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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[illegible]noma tissue, and normal colorectal tiss[illegible] PCR and Western blot were used to test [illegible]s of miR-375 and sp1 in HCT-116 cells after t[illegible]fection. MTT assay was performed to determ[illegible] HCT-116 cell proliferation.

RESULTS: Our data showed [illegible]375 was downregulated, while sp1 [illegible] ove[illegible]pressed in colorectal cancer tissu[illegible]ompare[illegible]ith that in para-carcinoma tissu[illegible] norm[illegible] < 0.05). MiR-375 leve[illegible]was[illegible] [illegible]while [illegible] mRNA was decline[illegible]er mi[illegible]mimic transfection ($p < 0.05$)[illegible]pared wit[illegible]-375 mimic group, the le[illegible]miR-375 and[illegible]howed no difference[illegible] sp1 group ($p > 0.05$). Of note, the i[illegible]ease [illegible]-375 and reduction of sp1 we[illegible]n a time-de[illegible]nt manner ($p < 0.05$). Th[illegible]ll proliferation r[illegible]miR-375 mimic gro[illegible]as sig[illegible]ficantly decreased compared with[illegible] in mi[illegible]control and blank group ($p < 0.0$[illegible] ce[illegible]oliferat[illegible] rate in miR-375 + sp1 group[illegible]nificant[illegible]gher than that miR-375 [illegible]up, b[illegible] lowe[illegible]an the control ($p < 0.05$). [illegible]rolife[illegible] gradually declined in a tim[illegible]pende[illegible]ner ($p < 0.05$).

C[illegible]CLUSION[illegible]: MiR-375 was decreased and sp[illegible]enhanced in colorectal cancer. [illegible]375 su[illegible]esses the proliferation of colorectal cancer cells via the inhibition of sp1 expression at posttranscriptional level.

Key[illegible]rds:
miR-375, Sp1, Colorectal Cancer, Proliferation.

Introduc[illegible]

Colore[illegible]a[illegible] a type of co[illegible]mon gastrointestinal ma[illegible]gnant t[illegible]with increasing morbidity year b[illegible]ar. There a[illegible]re than 140,000 new ca[illegible] United States [illegible]ry year[1]. MicroRNA ([illegible]NA) is a kind of single noncoding RNA that [illegible]haracterized [illegible]e length of about 22 nt. After [illegible] transcripte[illegible] RNA polymerase II, mature m[illegible]As form [illegible]r the effect of RNA enzymes III ([illegible]osha and Dicer enzyme[2]. It was reported [illegible]iRNA-375 widely existed in various [illegible]ssues or organs, and was downregulated in a variety [illegible]mors, especially in the digestive system tumors, [illegible]ch 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 amplifi[illegible]s from ABI (Vernon, CA, USA). TRIzol reag[illegible] from Invitrogen (Carlsbad, CA, USA). R[illegible]ell Park Memorial Institute (RPMI)-1640 me[illegible] and MTT were from Gibco (Rockville, MD, US[illegible]

Experimental Method

Routine Cell Cultivation

Human colorectal [illegible]cer [illegible]o cells were cultured in RPMI [illegible]0 mediu[illegible] maintained at 37°C and 5%[illegible]

Cell Transfection

HCT-1[illegible]ells were see[illegible]n culture plate overni[illegible] MiR-375 mimics, [illegible]R-375 inhibitor, mim[illegible]ntrol, [illegible]miR-375 + sp1 were transfected [illegible]CT-11[illegible]ng lip[illegible]fectamine® 2000. After 4-6 h[illegible]n, the [illegible] were cultured for 72 h [illegible]r me[illegible]exch[illegible]. Untransfected HCT-116 [illegible]ere c[illegible]lank control.

RT[illegible]Detection of miR-375 and sp1 [illegible]n HCT-116

[illegible]otal RNA was extracted by TRIzol according [illegible]e manual and qualified. A total of 200 ng RNA w[illegible]erse-transcripted to cDNA after poly A tail synth[illegible]sis. 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' [illegible] ward, 5'-TGGTGGGCAGTATGTTGT[illegible] 5'-GCTATTGGCATTGGTGAA-3'. [illegible] forward, 5'-CTCGCTTCGGCAGCACA-3' [illegible]se, 5'-AACGCTTCACGAATTTGCGT-3'. PCR [illegible]ion was consisted by 95°C for 30 s, fo[illegible]wed by [illegible]les of 95°C for 5 s and 60°C for [illegible] U6 was se[illegible] internal reference.

Western blot Det[illegible]n sp1 in HCT-11[illegible]ells

Total protei[illegible]as extrac[illegible]r[illegible]mmunoprecipitation [illegible](RIPA) and [illegible]ted by 8% sodium d[illegible]yl[illegible]e polyacryla[illegible]ide gel electrophoresi[illegible] (SDS-P[illegible]. After the membrane was bl[illegible]d at room t[illegible]rature for 1 h, it was in[illegible]with diluted pr[illegible]ary antibody (1:200, [illegible]in 1:500) at 4°C overnight. After the membrane was was[illegible]by Tris-buffered saline and [illegible]en 20 (TBS[illegible]it was further incubated in s[illegible]ary antil[illegible] (1:2000) for 1 h (Abcam, Cam[illegible]USA). Next, the membrane was added w[illegible]veloping solution A and B for 2 ml, [illegible]spectively. At last, the membrane was scanned [illegible]zed 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 8×10^4/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, washed by Tris-buffered saline Tween 20, the membrane was further incubated in secondary antibody (1:2000) for 1 h. Next, the membrane added with developing solution A and B for 2 respectively. At last, the membrane was scanned and analyzed by Quantity One.

Statistical Analysis

SPSS 17.0 software (SPSS Inc., 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 expression in HCT-116 cells. The results showed that miR-375 level was significantly increased, while sp1 mRNA 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).

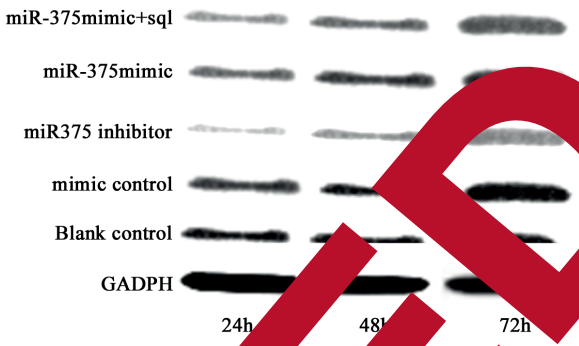

Figure 1. MiR-375 protein expression in HCT-116 after transfection.

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 II. 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'

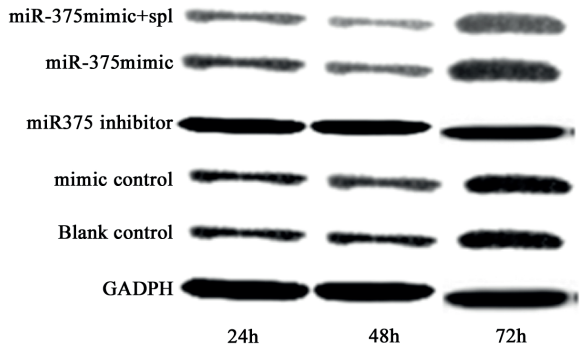

Figure 2. Sp1 protein expression in HCT-116 after transfection.

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 + sp1 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 [illegible] was statistically declined in HCT-116 cell[illegible]er sp1 3'-UTR and miR-375 mimic co-transfection ($p < 0.05$). Sp1 3'-UTR co-transfected with miR-375 obviously elevated luciferase acti[illegible]d suppressed miR-375 binding with sp[illegible]

RT-PCR Detection of miR-37[illegible]nd sp1 mRNA Expression in Colorec[illegible]ancer Tissue, Para-carcinoma [illegible]ssue, a[illegible] Normal Colorectal Tis[illegible]

RT-PCR was perform[illegible]o test m[illegible]R-375 an[illegible] mRNA expression in [illegible]orectal [illegible]er tissue, para-carcinoma tissue, a[illegible]rm[illegible]olorect[illegible]issue. Compared with [illegible]canc[illegible]tissue [illegible]normal colorectal tiss[illegible]miR-375 [illegible]ig[illegible]antly declined, while [illegible]RNA was ele[illegible]n colorectal cancer tis[illegible](p[illegible]). Additionall[illegible], miR-375 and sp1 expres[illegible]on leve[illegible]ved no statistical difference b[illegible]en para-ca[illegible]a tissue and normal co[illegible]issue ($p > 0.05$[illegible]able V, Figure 3).

Western blot[illegible]tection of miR-375 and sp1 Protein E[illegible]ession in Colorectal C[illegible]r Tissue[illegible]ara-Carcinoma tissue, and[illegible]lorectal Tissue

Weste[illegible]t was performed to detect miR-375 and sp1 protein expression in colorectal cancer [illegible]ara-carcinoma tissue, and normal col-

Table II. MiR-375 and sp1 expression in HCT-116 cells [illegible]sfection.

Item	miR-375 mimic+s[illegible]	[illegible]375 mim[illegible]	miR375 inhibitor	Mimic control	Blank control
miR-375					
24 h	1.321±0.029[illegible]	1.[illegible]	0.282±0.008[1236]	0.701±0.021	0.634±0.017
48 h	1.471±0.037[illegible]	[illegible]	0.201±0.005[12346]	0.713±0.019	0.651±0.015
72 h	1.698±0.[illegible]	[illegible]03±0.052[illegible]	0.137±0.002[123456]	0.721±0.022	0.647±0.019
sp1 mRNA					
24 h	1.18[illegible]31[12]	[illegible]0.027[12]	1.895±0.087[1236]	1.831±0.081	1.876±0.092
48 h	1.[illegible]	[illegible]014[124]	1.921±0.091[12346]	1.782±0.075	1.801±0.087
72 h	[illegible]±0.[illegible]	0.92[illegible]±0.008[1245]	1.983±0.096[123456]	1.801±0.078	1.785±0.079

1, $p < 0.05$, c[illegible]ared with mimi[illegible]l; 2, $p < 0.05$, compared with blank control; 3, $p < 0.05$, compared with miR-375 mimic group; 4, [illegible]05, compared with 2[illegible]; 5, $p < 0.05$, compared with 48 h; 6, $p < 0.05$, compared with miR-375 + sp1 group.

Tab[illegible] MiR-3[illegible]d sp1 protein expression in HCT-116 after transfection.

Item	miR-[illegible]mimic+sp1	miR-375 mimic	miR375 inhibitor	Mimic control	Blank control
[illegible]375 pr[illegible]					
2[illegible]	[illegible]2±0.018[123]	1.514±0.012[12]	0.233±0.008[1236]	0.689±0.021	0.634±0.017
48	[illegible]715±0.026[1234]	1.682±0.021[124]	0.189±0.005[12346]	0.724±0.019	0.651±0.015
[illegible]	1.866±0.057[12345]	1.912±0.042[1245]	0.124±0.002[123456]	0.733±0.022	0.647±0.019
[illegible]p1					
[illegible]4 h	1.672±0.093[123]	1.181±0.023[12]	1.905±0.085[1236]	1.742±0.079	1.767±0.081
[illegible]8 h	1.481±0.081[1234]	1.093±0.012[124]	1.932±0.087[12346]	1.773±0.064	1.782±0.076
	1.143±0.046[12345]	0.812±0.006[1245]	1.991±0.095[123456]	1.794±0.051	1.796±0.068

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.

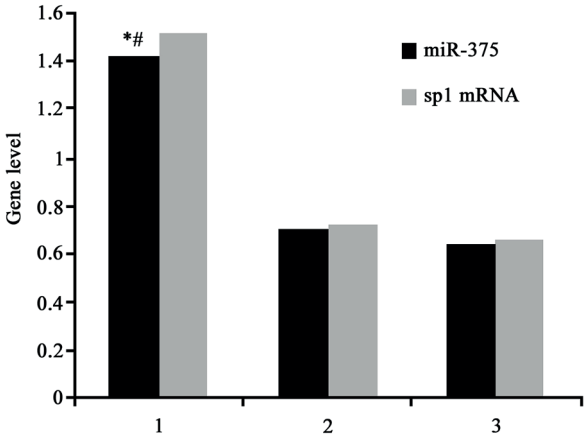

Figure 3. miR-375 and sp1 mRNA expression in colorectal cancer tissue, para-carcinoma tissue, and normal colorectal tissue. 1, Colorectal cancer tissue; 2, Para-carcinoma tissue; 3, Normal colorectal tissue. *$p < 0.05$, compared with para-carcinoma tissue; #$p < 0.05$, compared with normal colorectal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

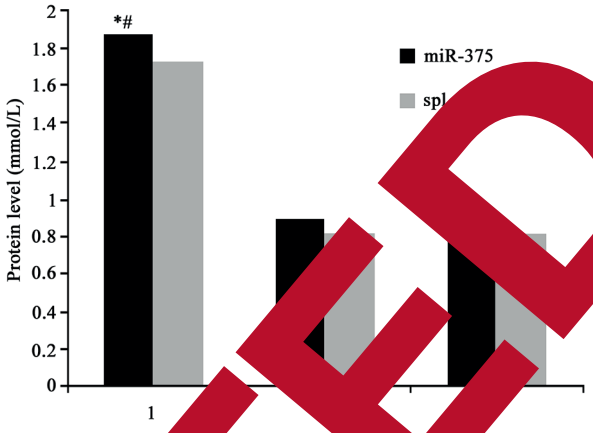

Figure 4. miR- d sp1 protein e in colorectal cancer tissue oma tissue, an rmal colorectal tissue. 1, C ctal c ssue; 2, Para-carcinoma tissue; 3, Normal colorectal tis < 0.05, compared with para-ra-car tissue; #$p <$ 0. pared with normal colo c.

Table IV. MTT assay detection of cell proliferation rate after tran ion.

Item	miR-375 mimic+sp1	miR-375 mimic	miR- ibitor	mic control	Blank control
OD value					
24 h	0.875±0.016[123]	0.792±0	29±0.018[1236]	1.021±0.012	1.012±0.017
48 h	0.801±0.013[1234]	0.724±0.	[12346]	1.084±0.031[4]	1.133±0.026[4]
72 h	0.068±0.013[12345]	0.403±0.0	[56]	1.131±0.042[45]	1.294±0.041[45]
Proliferation rate (%)					
24 h	114[123]		1291236	124	121
48 h	98[1234]		212346	1324	1384
72 h	92[12345]	84	123456	15845	16945

1, $p < 0.05$, compared with mi trol; 2 d with blank control; 3, $p < 0.05$, compared with miR-375 mimic group; 4, $p < 0.05$, compar wit 5 .05; ed with 48 h; 6, $p < 0.05$, compared with miR-375 + sp1 group.

Table V. MiR-375 RNA expressi lorectal cancer tissue, para-carcinoma tissue, and normal colorectal tissue.

Group	Cases	miR-375	sp1
Colorect cer	20	1.425±0.059*#	1.521±0.086*#
Para-c oma	20	0.707±0.023	0.709±0.026
Nor olorecta ue	10	0.643±0.026	0.656±0.024

*$p <$ 0 with pa rcinoma tissue; #$p < 0.05$, compared with normal colorectal tissue.

orec tissue. esults showed that, compared wit carcinoma tissue and normal colorectal t was decreased, while sp1 protein s enhanced in colorectal cancer tissue ($p <$). MiR-375 and sp1 expression level showed n ificant difference between para-carcinoma t sue 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
Para-carcinoma	20	0.892±0.037	0.8 027
Normal colorectal tissue	10	0.854±0.032	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 protein expression in colorectal cancer tissue, para-carcinoma tissue, and normal colorectal tissue. The results showed that miR-375 was decreased, while sp1 mRNA was elevated in colorectal cancer tissue compared with normal 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 regulated in colorectal cancer. Dai et al[16] found miR-375 level significantly declined in colorectal cancer tissue, 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 course, indicating 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 found that elevating miR-375 expression can suppress melanoma cell proliferation, invasion, and migration. Wang et al[20] reported 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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