

## ORIGINAL ARTICLE

# The role of miR-141/ Sirt1 in colon cancer

Yongliang Li, Feiteng Gu, Xi Lin

Second Department of Gastrointestinal Surgery, The Affiliated Hospital of Putian University, Putian, China.

## Summary

**Purpose:** To explore the effects of micro ribonucleic acid-141 (miR)-141 on the proliferation and apoptosis of colon cancer cells and its association with the sirtuin 1 (Sirt1) expression.

**Methods:** The samples of stage I, II, III and IV colon cancer were obtained, and the miRNA expression levels was analyzed, with normal colon tissues as controls. The expression of miR-141 and miR-34 was detected via quantitative reverse transcription-polymerase chain reaction (qRT-PCR), and the cell proliferation and apoptosis in each group were detected via cell counting kit-8 (CCK8) assay, respectively. Finally, the protein expressions of Sirt1, Caspase-3 and Caspase-8 were determined using Western blotting.

**Results:** The expressions of miR-141 and miR-34 (miR-34 is mentioned in previous methods. Furthermore, we found the expression of miR-141 increasing with the progression of colon cancer, which was higher in stage III than in stage

I-II and also higher in stage IV than in stage III. miR-34 was also highly expressed in stage IV colon cancer in our study were up-regulated in the progression of colon cancer. Overexpression of miR-141 could promote cell proliferation ( $p<0.05$ ) and inhibit apoptosis ( $p<0.05$ ), while inhibition on miR-141 expression could significantly weaken cell proliferation ( $p<0.05$ ) and promote apoptosis ( $p<0.05$ ). The results of luciferase reporter assay showed that miR-141 obviously inhibited Sirt1 ( $p<0.05$ ). SRT2183 reduced cell proliferation ( $p<0.05$ ) but up-regulated the protein expressions of Sirt1, Caspase-3 and Caspase-8 ( $p<0.05$ ), while EX 527 had the opposite effects ( $p<0.05$ ).

**Conclusions:** MiR-141 may promote proliferation and reduce apoptosis of colon cancer cells via targeting Sirt1.

**Key words:** colon cancer cells, miR-141, Sirt1, proliferation, apoptosis

## Introduction

Colon cancer is one of the most common cancers diagnosed [1-4], and it is also the third most common type of cancer [5-7], as well as the fourth most common cause of cancer-related death in the world [8]. The prognosis of colon cancer depends on the tumor stage, and its 5-year overall survival is 93% in stage I patients and 8% in stage IV patients [9]. Despite many efforts in the past few decades, the survival rate of colon cancer patients has not been significantly improved [10].

Micro ribonucleic acids (miRs) play regulatory roles in cell differentiation, cycle and apoptosis, which have become a research hotspot [11]. MiRs

are endogenous small non-coding RNAs with 18-25 nucleotides in length, and they can inhibit protein translation through binding to target mRNAs. According to bioinformatics and cloning research, it is estimated that miRs can regulate 30% of all human genes, and each miR can control hundreds of target genes [12,13]. Moreover, miRs exert important regulatory effects on the basic physiological processes, cell differentiation, proliferation and apoptosis [14,15]. Studies have found that the changes in the miR expression profile are observed in a variety of human tumors, including colon cancer. Functional studies have demonstrated that miRs

can serve either as tumor suppressors or oncogenes [16]. In this study, the mechanism of action of miR-141 in regulating the proliferation and apoptosis of colon cancer cells was explored, so as to better understand the pathogenesis of miR-141 and sirtuin 1 (Sirt1) in colon cancer.

## Methods

### Main materials

This study was approved by the Ethics Committee of The Affiliated Hospital of Putian University. Signed informed consents were obtained from all participants before the study entry. The following material has been used in this study: Stage I, II, III and IV colon cancer samples, human colorectal adenocarcinoma HT29 cells (American Type Culture Collection [ATCC], Manassas, VA, USA), Roswell Park Memorial Institute (RPMI)-1640 medium,  $\alpha$ -minimum essential medium (MEM), fetal bovine serum (FBS), trypsin, ethylene diamine tetraacetic acid (EDTA), and phosphate buffered solution (PBS) (Gibco, Rockville, MD, USA), SRT2183 and EX 527 (MCE, Monmouth Junction, NJ, USA), SYBR Green reverse transcription (RT) Master Mix kit (TaKaRa, Tokyo, Japan), Lipofectamine 2000 and TRIzol (Invitrogen, Carlsbad, CA, USA), Sirt1, Caspase-3, Caspase-8 and  $\beta$ -actin antibodies (Abcam, Cambridge, MA, USA), miRNA RT kit (Applied Biosystems, Foster City, CA, USA), luciferase reporter gene assay system (Promega, Madison, WI, USA), bioluminescent plate reader (Modulus™), and 0.22  $\mu$ m pinhole filter (Millipore, Billerica, MA, USA).

### Cell culture

After resuscitation, HT29 cells were cultured with RPMI-1640 complete medium containing 10% FBS in an incubator with 5% CO<sub>2</sub> at 37°C, followed by passage when the confluence reached about 90%. After the original medium was discarded, the cells were washed twice with PBS, and digested with an appropriate amount of 2.5% trypsin containing 0.02% EDTA in the incubator at 37°C for 2-3 min. After an appropriate amount of complete medium was added to terminate the digestion, the cells were carefully blown into single cells, followed by subculture at 1:3. Besides, SRT2183 or EX 527 was dissolved in dimethyl sulfoxide (DMSO) into mother solution (Sigma-Aldrich, St. Louis, MO, USA), respectively. Then, an appropriate amount of SRT2183 or EX 527 was dissolved in complete medium at a final concentration of 2  $\mu$ M or 10  $\mu$ M, and used to culture cells for 24 h or 48 h.

### Cell transfection

The well-growing cells were inoculated into 24-well plates (2×10<sup>4</sup> cells/mL), and transfected with miR-141 inhibitor, miR-141 mimics and miR-negative control (NC) when the confluence reached 90%: 4 portions of 250  $\mu$ L  $\alpha$ -MEM was added and mixed evenly with 5  $\mu$ L of miR-141 inhibitor, miR-141 mimics or miR-NC at a concentration of 20  $\mu$ M, followed by incubation at room temperature for 5 min. Then, the mixed transfection

solution was added into the above 24-well plates and shaken evenly, followed by incubation in the incubator with 5% CO<sub>2</sub> at 37°C. After 48 h, the miR-141 expression level in each group was determined.

### Total RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

The colon cancer tissues or normal colon tissues were ground in liquid nitrogen, or the above cultured cells were added and mixed evenly with an appropriate amount of TRIzol, placed at room temperature for 5 min, added with 200  $\mu$ L of chloroform for a while, followed by centrifugation at 12000 rpm and 4°C for 10 min. The supernatant was separated, added with an equal amount of isopropanol, and placed at room temperature for 10 min, followed by centrifugation at 12000 rpm and 4°C for 15 min. After the supernatant was discarded, the samples were washed twice with freshly-prepared 75% diethyl pyrocarbonate (DEPC)-treated ethanol (Beyotime, Shanghai, China), dried and dissolved with an appropriate amount of DEPC-treated water, and the concentration was determined using a NanoDrop spectrophotometer. The RNA was extracted from tissue samples for transcriptome sequencing. The primers were designed online using the Primer Design Tool and synthesized by Sangon (Shanghai), and the primer sequences of miR-141, miR-34 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown in Table 1. The first-strand complementary deoxyribonucleic acid (cDNA) of miR-141 and miR-34 was synthesized using the qScript microRNA cDNA kit, and the total RNA was synthesized into cDNA using the random primers in RT Master Mix kit for GAPDH. Then qRT-PCR was performed using SYBR Green Real-Time PCR Master Mix and ABI 7500 sequence detection system according to the manufacturer's instructions, and the transcriptional level was assessed using the cycle threshold. The expressions of target genes normalized into endogenous reference were determined using the 2<sup>- $\Delta\Delta$ Ct</sup> method.

### Luciferase reporter assay

At 48 h after transfection of HT29 cells in each group, the medium was discarded, and the cells were washed with phosphate buffered saline (PBS) for 3 times, and lysed with 50  $\mu$ L of freshly-prepared RIPA lysis buffer in each well for 30 min. Then, 10  $\mu$ L of lysate were taken and added with 100  $\mu$ L of luciferase

**Table 1.** Primer sequences of miR-141, miR-34 and GAPDH

Gene	Sequence
miR-141 F	5'-GGTAGAAATGGTCTGTCAAAAT-3'
miR-141 R	5'-CACAGCTCGTAGAACAGGAGG-3'
miR-34 F	5'-TGGCAGTGTCTTAGCTGGTTGT-3'
miR-34 R	5'-CACAGCTCGTAGAACAGGAGG-3'
GAPDH F	5'-GTCAAGGCTGAGAACGGGAA-3'
GAPDH R	5'-AAATGAGCCCCAGCCTTCTC-3'

assay reagent prepared in advance. The luciferase activity was measured using the luciferase reporter gene assay system and bioluminescent plate reader. Each experiment was repeated 3 times independently, with 3 parallel controls.

#### One-step terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

After culture in each group, the cells were washed twice with PBS, fixed with 4% paraformaldehyde for 15 min, washed with PBS 3 times, permeabilized with 0.1% Triton X-100 for 2 min, and washed again with PBS 3 times. Then, 50  $\mu$ L of TUNEL assay buffer was added for incubation at room temperature for 1 h, and the cells were washed with PBS 3 times, added with an appropriate amount of anti-fade solution and sealed. Finally, the cells were observed and photographed under a fluorescence microscope.

#### Cell counting kit-8 (CCK8) assay

The cells in good growth status were inoculated into a 96-well plate ( $4 \times 10^5$  cells/mL), with 6 replicates in each group, and cultured in the incubator with 5% CO<sub>2</sub> at 37°C. After adherence, the cells were cultured with different media, including RPMI-1640 medium and MEM for 24 h or 48 h. Then, the RPMI-1640 medium or MEM was discarded, and 100  $\mu$ L of freshly-prepared complete medium containing 10  $\mu$ L of CCK8 assay buffer was added in each well for incubation in the incubator with 5% CO<sub>2</sub> at 37°C for 4 h. Finally, the optical density

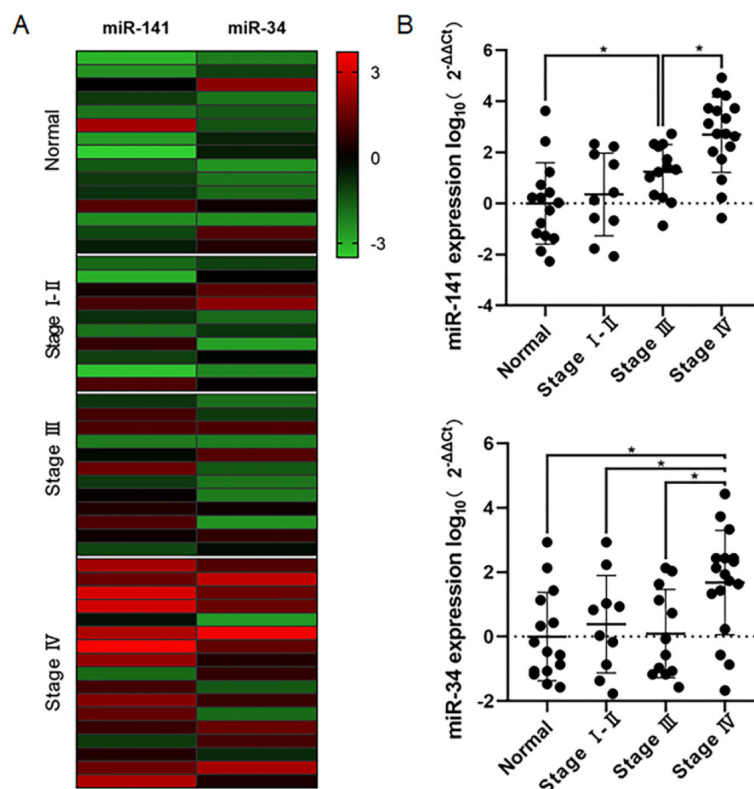
(OD) was measured at 450 nm using a microplate reader. The experiment was repeated 3 times, and the average was taken as the final result.

#### Western blotting

After discarding the RPMI-1640 medium and MEM the cells were lysed with an appropriate amount of RIPA lysis buffer at 4°C overnight. The total protein was extracted *via* centrifugation at 13,000 rpm, and the protein concentration was determined using the bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). After separation *via* 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis, the protein was transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA), sealed with 5% skim milk powder and 0.1% Tween-20 in Tris-buffered saline, and incubated with Sirt1, Caspase-3, Caspase-8 and  $\beta$ -actin primary antibodies at 4°C overnight. Then the protein was incubated again with horse radish peroxidase (HRP)-labeled secondary antibodies, followed by exposure with electrochemiluminescence (ECL) reagent.

#### Statistics

SPSS 22.0 software (IBM, Armonk, NY, USA) was used for data analyses. The data in each group were expressed as mean  $\pm$  standard deviation, and independent-samples *t*-test was used for the intergroup comparison. Survival curves were plotted according to Kaplan-Meier method and compared with log-rank test.  $P < 0.05$  suggested that the difference was statistically significant.



**Figure 1.** Expressions of miR-141 and miR-34 in normal tissues and stage I, II, III and IV colon cancer tissues. **A:** Heat map analysis of miR-141 and miR-34 expressions in different tissues. **B:** Relative quantitative analysis showing that the expression of miR-141 and miR-34 in normal tissues were significantly lower than those in colon cancer tissues (\* $p < 0.05$ ).

## Results

### MiR expressions in colon cancer tissues at different stages

According to the heat map analysis, there were differences in the expressions of miR-141 and miR-34 in colon cancer tissues at different stages (Figure 1A). The expressions of miR-141 and miR-34 in normal tissues were lower than those in colon cancer tissues ( $p < 0.05$ ) (Figure 1B). With the increase of stage of colon cancer, the expression of miR-141 gradually rose, which was significantly higher at stage III than that at stage I-II ( $p < 0.05$ ) and also remarkably higher at stage IV than at stage III ( $p < 0.05$ ). Besides, the expression of miR-34 was obviously up-regulated at stage IV ( $p < 0.05$ ) (Figure 1B).

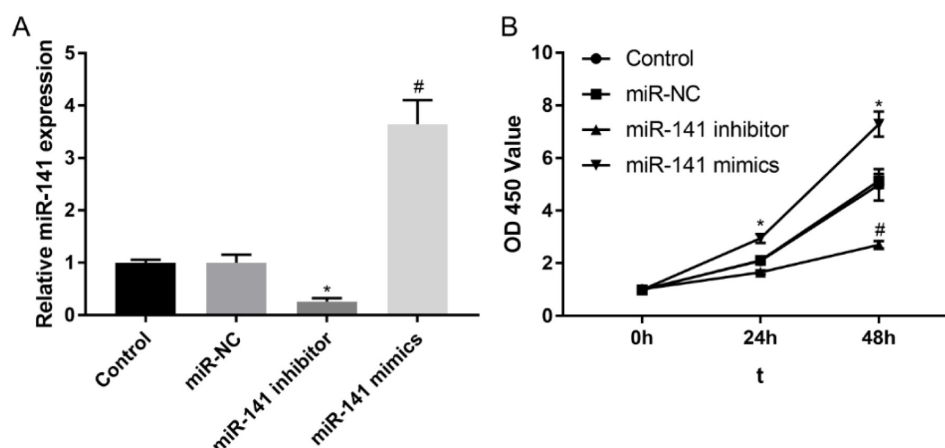
### Effect of miR-141 on the proliferation of HT29 cells

The results of qRT-PCR revealed that the expression of miR-141 in HT29 cells was obviously up-regulated after transfection with miR-141 mim-

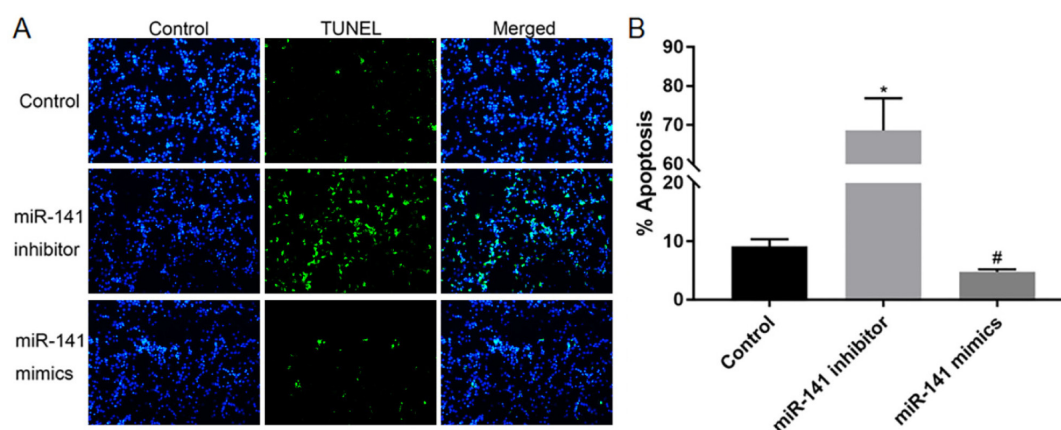
ics ( $p < 0.05$ ), while it was notably down-regulated after transfection with miR-141 inhibitor ( $p < 0.05$ ), but transfection with miR-NC had no significant influence on the expression of miR-141 ( $p > 0.05$ ) (Figure 2A). The results of CCK8 assay showed that the cell proliferation ability was evidently stronger at 24 h and 48 h after transfection with miR-141 mimics than in other groups ( $p < 0.05$ ), while it was evidently weaker at 48 h after transfection with miR-141 inhibitor than in other groups ( $p < 0.05$ ), but no significant influence of transfection with miR-NC on the proliferation ability of HT29 cells was observed ( $p > 0.05$ ) (Figure 2B).

### Effect of miR-141 on the apoptosis of HT29 cells

HT29 cell apoptosis was determined using one-step TUNEL assay, which found that the apoptosis level declined after transfection with miR-141 mimics ( $p < 0.05$ ) but rose after transfection with miR-141 inhibitor compared with normal cells ( $p < 0.05$ ) (Figure 3).



**Figure 2.** Effect of miR-141 on proliferation of HT29 cells. **A:** Expression level of miR-141 in each group detected using qRT-PCR. **B:** Cell proliferation ability in each group at 0, 24 and 48 h after transfection detected using CCK8 assay (\*#There is a significant difference compared with other groups;  $p < 0.05$ ).



**Figure 3.** Apoptosis detected using one-step TUNEL assay. **A:** Fluorescence diagram of apoptosis. (magnification: 400 $\times$ ). **B:** Comparison of apoptosis rate. (\*#There is a significant difference compared with other groups;  $p < 0.05$ ). Scale bar: 100  $\mu\text{m}$ .

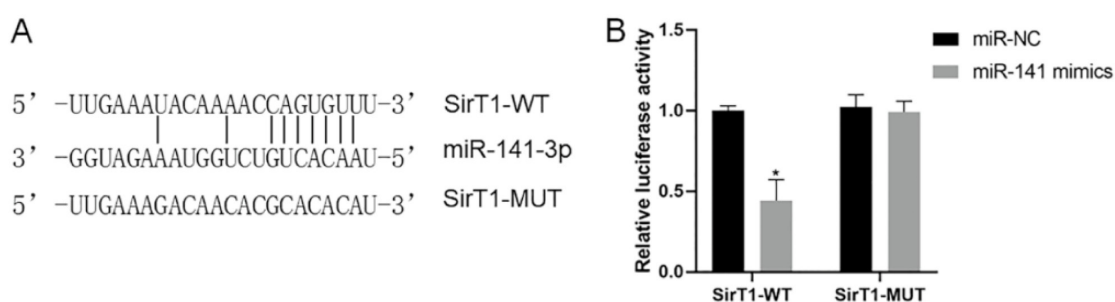


Association between miR-141 and Sirt1

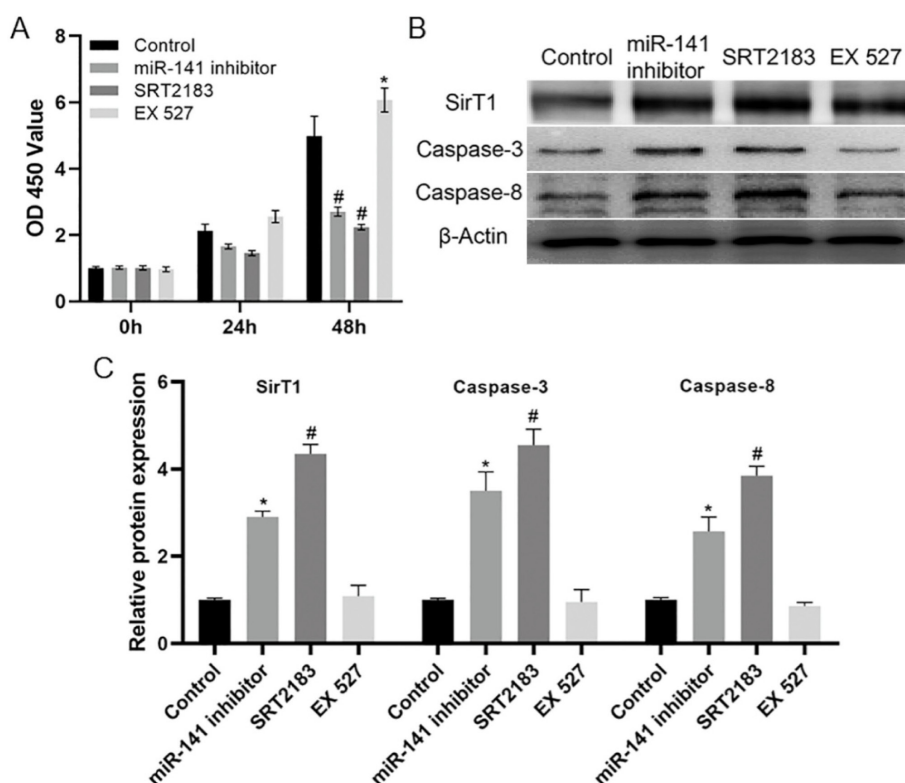
To further verify the regulatory relation between miR-141 and Sirt1, the Sirt1 luciferase reporter vectors containing predicted target sites of miR-141 were constructed based on the software prediction results. The target gene sequences are shown in Figure 4A. HT29 cells were co-transfected with wild-type Sirt1 (Sirt1-WT) or mutant-type Sirt1 (Sirt1-MUT) plasmids and miR-141 mimics or miR-NC. The results manifested that the Sirt1-WT luciferase activity was markedly inhibited by miR-141 mimics ( $p < 0.05$ ), while the Sirt1-MUT luciferase activity was not markedly inhibited ( $p > 0.05$ ) (Figure 4B).

Effects of miR-141 and Sirt1 on cell proliferation and apoptosis

To further verify the effects of miR-141 and Sirt1 on the proliferation and apoptosis of HT29 cells, the cells were transfected with the miR-141 inhibitor, Sirt1 activator SRT2183 and Sirt1 inhibitor EX 527, respectively. The results of CCK8 assay showed that compared with that in the control group, the proliferation of HT29 cells was remarkably weakened by miR-141 inhibitor and SRT2183 ( $p < 0.05$ ), while it was enhanced by EX 527 ( $p < 0.05$ ) (Figure 5A). Moreover, the protein expressions of Sirt1, Caspase-3 and Caspase-8 were determined via Western blotting, and it was



**Figure 4.** Association between miR-141 and Sirt1 determined via luciferase reporter gene assay system. **A:** Comparison of target gene sequences of miR-141 and Sirt1. **B:** Relative luciferase activity. (\*There is a significant difference between two groups;  $p < 0.05$ ).



**Figure 5.** Effects of miR-141 and Sirt1 on cell proliferation and apoptosis. **A:** Cell proliferation detected via CCK8 assay. **B:** Protein expressions of Sirt1, Caspase-3 and Caspase-8 detected via Western blotting. **C:** Relative quantitative analysis of Sirt1, Caspase-3 and Caspase-8 protein. (\*#There is a significant difference compared with other groups;  $p < 0.05$ ).

confirmed that miR-141 inhibitor and SRT2183 remarkably up-regulated the protein expressions of Sirt1, Caspase-3 and Caspase-8 ( $p < 0.05$ ), while EX 527 had the opposite effects ( $p < 0.05$ ) (Figure 5B & 5C).

## Discussion

Colon cancer is the third most common cancer in the world. Despite progress in diagnosis and treatment techniques, the 5-year survival rate of patients with colon cancer is still 40%, and about 50% of patients will die of cancer cell metastasis [5,17-20]. MiRs are involved in the regulation of cell differentiation, cell cycle progression and apoptosis through small sequences (18-25 nucleotides) [5,11]. Studies have revealed that miR-141 is related to the occurrence and development of many types of cancers [21]. Brunet et al [22] identified that more than 11 miRs in the miR expression profile have abnormal expression in stage III colon cancer, in which miR-141 is significantly up-regulated. Cheng et al [23] found that miR-141 is obviously correlated with stage IV colon cancer in 102 plasma samples, and they evaluated the sensitivity and specificity of miR markers in candidate plasma *via* ROC analysis. As a result, the combination of miR-141 and carcinoembryonic antigen labeling colon cancer was observed, further improving the accuracy of the detection, and confirming that the high-level miR-141 in plasma is prominently associated with poor survival. In addition, Ding et al [24] argued that the expression of miR-141 markedly rises in clinical samples of colon cancer compared with that in adjacent normal tissues. In this study, the expression levels of miR-141 and miR-34 were analyzed in colon cancer at different stages. It was found that the expression of miR-141 rose with the progression of colon cancer, which was higher in stage III than in stage I-II and also higher in stage IV than in stage III. MiR-34 was also highly expressed in stage IV colon cancer. To sum up, it can be seen combined with previous studies that miR-141 may play an important role in promoting the progression of colon cancer.

Ding et al [24] studied and found that the expression of mitogen-activated protein kinase 4 (MAP2K4) is down-regulated in rectal cancer, and overexpression of miR-141 can promote the proliferation of rectal cancer cells *via* repressing MAP2K4 activity according to *in vitro* studies. The research results of Yang et al [25] manifested that miR-141 targets Sirt1 and inhibits autophagy, thus reducing hepatitis B virus (HBV) replication. The

above findings suggest that miR-141 can target a variety of mRNAs. In this study, the expressions of miR-141 and Sirt1 were compared in human colorectal adenocarcinoma cell lines, and a negative correlation between them was found. Based on previous studies, it is speculated that there may be a targeted relation between expressions of miR-141 and Sirt1. The gene sequences of miR-141 and Sirt1 were analyzed using the prediction software, and the corresponding luciferase reporter vectors were constructed. Then the results of luciferase reporter gene assay revealed that the Sirt1 luciferase activity in HT29 cells declined by more than 55% after transfection with miR-141 plasmids.

There are some studies proving that Sirt1 may exert an anti-cancer effect, which can inhibit the androgen receptor-dependent proliferation of prostate tumor cells [26], whereas Sirt1 deficiency can lead to tumor formation in mice [27]. According to the research results of Kabra et al [28], knockdown of Sirt1 accelerates the formation of tumor xenografts, while overexpression of Sirt1 inhibits the tumor formation. In addition, the cell proliferation can be stimulated by Sirt1 due to its pharmacological effect under deprivation of growth factors. The results of immunohistochemical staining showed that there is a high level of Sirt1 in normal colonic mucosa and benign adenoma [28]. Overexpression of Sirt1 is observed in about 25% of stage I/II/III colorectal adenocarcinoma, but it is rarely seen in advanced tumor (stage IV). Moreover, the expression of Sirt1 is lower than normal in about 30% of cancers [28]. In this study, the Sirt1 expression was inhibited by the Sirt1 specific inhibitor EX 527, after which the proliferation of human colorectal adenocarcinoma cells was enhanced but the apoptosis rate was reduced, consistent with the effects of overexpression of miR-141. On the contrary, after the Sirt1 expression was activated by the Sirt1 activator SRT2183, the proliferation of human colorectal adenocarcinoma cells was weakened but the apoptosis rate was raised, similar to the effects of inhibition on miR-141. Overall, it can be seen that Sirt1 can inhibit the development of human colon cancer.

## Conclusions

In conclusion, miR-141 may promote the proliferation and inhibit the apoptosis of colon cancer cells *via* targeting Sirt1.

## Conflict of interests

The authors declare no conflict of interests.

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