 nude mice were randomly divided into 2 groups, consistent with the cell grouping. Matrigel and PBS were mixed into the mixed solution at 1:1, and cells in the two groups were re-suspended into single cell suspension. After nude mice in both groups were anesthetized with diethyl ether, 10⁷ of cells were inoculated under the soft skin at the back of the right forelimb. After 6 weeks, the mice were sacrificed by cervical dislocation. Finally, the tumor mass was separated and measured. This study was approved by the Animal Ethics Committee of Jinan University Animal Center.

Immunohistochemical Staining

Paraffin sections of human colon cancer tissues and adjacent normal tissues were first deparaffinized in an incubator at 60°C for 30 min, followed by antigen retrieval with citrate buffer under high pressure. Subsequently, the tissues were incubated with 3% hydrogen peroxide for 20 min and blocked with 8% sheep serum for 30 min. Next, the tissues were incubated with CDK1 primary antibody (diluted with PBS at 1:100) in a refrigerator at 4°C overnight. On the next day, after rewarming, the tissues were incubated with secondary antibody B solution at room temperature for 30 min. After washing, diaminobenzidine (DAB) developer (Solarbio, Beijing, China) was added dropwise. The time of color development was strictly controlled under a light microscope. Finally, the sections were counterstained with hematoxylin, dehydrated with gradient ethanol and mounted. The sections were photographed in 10 randomly-selected non-repeated fields under a light microscope (200 \times). The positive rate of protein in each group was analyzed using ImageJ software.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software was used for all statistical analysis. Measurement data were expressed as mean \pm standard deviation. The *t*-test was used for the comparison of data between two groups. *p*<0.05 was considered statistically significant.

Results

Expression of MiR-490-5p in Colon Cancer Tissues and Adjacent Normal Tissues

The expression of miR-490-5p in 30 pairs of colon cancer tissues and adjacent normal tissues

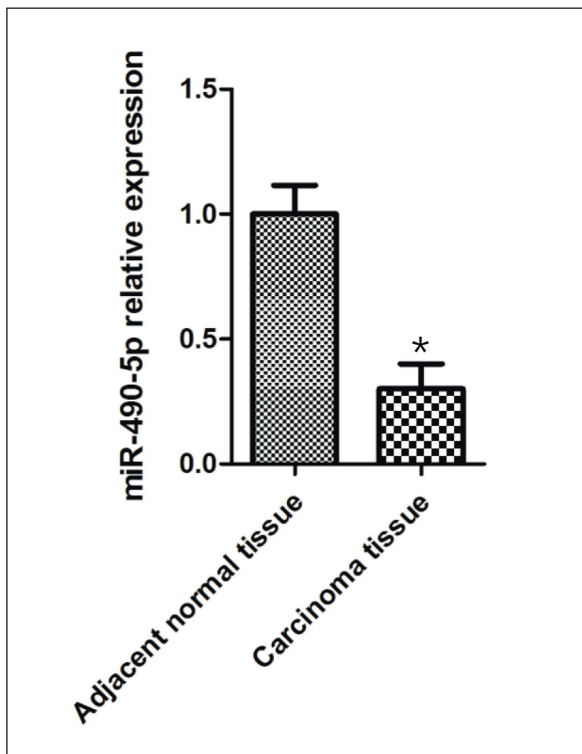


Figure 1. Expression of miR-490-5p in colon cancer tissues and adjacent normal tissues. Adjacent normal tissues: para-carcinoma normal tissues, Carcinoma tissue: colon cancer tissues. * $p < 0.05$: a statistically significant difference vs. para-carcinoma normal tissues.

was detected *via* RT-PCR. As shown in Figure 1, the expression of miR-490-5p in colon cancer tissues was significantly lower than that in adjacent normal tissues (about 0.32 times) ($p < 0.05$).

Effect of MiR-490-5p Overexpression on Proliferation of Human Colon Cancer Cells

EdU staining showed that the proportion of EdU-positive cells in miR-490-5p mimic group was significantly lower than that in Control group ($p < 0.05$) (Figure 2A), indicating that miR-490-5p can inhibit the DNA replication of colon cancer cells.

Effect of MiR-490-5p Overexpression on Colony Formation of Human Colon Cancer Cells

Colony formation assay revealed that the number of colonies in miR-490-5p mimic group was significantly less than that in Control group [(42±6) vs. (178±12)] ($p < 0.05$) (Figure 2B), suggesting that miR-490-5p is able to inhibit the colony formation ability of colon cancer cells.

Effect of MiR-490-5p Overexpression on Apoptosis of Human Colon Cancer Cells

Western blotting results indicated that Bax/Bcl-2 and C-Caspase3/T-Caspase3 ratios rose markedly in miR-490-5p mimic group compared with Control group (Figure 2C). It can be inferred that miR-490-5p is able to promote apoptosis of colon cancer cells.

Effect of MiR-490-5p Overexpression on Extracellular Signal-Regulated Kinase (ERK) Signaling Pathway in Human Colon Cancer Cells

Previous evidence¹² has shown that miR-490-5p exerts an anti-tumor effect through the inhibition on ERK. Therefore, the expressions of P-ERK1/2 and T-ERK1/2 in the two groups were detected. P-ERK1/2/T-ERK1/2 ratio declined markedly in colon cancer cells after overexpression of miR-490-5p ($p < 0.05$). At the same time, the protein expression of CDK1, one of the targets of miR-490-5p, in the upstream of ERK was determined. It was found that the protein expression of CDK1 in colon cancer cells was remarkably inhibited by overexpression of miR-490-5p ($p < 0.05$) (Figure 3).

Effect of MiR-490-5p Overexpression on Subcutaneous Tumorigenesis of Nude Mice

Subcutaneous tumorigenesis assay confirmed that the *in vitro* subcutaneous tumorigenesis of colon cancer cells was markedly inhibited by overexpression of miR-490-5p ($p < 0.05$). The above findings further prove that overexpression of miR-490-5p suppresses the growth of colon cancer cells (Figure 4A).

Immunohistochemical Staining Results of CDK1 in Colon Cancer Tissues

The protein expression of CDK1 was further detected in colon cancer tissues and adjacent normal tissues. The results of immunohistochemical staining manifested that the protein expression of CDK1 in colon cancer tissues was remarkably higher than that in adjacent normal tissues ($p < 0.05$) (Figure 4B). It can be seen that the anti-colon cancer effect of miR-490-5p may be mediated by CDK1-dependent ERK signaling pathway.

Discussion

As a common malignant tumor of the digestive tract, the mortality of colon cancer is

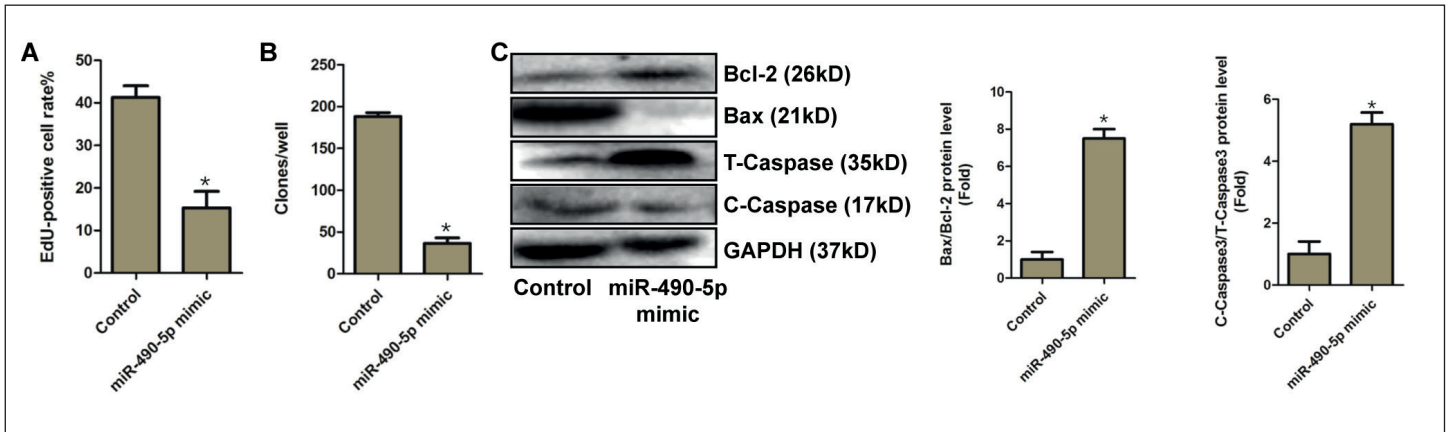


Figure 2. The inhibitory effects of miR-490-5p on the growth of human colon cancer cells. **(A)** Effect of miR-490-5p overexpression on proliferation of human colon cancer cells; **(B)** Effect of miR-490-5p overexpression on colony formation of human colon cancer cells; **(C)** Effect of miR-490-5p overexpression on apoptosis of human colon cancer cells. (Control: Control group, miR-490-5p mimic: miR-490-5p mimic group. * $p < 0.05$: a statistically significant difference vs. Control group).

increasing year by year¹³. The occurrence and development of colon cancer have been found closely related to genetic changes¹⁴. With the development of modern molecular biology and bioinformatics, increasingly more oncogenes and tumor suppressor genes have been confirmed to be involved in the occurrence and development of colon cancer¹³. Currently, the clinical symptoms and diagnostic criteria for colon cancer have been clarified and standardized. However, the mechanisms of biological behaviors of colon cancer cells, including proliferation, migration, apoptosis and angiogen-

esis, remain unclear. Therefore, it is necessary to clarify the abnormal gene regulatory network during the occurrence and development of colon cancer, so as to provide certain basis for targeted gene therapy or drug therapy in the future.

MiRNAs are a class of endogenous small non-coding RNAs with 20-24 nucleotides in length. They can regulate the expression of a variety of genes at the post-transcriptional level¹⁵. In terms of the mechanism, miRNAs can bind to the 3'UTR of target genes to inhibit the translation of corresponding proteins, thereby

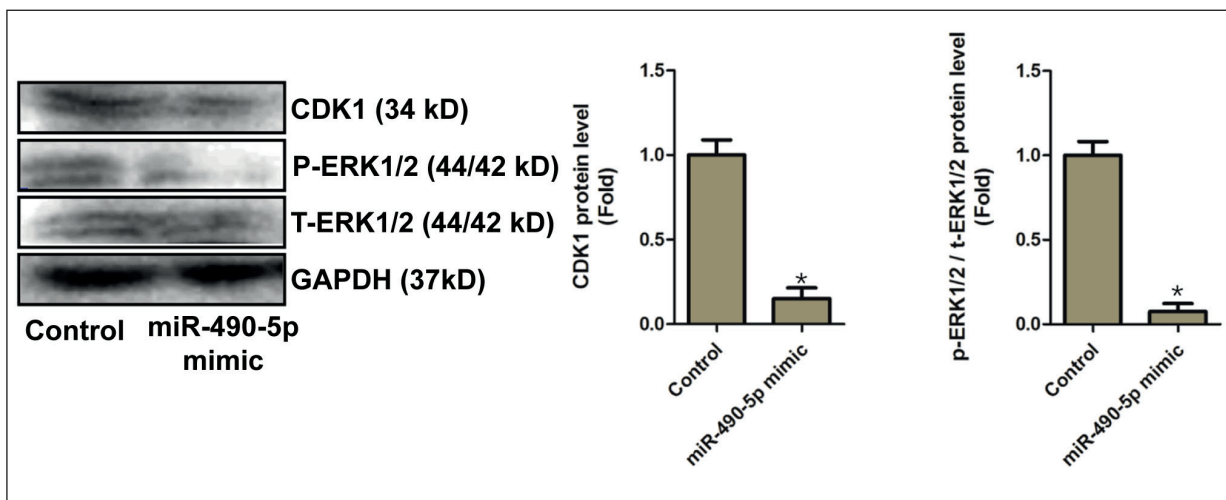


Figure 3. Effect of miR-490-5p overexpression on ERK signaling pathway in human colon cancer cells. Control: Control group, miR-490-5p mimic: miR-490-5p mimic group. * $p < 0.05$: a statistically significant difference vs. Control group.

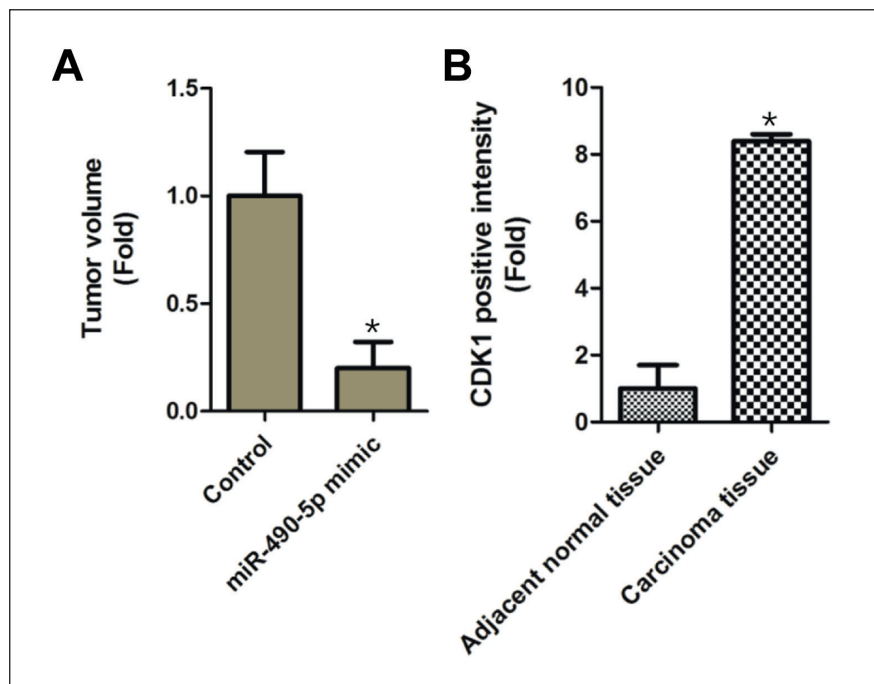


Figure 4. The *in vivo* effects of miR-490-5p. (A) Effect of miR-490-5p overexpression on subcutaneous tumorigenesis of nude mice (Control: Control group, miR-490-5p mimic: miR-490-5p mimic group. * $p < 0.05$: a statistically significant difference vs. Control group); (B) Immunohistochemical quantitative results of CDK1 in colon cancer and the adjacent normal tissues (Adjacent normal tissues: para-carcinoma normal tissues, Carcinoma tissues: colon cancer tissues. * $p < 0.05$: a statistically significant difference vs. para-carcinoma normal tissues).

playing important roles in various life activities of cells¹⁶. Stenvang et al¹⁷ have revealed that the abnormal expression of nucleases Drosha and Dicer and 3'-5' exonuclease can enhance the tumor susceptibility in the process of miRNA generation. During the occurrence and development of colon cancer, a variety of miRNAs have been proved to be involved in cell growth. For example, miR-34a can induce the senescence of human colon cancer cells *via* regulating the E2F pathway, ultimately exerting an anti-tumor effect¹⁸. In colon adenoma and colon epithelial tumors, the transcriptional level of miR-92a increases significantly¹⁹. The possible underlying mechanism is that miR-92a can directly interact with the anti-apoptotic gene Bcl-2 to inhibit cell apoptosis. Moreover, down-regulation of miR-1, jointly with MACC1²⁰, promotes epithelial-mesenchymal transition of human colon cancer. In this study, it was found that the transcriptional level of miR-490-5p in colon cancer tissues declined remarkably. Overexpression of miR-490-5p remarkably inhibited proliferation and promoted apoptosis of colon cancer cells. All these findings indicate that miR-490-5p possesses an anti-colon cancer effect.

Extracellular signals are transmitted into cells under the cooperation of a large number of interacting proteins. In the past few decades, the mitogen-activated protein kinase (MAPK) signaling cascade has attracted increasing attention. In mammals, there are dozens of MAPK enzymes that coordinately regulate cell proliferation, differentiation, motility and survival. Currently, the most widely-studied members of the MAPK family include ERK1/2, JNK1-3, P38 (α , β and γ) and ERK5. In addition, there are some atypical MAPK family members, including ERK3/4, ERK7/8 and Nemo-like kinase^{21,22}. Among them, ERK/MAPK is a critical player in the proliferation, apoptosis, migration and endothelial-mesenchymal transition of colon cancer cells. As a highly conserved anti-apoptotic protein, Bcl-2 initiates anti-apoptotic response through ERK1/2-mediated pathways. Therefore, targeted inhibition on the ERK1/2 signaling pathway can effectively promote tumor cell apoptosis²³. CDK1 is a cell cycle regulatory protein that plays an important regulatory role in tumor cell cycle. Previously, Chen et al²⁴ have found that miR-490-5p targeting CDK1 participates in regulating gastric cancer cell

cycle by inhibiting the ERK1/2 signaling pathway. In this study, the results revealed that the expression of CDK1 rose significantly in colon cancer tissues. After *in vitro* overexpression of miR-490-5p, the expression of CDK1 and the activation of ERK1/2 signaling pathway were significantly suppressed. It can be seen that the anti-proliferation and pro-apoptosis effects of miR-490-5p on colon cancer cells may be related to the CDK1/ERK1/2 axis.

Conclusions

MiR-490-5p was lowly expressed in colon cancer tissues. Overexpression of miR-490-5p exerted an anti-tumor effect *via* targeted inhibition on the CDK1/ERK1/2 axis.

Conflict of Interest

The authors declare that they have no conflict of interest.

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