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# The inhibitory effect of miR-375 targeting sp1 in colorectal cancer cell proliferation

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**Abstract. – OBJECTIVE: Sp1 is a member of super zinc finger structure family that participates in cancer cells' apoptosis, proliferation, survival, and differentiation. This study detected the expressions of miR-375 and sp1 in colorectal cancer tissue and cells to analyze their impact on cell proliferation.**

**PATIENTS AND METHODS: Colorectal cancer patients in our hospital were enrolled. HCT-116 cell was transfected with miR-375 mimics, mimics control, and miR-375 + sp1, respectively. RT-PCR and Western blot were applied to detect expressions of miR-375 and sp1 at mRNA and protein level in colorectal cancer tissue, para-carcinoma tissue, and normal colorectal tissue. RT-PCR and Western blot were used to test levels of miR-375 and sp1 in HCT-116 cells after transfection. MTT assay was performed to determine HCT-116 cell proliferation.**

**RESULTS: Our data showed that miR-375 was downregulated, while sp1 was overexpressed in colorectal cancer tissue compared with that in para-carcinoma tissue and normal control ($p < 0.05$). MiR-375 level was elevated, while sp1 mRNA was declined after miR-375 mimic transfection ($p < 0.05$). Compared with miR-375 mimic group, the levels of miR-375 and sp1 showed no difference in miR-375 + sp1 group ($p > 0.05$). Of note, the increase of MiR-375 and reduction of sp1 were in a time-dependent manner ($p < 0.05$). The cell proliferation rate in miR-375 mimic group was significantly decreased compared with that in mimic control and blank group ($p < 0.05$). The cell proliferation rate in miR-375 + sp1 group was significantly higher than that miR-375 group, but still lower than the control ($p < 0.05$). The proliferation rate gradually declined in a time dependent manner ($p < 0.05$).**

**CONCLUSIONS: MiR-375 was decreased and sp1 level was enhanced in colorectal cancer. MiR-375 suppresses the proliferation of colorectal cancer cells via the inhibition of sp1 expression at posttranscriptional level.**

*Key Words:*
miR-375, Sp1, Colorectal Cancer, Proliferation.

## Introduction

Colorectal cancer is a type of common gastrointestinal malignant tumor with increasing morbidity year by year. There are more than 140,000 new cases in the United States every year[1]. MicroRNA (miRNA) is a kind of single noncoding RNA that is characterized as the length of about 22 nt. After being transcripted by RNA polymerase II, mature miRNAs form under the effect of RNA enzymes III (RNase III) Drosha and Dicer enzyme[2]. It was reported that miRNA-375 widely existed in various tissues or organs, and was downregulated in a variety of tumors, especially in the digestive system tumors, such as liver cancer, esophageal cancer, gastric cancer, and pancreatic cancer[3,4]. Transcription factor specific protein (SP) family is highly related to transcription, which includes eight important members Sp1-Sp8 with homologous sequence of three series of zinc finger structures on the c-terminal. Each family member recognizes GC box and GT box to regulate gene transcription[5]. Sp1, as a basic transcription factor, has strong affinity to GC box to participate in cell proliferation, apoptosis, differentiation, and transformation[6]. This article enrolled colorectal cancer patients in our hospital to test miR-375 and sp1 expression in cancer tissue, para-carcinoma tissue, and normal control, to investigate the targeting effect of miR-375 on sp1 in colorectal cancer cell proliferation.

## Patients and Methods

### Patients

A total of 20 cases of colorectal cancer patients who received surgery in Xiangyang Hospital between January 2014 and January 2016 and that were diagnosed by pathology test, were enrolled, including 11 males and 9 females. There were 5 cases in stage I, 9 cases in stage II, 5 cases in stage III, and 1 case

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in stage IV. 11 cases were well differentiated, 7 cases were moderately differentiated, and 2 cases were poorly differentiated. The mean age of enrolled patients was 43.1 ± 6.2 (19-62) years old. All the cancer samples were preserved at -70°C. No patients received chemotherapy or radiotherapy before surgery. Another 20 cases of patients with benign colorectal disease that received surgery or biopsy were selected as normal control with mean age at 40.8 ± 5.3 (28-55) years old. No significant difference about gender and age was observed between two groups ($p > 0.05$). The study protocol was approved by the Research Ethics Committee of Xiangyang Hospital, and all patients gave their informed consent before study commencement.

### Cells and Reagents

Human colorectal cancer HCT-116 cell line was offered by Department of Cell Biology, China Medical University (Beijing, China). MiR-375 mimics and inhibitor were from GenePharma (Shanghai, China). Lipofectamine® 200 was purchased from Invitrogen (Carlsbad, CA, USA). RT-PCR kit for miR-375, sp1, and β-actin was from TaKaRa (Otsu, Shiga, Japan). PCR amplifier was from ABI (Vernon, CA, USA). TRIzol reagent was from Invitrogen (Carlsbad, CA, USA). Roswell Park Memorial Institute (RPMI)-1640 medium and MTT were from Gibco (Rockville, MD, USA).

### Experimental Method

#### Routine Cell Cultivation

Human colorectal cancer HCT-116 cells were cultured in RPMI-1640 medium and maintained at 37°C and 5% $CO_2$.

### Cell Transfection

HCT-116 cells were seeded in culture plate overnight. MiR-375 mimics, miR-375 inhibitor, mimic control, and miR-375 + sp1 were transfected to HCT-116 using lipofectamine® 2000. After 4-6 h incubation, the cells were cultured for 72 h after medium exchange. Untransfected HCT-116 cells were chosen as blank control.

### RT-PCR Detection of miR-375 and sp1 Expression in HCT-116

Total RNA was extracted by TRIzol according to the manual and qualified. A total of 200 ng RNA was reverse-transcripted to cDNA after poly A tail synthesis. The cDNA was used as template for PCR amplification. The primer sequences used in the experiments were listed as follows: miR-375, forward, 5'-AGCCGTCAAGAGCAATAACGAA-3', reverse, 5'-GTGCAGGGTCCGAGGT-3'. Sp1, forward, 5'-TGGTGGGCAGTATGTTGT-3', reverse, 5'-GCTATTGGCATTGGTGAA-3'. U6, forward, 5'-CTCGCTTCGGCAGCACA-3', reverse, 5'-AACGCTTCACGAATTTGCGT-3'. PCR reaction was consisted by 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. U6 was selected as internal reference.

### Western blot Detection of sp1 in HCT-116 Cells

Total protein was extracted by radioimmunoprecipitation assay (RIPA) and separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After the membrane was blocked at room temperature for 1 h, it was incubated with diluted primary antibody (1:200, β-actin 1:500) at 4°C overnight. After the membrane was washed by Tris-buffered saline and Tween 20 (TBST), it was further incubated in secondary antibody (1:2000) for 1 h (Abcam, Cambridge, MA, USA). Next, the membrane was added with developing solution A and B for 2 ml, respectively. At last, the membrane was scanned and analyzed by Quantity One software.

### MTT Assay

The cells transfected with miR-375 mimics, miR-375 inhibitor, mimic control, or miR-375 mimic + sp1 in logarithm phase were seeded in 12-well plate at $8\times10^4$/well and culture at 37°C and 5% $CO_2$. Cell viability was determined at 24 h, 48 h, and 72 h by 20 μl MTT (5 mg/ml). After 4 h incubation, the cell reaction was stopped by 150 μl dimethyl sulfoxide (DMSO) for 10 min. At last, the plate was read at 570 nm for OD value to draw the proliferation curve.

### Luciferase Assay

Sp1 3'-UTR in genome DNA was amplified by PCR and inserted to pGL3 control vector. Mutated sp1 3'-UTR vector was selected as control. MiR-375 was co-transfected to the cells by lipofectamine® 2000. After 24 h, the transfected HCT-116 was detected by luciferase assay according to the manual.

### RT-PCR Detection of miR-375 and sp1 mRNA expression in Colorectal Cancer Tissue, Para-carcinoma Tissue, and Normal Colorectal Tissue

Total RNA was extracted by TRIzol according to the manual and qualified. A total of 200 ng

RNA was reversing transcripted to cDNA after poly A tail synthesis. The primer sequences used in the experiments were listed in Table I. Reverse transcription system contained 2 μl RNA and 1 μl primer. A total of 3 μl cDNA together with 1 μl primers and 0.2 μl Taq DNA polymerase were applied for PCR amplification. PCR reaction was consisted by 94°C for 3 min, followed by 30 cycles of 94°C for 40 s, 56°C for 1 min, and 72°C for 1 min. The PCR product was scanned on gel imaging system and analyzed by Quantity One software.

### Western Blot Detection of sp1 in Colorectal Cancer Tissue, Para-carcinoma Tissue, and Normal Colorectal Tissue

Total protein was treated with radioimmunoprecipitation assay buffer (RIPA) and the supernatant was moved to new Ep tubes after it was centrifuged at 300 g for 20 min. A total of 40 μg protein was separated by 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After blocked at room temperature for 1 h, the membrane was incubated with diluted primary antibody (1:200, β-actin 1:500) at 4°C overnight. After washed by Tris-buffered saline Tween 20 (TBST), the membrane was further incubated in secondary antibody (1:2000) for 1 h. Next, the membrane was added with developing solution A and B for 2 ml, respectively. At last, the membrane was scanned and analyzed by Quantity One software.

### Statistical Analysis

SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was applied for data analysis. All data was presented as mean ± standard deviation. Enumeration data was compared by chi-square test, while measurement data was compared by *t*-test. $p < 0.05$ was considered as statistical significance.

## Results

### RT-PCR Detection of miR-375 and sp1 mRNA Expression in HCT-116 Cells

RT-PCR was applied to test miR-375 and sp1 mRNA expression in HCT-116 cells. The results showed that miR-375 level was significantly increased, while sp1 expression was reduced in miR-375 mimic group compared with that in miR-375 inhibitor, mimic control, and blank control group ($p < 0.05$). The expression of miR-375 showed no difference between miR-375 mimic group and miR-375 + sp1 group ($p > 0.05$). However, statistically lower level of miR-375 and higher level of sp1 mRNA were found in miR-375 inhibitor group ($p < 0.05$) (Table II).

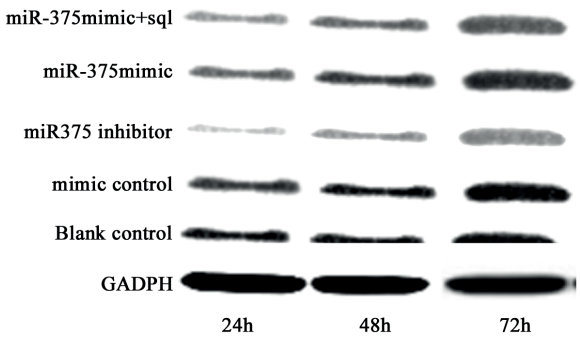


**Figure 1.** MiR-375 protein expression in HCT-116 after transfection.

### Western Blot Detection of miR-375 and sp1 Expression in HCT-116 Cells

Western blot was used to test miR-375 and sp1 expression in HCT-116 cells. It was revealed that miR-375 level was upregulated, while sp1 level was declined in miR-375 mimic group compared with that in miR-375 inhibitor, mimic control, and blank control group ($p < 0.05$). No difference of miR-375 expression was shown between miR-375 mimic group, miR-375 + sp1 group showed ($p > 0.05$). Of note, the miR-375 was increased and sp1 protein was reduced in miR-375 + sp1 group and miR-375 mimic group in a time dependent manner ($p < 0.05$). Nevertheless, the miR-375 was declined, and sp1 mRNA was enhanced in miR-375 inhibitor group ($p < 0.05$) (Table III, Figures 1 and 2).

### MTT Assay Determination of HCT-116 Cell Proliferative Ability

MTT assay was performed to determine cell viability. It demonstrated that significantly de-

**Table I.** Primer sequence.

| Gene | Sense | Anti-sense |
|---|---|---|
| miR-375 | 5'-AAATCACCACCTTCACAGCC-3' | 5'-GTTGTAATGGTTCTCCTCCAGC-3' |
| sp1 | 5'-ATGGCAGCCGGG AGCATCACC-3' | 5'-CACACACTCCTTTGATAGACACAA-3' |
| β-actin | 5'-GAAACTACCTTCAACTCCATC-3' | 5'-CTAGAAGCATTTGCGGTGGACGAT GGAGGGGCC-3' |

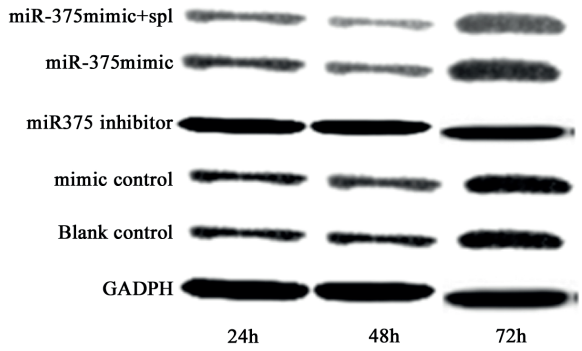


**Figure 2.** Spl protein expression in HCT-116 after transfection.

creased proliferation rate in miR-375 mimic group was presented compared with that in mimic control and blank group ($p < 0.05$). However, markedly higher proliferation rate in miR-375 + spl group was exhibited than that in miR-375 group, but it was still lower than the control ($p < 0.05$). The proliferation rate was gradually declined in a time dependent manner ($p < 0.05$) (Table IV).

### MiR-375 Targeting sp1 3'-UTR

Luciferase assay demonstrated that its activity was statistically declined in HCT-116 cells after sp1 3'-UTR and miR-375 mimic co-transfection ($p < 0.05$). Sp1 3'-UTR co-transfected with miR-375 obviously elevated luciferase activity and suppressed miR-375 binding with sp1 3'-UTR.

### RT-PCR Detection of miR-375 and sp1 mRNA Expression in Colorectal Cancer Tissue, Para-carcinoma tissue, and Normal Colorectal Tissue

RT-PCR was performed to test miR-375 and sp1 mRNA expression in colorectal cancer tissue, para-carcinoma tissue, and normal colorectal tissue. Compared with para-carcinoma tissue and normal colorectal tissue, miR-375 was significantly declined, while sp1 mRNA was elevated in colorectal cancer tissue ($p < 0.05$). Additionally, miR-375 and sp1 expression level showed no statistical difference between para-carcinoma tissue and normal colorectal tissue ($p > 0.05$) (Table V, Figure 3).

### Western blot Detection of miR-375 and sp1 Protein Expression in Colorectal Cancer Tissue, Para-Carcinoma tissue, and Normal Colorectal Tissue

Western blot was performed to detect miR-375 and sp1 protein expression in colorectal cancer tissue, para-carcinoma tissue, and normal col-

**Table II.** MiR-375 and sp1 expression in HCT-116 cells after transfection.

| Item | miR-375 mimic+sp1 | miR-375 mimic | miR375 inhibitor | Mimic control | Blank control |
|---|---|---|---|---|---|
| miR-375 | | | | | |
| 24 h | $1.321\pm0.029^{123}$ | $1.316\pm0.038^{12}$ | $0.282\pm0.008^{1236}$ | $0.701\pm0.021$ | $0.634\pm0.017$ |
| 48 h | $1.471\pm0.037^{1234}$ | $1.452\pm0.046^{124}$ | $0.201\pm0.005^{12346}$ | $0.713\pm0.019$ | $0.651\pm0.015$ |
| 72 h | $1.698\pm0.068^{12345}$ | $1.703\pm0.052^{1245}$ | $0.137\pm0.002^{123456}$ | $0.721\pm0.022$ | $0.647\pm0.019$ |
| sp1 mRNA | | | | | |
| 24 h | $1.183\pm0.031^{12}$ | $1.172\pm0.027^{12}$ | $1.895\pm0.087^{1236}$ | $1.831\pm0.081$ | $1.876\pm0.092$ |
| 48 h | $1.112\pm0.023^{124}$ | $1.101\pm0.014^{124}$ | $1.921\pm0.091^{12346}$ | $1.782\pm0.075$ | $1.801\pm0.087$ |
| 72 h | $1.055\pm0.011^{1245}$ | $0.923\pm0.008^{1245}$ | $1.983\pm0.096^{123456}$ | $1.801\pm0.078$ | $1.785\pm0.079$ |

1, $p < 0.05$, compared with mimic control; 2, $p < 0.05$, compared with blank control; 3, $p < 0.05$, compared with miR-375 mimic group; 4, $p < 0.05$, compared with 24 h; 5, $p < 0.05$, compared with 48 h; 6, $p < 0.05$, compared with miR-375 + sp1 group.

**Table III.** MiR-375 and sp1 protein expression in HCT-116 after transfection.

| Item | miR-375 mimic+sp1 | miR-375 mimic | miR375 inhibitor | Mimic control | Blank control |
|---|---|---|---|---|---|
| miR-375 protein | | | | | |
| 24 h | $1.532\pm0.018^{123}$ | $1.514\pm0.012^{12}$ | $0.233\pm0.008^{1236}$ | $0.689\pm0.021$ | $0.634\pm0.017$ |
| 48 h | $1.715\pm0.026^{1234}$ | $1.682\pm0.021^{124}$ | $0.189\pm0.005^{12346}$ | $0.724\pm0.019$ | $0.651\pm0.015$ |
| 72 h | $1.866\pm0.057^{12345}$ | $1.912\pm0.042^{1245}$ | $0.124\pm0.002^{123456}$ | $0.733\pm0.022$ | $0.647\pm0.019$ |
| sp1 protein | | | | | |
| 24 h | $1.672\pm0.093^{123}$ | $1.181\pm0.023^{12}$ | $1.905\pm0.085^{1236}$ | $1.742\pm0.079$ | $1.767\pm0.081$ |
| 48 h | $1.481\pm0.081^{1234}$ | $1.093\pm0.012^{124}$ | $1.932\pm0.087^{12346}$ | $1.773\pm0.064$ | $1.782\pm0.076$ |
| 72 h | $1.143\pm0.046^{12345}$ | $0.812\pm0.006^{1245}$ | $1.991\pm0.095^{123456}$ | $1.794\pm0.051$ | $1.796\pm0.068$ |

1, $p < 0.05$, compared with mimic control; 2, $p < 0.05$, compared with blank control; 3, $p < 0.05$, compared with miR-375 mimic group; 4, $p < 0.05$, compared with 24 h; 5, $p < 0.05$, compared with 48 h; 6, $p < 0.05$, compared with miR-375 + sp1 group.

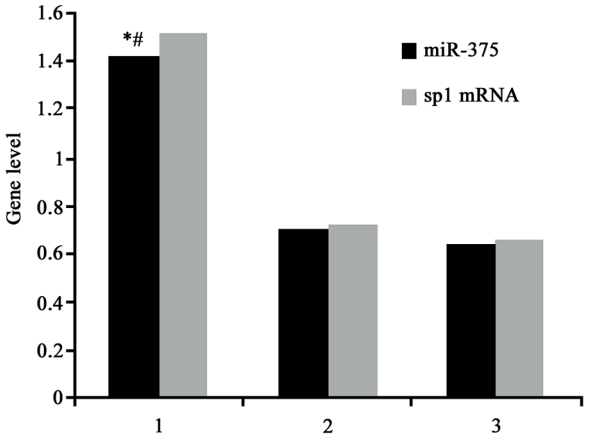


**Figure 3.** miR-375 and sp1 mRNA expression in colorectal cancer tissue, para-carcinoma tissue, and normal colorectal tissue. 1, Colorectal cancer tissue; 2, Para-carcinoma tissue; 3, Normal colorectal tissue. *$p < 0.05$, compared with para-carcinoma tissue; #$p < 0.05$, compared with normal colorectal tissue.

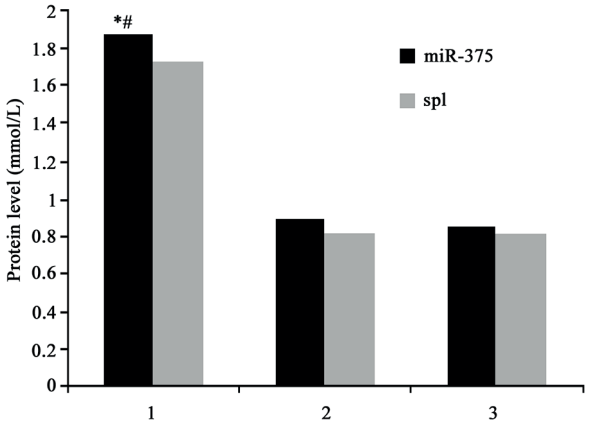


**Figure 4.** miR-375 and sp1 protein expression in colorectal cancer tissue, para-carcinoma tissue, and normal colorectal tissue. 1, Colorectal cancer tissue; 2, Para-carcinoma tissue; 3, Normal colorectal tissue. *$p < 0.05$, compared with para-carcinoma tissue; #$p < 0.05$, compared with normal colorectal tissue.

**Table IV.** MTT assay detection of cell proliferation rate after transfection.

| Item | miR-375 mimic+sp1 | miR-375 mimic | miR375 inhibitor | mimic control | Blank control |
|---|---|---|---|---|---|
| OD value | | | | | |
| 24 h | 0.875±0.016[123] | 0.792±0.012[12] | 1.029±0.018[1236] | 1.021±0.012 | 1.012±0.017 |
| 48 h | 0.801±0.013[1234] | 0.724±0.017*[124] | 1.139±0.025[12346] | 1.084±0.031[4] | 1.133±0.026[4] |
| 72 h | 0.068±0.013[12345] | 0.403±0.012[1245] | 1.324±0.043[123456] | 1.131±0.042[45] | 1.294±0.041[45] |
| Proliferation rate (%) | | | | | |
| 24 h | 114[123] | 112[12] | 1291236 | 124 | 121 |
| 48 h | 98[1234] | 92[124] | 14212346 | 1324 | 1384 |
| 72 h | 92[12345] | 84 | 171123456 | 15845 | 16945 |

1, $p < 0.05$, compared with mimic control; 2, $p < 0.05$, compared with blank control; 3, $p < 0.05$, compared with miR-375 mimic group; 4, $p < 0.05$, compared with 24 h; 5, $p < 0.05$, compared with 48 h; 6, $p < 0.05$, compared with miR-375 + sp1 group.

**Table V.** MiR-375 and sp1 mRNA expression in colorectal cancer tissue, para-carcinoma tissue, and normal colorectal tissue.

| Group | Cases | miR-375 | sp1 |
|---|---|---|---|
| Colorectal cancer | 20 | 1.425±0.059*# | 1.521±0.086*# |
| Para-carcinoma | 20 | 0.707±0.023 | 0.709±0.026 |
| Normal colorectal tissue | 10 | 0.643±0.026 | 0.656±0.024 |

*$p < 0.05$, compared with para-carcinoma tissue; #$p < 0.05$, compared with normal colorectal tissue.

orectal tissue. The results showed that, compared with para-carcinoma tissue and normal colorectal tissue, miR-375 was decreased, while sp1 protein was enhanced in colorectal cancer tissue ($p < 0.05$). MiR-375 and sp1 expression level showed no significant difference between para-carcinoma tissue and normal colorectal tissue ($p > 0.05$) (Table VI, Figure 4).

## Discussion

Colorectal cancer is a kind of common digestive tract malignant tumor that accounts for the fourth of cancer death[7]. At present, the incidence of colorectal cancer increase with the change of living inhabits. Compared with 1970s, the current prevalence of colorectal cancer rises to

**Table VI.** MiR-375 and sp1 protein expression in colorectal cancer tissue, para-carcinoma tissue, and normal colorectal tissue.

| Group | Cases | miR-375 | sp1 |
|---|---|---|---|
| Colorectal cancer | 20 | 1.878±0.069*# | 1.732±0.075*# |
| Para-carcinoma | 20 | 0.892±0.037 | 0.811±0.027 |
| Normal colorectal tissue | 10 | 0.854±0.032 | 0.816±0.021 |

*$p < 0.05$, compared with para-carcinoma tissue; #$p < 0.05$, compared with normal colorectal tissue.

32.0% in city and 8.5% in rural areas[8,9]. Early detection and treatment can improve the 5-year survival rate of patients with colorectal cancer, especially for tumor confined to the intestinal wall, up to 90%[10]. MiRNA is a kind of important gene regulating factor that has many important roles in regulating cell growth, proliferation, differentiation, and apoptosis. It participates in malignant tumor occurrence and development[11]. The expressional change of miRNA has effect on the development of carcinogenesis[12]. A previous study[13] found that miR-375 can inhibit tumor cell proliferation, invasion, metastasis, and induce apoptosis. Sp1 is a specific DNA binding protein which is abnormally expressed in multiple tumors. It is involved in various biological process, including cell proliferation, invasion, and angiogenesis[14]. Sp1 was reported to be positively correlated with cancer and impacted by posttranscriptional modification[15].

In this study, we enrolled colorectal cancer patients as experimental group, and patients with benign colorectal disease were set as control. RT-PCR was applied to test miR-375 and sp1 mRNA expression in colorectal cancer tissue, para-carcinoma tissue, and normal colorectal tissue. The results showed that miR-375 was declined, while sp1 mRNA was elevated in colorectal cancer tissue compared with that in control. Western blot also discovered that miR-375 was decreased and sp1 protein was enhanced in cancer tissue. It suggested that miR-375 was reduced and sp1 was upregulated in colorectal cancer. Dai et al[16] found miR-375 level was significantly declined in colorectal cancer, para-carcinoma tissue, human colorectal cancer cell line, and human normal colorectal mucous tissue, which was in accordance with our results. To explore miR-375 and sp1 expression changes in colorectal cancer and related mechanism, we transfected miR-375 mimics, miR-375 inhibitor, mimic control, and miR-375 mimic + sp1 to HCT-116 cells. RT-PCR revealed that miR-375 level was elevated, while sp1 mRNA was declined after miR-375 mimic transfection. Western blot demonstrated that sp1 protein was reduced in miR-375 + sp1 group following time extension. It indicated that sp1 mRNA showed no significant changes, while sp1 protein significantly was downregulated after miR-375 transfection in colorectal cancer tissue. It suggested that miR-375 can suppress sp1 expression at posttranscriptional level[17]. It suggested that miR-375 transfection may inhibit HCT-116 cell viability through suppressing sp1 expression. A previous study[18] reported that miR-375 downregulated in gastric cancer cells to play a cancer suppressor gene function. Mazar et al[19] found that elevating miR-375 expression can suppress melanoma cell proliferation, invasion, and migration. Wang et al[20] reported that miR-375 plays a cancer suppressor gene role in squamous carcinoma of the cervix through targeting sp1. Accumulative evidence showed that microRNAs such as miR-143 and miR-874, were associated with colorectal cancer progression[21,22]. In this paper, we demonstrated that another miRNA, miR-375, also participated in the regulation of the development of colorectal cancer.

## Conclusions

miR-375 decreased and sp1 level enhanced in colorectal cancer. MiR-375 can inhibit sp1 expression at posttranscriptional level to suppress colorectal cancer proliferation. MiR-375 may play an important role in colorectal cancer occurrence and development, and it may be a new biomarker for colorectal cancer treatment in clinic.

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### Conflict of Interest

The Authors declare that they have no conflict of interests.

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