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Original Article

Epigenetic regulation of miR-375 in human colorectal cancer cells: a key role of DNA methylation

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Abstract: The methylation status of miR-375 promoter in human colorectal cancer (CRC) and the biological roles of dysregulated miR-375 in CRC cells remain unknown. Bioinformatics analysis combined with methylation specific PCR (MSP) and regular quantitative real-time PCR (qRT-PCR) were performed to determine the expression as well as the methylation status of miR-375. The correlation of miR-375 expression and its methylation with the clinicopathological features of CRC patients was statistically analyzed. CRC cell lines were treated with 5-aza-2'-deoxycytidine (5-AzadC, DAC) to make the methylation inactivation. MTT and colony formation assays were carried out to evaluate the effects of miR-375 on CRC cells. Finally luciferase assay and western blot were performed to validate the targeting of YAP1 by miR-375. We found that MiR-375 was decreased, and DNA methylation in its promoter occured more frequently in cancerous CRC tissues than in adjacent normal tissues. Statistical analysis results showed a significant correlation between DNA methylation frequency and differentiation extent of CRC tumors. In addition, demethylation of miR-375 by DAC treatment revealed that miR-375 expression is epigenetically regulated in CRC cells. Importantly, we proved that miR-375 significantly inhibited cell growth, which might be mediated by one of its target, YAP1. Our data indicated that miR-375 functions as a tumor suppressor by inhibiting cell growth, and is partially silenced by DNA hypermethylation in CRC, which may provide a potential therapeutic strategy for human CRC treatment.

Keywords: miR-375, clinicopathological features, DNA methylation, colorectal cancer, cell proliferation

Introduction

Colorectal Cancer (CRC) is the third commonest malignancy all around the world and the third leading cause of cancer-related morbidity and mortality in the United States of America, in both males and females [1]. According to the Chinese Cancer Registry Annual Report (2012), CRC ranks the fourth leading cause of cancer death in China with an increasing tendency [2]. Despite of substantial advancement with regard to the available treatment options in recent years, the prognosis of CRC patients remains poor. The post-operative 5-year survival rate is still hovering at 30%-50%. Generally speaking, the difficulties in early diagnosis, prognostic assessments, especially relapse and/or metastasis prevention, which has become a critical option that decide whether the therapy is successful or not, all contribute to the present puzzle of CRC treatment. Approximately 90% of CRC-related mortalities are due to metastasis, yet the mechanism underlying this process remains unclear [3]. Thus, understanding the underlying molecular genesis of CRC progression and metastasis, as well as exploring valid biomarkers for early diagnosis and prognosis prediction are of fundamental clinical values in CRC therapy nowadays.

MiRNAs (miRNAs) are a class of small, endogenous, non-coding RNA molecules that negatively regulate gene expression by base pairing to the 3'-untranslated region (3'-UTR) of targeted messenger RNA (mRNA), in a post-transcriptional manner. They have been intensively studied in recent years and reported as critical factors in various physiological and pathological processes [4]. Increasing evidence suggests that aberrant expression of specific miRNAs

Table 1. Summary of patients and the relevant clinicopathological features characteristic

Patient No.	Gender	Age (years)	Differentiation extent	TNM stage	Lymphnode involvement	Organ metastasis	Tumor location	CEA mg/L
1	F	41	Low	IV	N	N	Colon	1.89
2	F	49	Medium	I	N	N	Rectum	3.90
3	F	52	Low	IV	P	N	Rectum	19.20
4	M	69	High	I	N	N	Rectum	8.06
5	M	44	High	I	N	N	Colon	5.20
6	M	36	Low	II	N	N	Colon	580.20
7	M	62	High	I	N	N	Rectum	2.20
8	M	50	Low	II	N	N	Colon	1.70
9	F	44	Low	IV	N	P	Colon	200.90
10	F	72	Low	II	P	N	Rectum	1.61
11	M	68	Low	II	N	N	Rectum	4.40
12	M	52	Low	II	P	N	Rectum	3.10
13	F	55	Low	II	N	N	Rectum	2.00
14	M	57	Medium	III	N	N	Rectum	8.89
15	M	36	Low	II	N	N	Colon	63.18
16	M	53	Low	II	N	N	Rectum	1.38
17	M	48	Medium	I	N	N	Rectum	3.80
18	M	76	Low	III	P	N	Rectum	5.10
19	F	61	Medium	III	N	N	Rectum	3.97
20	M	78	Low	IV	P	N	Rectum	7.50
21	F	54	Low	IV	P	N	Colon	8.40
22	M	40	High	I	N	N	Rectum	0.88
23	F	73	Medium	I	N	N	Colon	3.20
24	F	73	Low	II	P	N	Rectum	3.60
25	F	80	Medim	I	N	N	Rectum	3.70
26	M	63	Low	II	P	N	Rectum	3.00
27	F	82	Low	III	N	N	Colon	80.20
28	M	50	Low	II	N	N	Rectum	1.70
29	M	67	High	I	N	N	Rectum	18.50
30	F	41	Low	III	P	N	Rectum	8.20
31	M	80	High	II	N	N	Rectum	2.10
32	M	52	Low	IV	P	N	Colon	3.30
33	F	37	Low	IV	P	N	Colon	16.50
34	F	74	Low	II	P	N	Rectum	3.40
35	M	79	Low	II	N	N	Rectum	9.20
36	M	50	Low	III	P	N	Colon	87.20
37	F	71	Low	IV	P	P	Rectum	99.20
38	F	49	Low	II	N	N	Colon	8.30
39	M	56	High	III	P	N	Rectum	1.40
40	F	41	Low	IV	P	P	Colon	12.43
41	F	51	Medium	II	N	N	Rectum	3.80
42	M	69	Low	III	P	N	Rectum	3.40
43	M	58	Medium	I	N	N	Rectum	2.10
44	F	53	High	I	N	N	Rectum	0.30
45	F	67	Low	II	N	N	Colon	2.30
46	M	62	Medium	I	N	N	Colon	3.30

47	F	66	Low	IV	P	N	Colon	3.20
48	F	43	Low	IV	P	N	Rectum	1.70
49	F	73	Medium	I	N	N	Rectum	1.00
50	F	63	Low	IV	P	P	Colon	>1000
51	F	40	Low	II	P	N	Colon	11.96
52	M	79	Low	IV	N	P	Colon	30.80
53	M	77	High	I	N	N	Colon	6.30
54	F	68	Low	IV	N	N	Rectum	41.80
55	M	40	Low	II	N	N	Colon	2.00
56	F	60	Low	III	N	N	Colon	3.20
57	F	72	High	III	P	N	Colon	17.62
58	M	67	Low	II	P	N	Colon	3.40
59	M	48	Low	III	N	N	Colon	3.77
60	F	65	Low	II	N	N	Colon	35.90
61	F	40	Low	III	P	P	Rectum	3.40
62	M	42	Low	III	P	P	Rectum	3.40
63	F	61	Medium	II	N	N	Rectum	10.50
64	M	44	Medium	II	N	N	Rectum	5.82
65	M	71	Low	II	N	N	Rectum	1.78
66	M	61	Low	II	P	N	Colon	75.10
67	M	39	Low	IV	P	P	Colon	20.44
68	F	62	High	III	N	N	Colon	5.20
69	F	37	Low	II	N	N	Rectum	1.50
70	M	54	Low	I	N	N	Rectum	10.47
71	F	57	Low	II	P	N	Colon	6.71
72	M	60	Low	II	P	N	Rectum	254.10
73	M	51	Low	IV	P	P	Rectum	1.85
74	M	50	Medium	I	N	N	Rectum	21.08
75	F	53	Low	III	P	P	Colon	10.44
76	M	64	Low	II	N	N	Rectum	10.54
77	M	57	Low	III	P	P	Colon	3.14
78	F	64	High	I	N	N	Rectum	3.10
79	F	40	Low	IV	P	P	Rectum	3.70
80	M	73	Medium	II	P	N	Rectum	6.72
81	F	80	High	I	N	N	Colon	2.80
82	F	71	Medium	II	N	N	Rectum	111.58
83	M	70	Low	I	N	N	Rectum	10.20
84	M	80	Low	I	N	N	Rectum	2.98
85	F	63	Low	II	P	N	Colon	20.70
86	M	47	Medium	II	P	N	Rectum	3.85
87	F	49	Low	IV	P	P	Colon	12.87
88	F	55	High	I	N	N	Rectum	10.20
89	M	64	Low	II	P	N	Rectum	5.12
90	F	54	High	III	N	N	Rectum	2.20
91	F	61	Medium	I	N	N	Colon	80.70
92	F	57	High	I	N	N	Rectum	2.60
93	F	60	High	II	N	N	Colon	3.97
94	M	48	Medium	I	N	N	Rectum	4.31
95	F	45	High	I	N	N	Rectum	2.20

96	F	58	Low	IV	P	P	Colon	11.84
97	M	55	Low	III	P	P	Colon	10.58
98	M	67	Medium	I	N	N	Colon	2.10
99	F	69	Low	II	N	N	Rectum	0.17
100	M	62	Low	IV	P	P	Colon	1.65

Note: F: Female; M: Male; N: Negative; P: Positive.

might leads to CRC development and progression [5-9]. And specific signatures of deregulated miRNAs highlight the putative diagnostic and therapeutic potentials in CRC [10, 11].

MicroRNA-375 (miR-375) was first identified as a pancreatic islet-specific miRNA regulating insulin secretion [12]. However, further studies revealed that miR-375 is a multifunctional miRNA participating in pancreatic islet development, glucose homeostasis, mucosal immunity, lung surfactant secretion and more importantly, tumorigenesis [13]. Recently, miR-375 has been proved to be down-regulated in multiple types of human cancers, including pancreatic cancer, gastric cancer and CRC [14-16]. Epigenetic regulation is an important mechanism governing miRNA expression. It was reported that aberrant promoter methylation of miR-375 gene add to the reasons why miR-375 is frequently dysregulated in different cancer types [13, 17-19]. While the exact mechanisms that determine its down-regulation in CRC are still elusive.

In this study, we performed bioinformatics analysis as well as DNA methylation-specific PCR to characterize its methylation status of its CpG islands in both CRC tissues and cell lines. We further proved that miR-375 significantly repressed cell growth *in vitro*, and validated YAP1 as one of its direct targets. Our findings demonstrated that epigenetic modifying constitutes an important pathway for the decreased expression of tumor suppressor miR-375 in CRC, which may eventually guide the future treatment for CRC.

Methods

Patients

A total of 100 patients with CRC who underwent surgery without preoperative treatment at the Affiliated Tumor Hospital of Zhengzhou University (Tumor Hospital of Henan Province), from March 2005 to December 2008, were included in this study. The receiving tumor tissues and paired adjacent normal tissues were immediately frozen in liquid nitrogen and stored at -80°C. All surgical tumor tissues were examined by a pathologist and final surgical pathology reports were obtained and recorded. The clinicopathological features of the patients, including gender, age, differentiation extent, TNM stage *et al.* were shown in **Table 1**. All samples were obtained with informed written consent prior to the start of the study. This study was approved by the medical ethics committee of the Affiliated Tumor Hospital of Zhengzhou University.

Cell culture

Two human CRC cell lines (HCT116 and SW620) and a non-malignant colon mucosa cell line FHC were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were routinely cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 100 U/ml penicillin and 0.1 mg/ml streptomycin, in a humidified atmosphere of 5% CO_2 at 37°C. Cells were cultured in 10 cm culture dishes and allowed to grow approximately 70% confluence before experimentation.

Nucleic acid isolation

Total RNA was isolated by using Trizol (Invitrogen, USA), according to the manufacturer's instructions and was quantified using absorption measurements at 260 nm. cDNA was synthesized by using an Expand Reverse Transcriptase Kit (Takara, China), using the indicated reverse transcripting primers. Genomic DNA was extracted from 25 mg of frozen tissues using the AxyPrep Multisource Genomic DNA Miniprp Kit (Axygen, China) and was quantified spectrophotometrically at 260 nm.

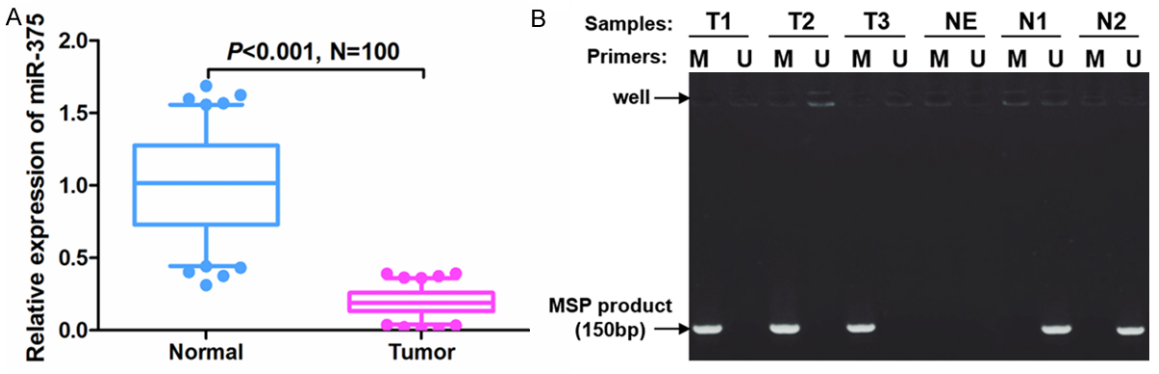

Figure 1. Decreased expression of miR-375 and its promoter methylation status in paired clinical CRC tissues. A: qRT-PCR detection of miR-375 in 100 paired normal and tumoral CRC tissues showed its significantly decreased expression. Results are presented as fold differences based on 2^{-DDCt} calculations, obtained from three independent experiments. Error bars represent the standard deviation (SD). P<0.001, N=100. B: U- and M-MSP analysis of miR-375 gene promoter showed that 3 CRC tumors (T1-T3) was completely methylated while 2 normal controls (N1, N2) were completely unmethylated. NE indicates a negative control.

Quantitative real-time PCR (qRT-PCR)

Quantitative Real-time PCR (qRT-PCR) was performed to analyze the expression of mature miR-375, using an ABI Prism 7500 Fast Real-time PCR system (Applied Biosystems, Foster City, CA, USA) with the SYBR Green PCR Master Mix (Applied Biosystems, USA) in accordance with the manufacturer's instructions. U6 snRNA was used as the internal control. The data was analyzed using comparative $2^{-\Delta\Delta Ct}$ method.

5-Aza-2'-deoxycytidine (5-AzadC, DAC) treatment

HCT116 and SW620 cells were seeded into 6-well plates at 50-60% confluence and cultured with 1 μM of DAC (Sigma-Aldrich, USA) for 3 days respectively. Fresh DAC containing medium was replaced every 24 hours. These two cell lines untreated or treated by DAC were harvested, for following experiments, including MSP and qRT-PCR.

Methylation-specific PCR (MSP)

Prior to the MSP detection, DNA extracted in the above steps were treated with bisulfite for conversion of unmethylated cytosine (C) to uracil (U) (but unaffecting methylated cytosine) by using the EpiTect Bisulfite Kit (QIAGEN, Germany). Then MSP procedures for aberrant DNA methylation was performed as described previously [20]. Briefly, after purification of genomic DNAs, each sample was amplified with two sets of primers. One set was for methylated DNA (M-MSP), and another set was for unmethylated DNA (U-MSP). These primers were designed by an online tool named MethPrimer (http://www.urogene.org/methprimer/). Sequences were in detail as follows: M-MSP Forward: 5'-AGCGGCGTATAGTTTTTTTTATTC-3'; M-MSP Reverse: 5'-CGAACCTAAACGTTTTATTCGTT-3'; U-MSP Forward: 5'-TGGAGTGGTGTATAGTTTTTTTTATTT-3'; U-MSP Reverse: 5'-ACCAAACCTAAACATTTTATTCATT-3'.

MTT assay and colony formation assay

Cells were seeded at a density of 2×10^5 cells/mL in a 6-well plate and incubated with 100 nM of miRNA control or miR-375 mimics, or mocked for 48 hr at 37°C. Afterwards, these transfected cells were seeded into 96-well plates for MTT assay. 20 μL MTT (5 mg/ml) was added to each well and the cells were incubated for another 4 h. Absorbance was read at 490 nm with a 96-well plate reader following the addition of DMSO. For colony formation assay, cells incubated with miRNA control or miR-375 mimic, or mocked, were seeded at a low density of 2×10^3 cells/well in 6-well plates. 10 days later, cells were fixed with methanol and stained with 0.1% crystal violet. Pictures were obtained by a digital camera. miRNA control or miR-375 mimics were synthesized by Shanghai GenePharma Co., Ltd (Shanghai, China).

Transfection, plasmid construction and luciferase assay

Cell transfection was performed using Lipofectamine 2000 (Invitrogen, USA), following the

Table 2. Association between the expression of miRNA-375 with clinicopathological features of CRC patients

Features	No.	Expression of miRNA-375[a]	*P* value
Gender			
Male	50	0.0482±0.0923	0.560
Female	50	0.0361±0.1135	
Age (years)			
<50	26	0.0377±0.0249	0.282
≥50	74	0.0512±0.0986	
Differentiation extent			
High-Medium	37	0.0845±0.0938	0.002
Low	63	0.0311±0.0241	
TNM stage			
I+II	62	0.0527±0.0715	0.209
III+IV	38	0.0294±0.0985	
Lymphnode involvement			
Positive	41	0.0491±0.1004	0.390
Negative	59	0.0665±0.0973	
Organ metastasis			
Positive	16	0.0223±0.0514	0.078
Negative	84	0.0513±0.0912	
Tumor location			
Colon	42	0.0409±0.1107	0.548
Rectum	58	0.0523±0.0612	
CEA			
<3.5 μg/L	41	0.0475±0.0614	0.506
≥3.5 μg/L	59	0.0564±0.0711	

Note: a, Expression of miR-375 was determined by qRT-PCR and calculated by the 2^{-DDCt} method.

Table 3. Methylation status of miR-375 gene promoter in CRC tissues

Group	No.	Methylation status		χ^2	*P* value
		Positive	Negative		
CRC tumors	100	66	34	27.4486	.000
CRC controls	100	29	71		

manufacturer's instruction. Selected fragments of the wide-type 3'-UTRs of YAP1 containing predictive miR-375 binding sites, and the mutated isoforms were synthesized downstream of the firefly luciferase gene in the pmirGLO vector (Promega, USA). For the luciferase reporter assays, cells were seeded in 24-well plates. Luciferase reporter vectors were cotransfected with miRNA control or miR-375 mimics using Lipofectamine 2000. 48 hrs later, the cells were harvested and assayed with the Dual-Luciferase Assay (Promega, USA). Experiments were performed at least three times and the results are expressed as relative luciferase activity (Firefly luciferase activity/Renilla luciferase activity).

Western blot

Cells were incubated with miR-375 mimics for 48 h, washed with PBS, and the cell pellets were prepared in RIPA buffer added with protease inhibitors Na_3VO_4 and leupeptin. Lysates were electrophoresed on SDS-PAGE and transferred to PVDF membranes. Membranes were blocked for 1 h at room temperature with 5% milk protein, 0.1% Tween-20 in PBS, and then were probed with rabbit anti-YAP1, or mouse anti-Tubulin antibodies at 1:1000 dilution overnight at 4°C. Both antibodies were purchased from Santa Cruz Biotechnology. After washing, the membranes were probed with HRP-conjugated goat anti-rabbit/mouse antibody at 1:5000 dilution for 1 h. The blots were finnaly visulized with the Phototope HRP Western Blot Detection system (Cell Signaling Technology, USA).

Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Each experiment was repeated at least three times. The χ^2 test was used for analyzing the association of miR-375 methylation status with continuous variables (including gender, age, differentiation extent, TNM stage, lymphnode involvement, organ metastasis, tumor location and CEA levels). The two-tailed Student's t-test was used to assess the differences between two groups of data. Statistical analysis was performed using SPSS 18.0 software (SPSS Inc., USA). Statistical significance was accepted at $P<0.05$.

Results

Expression of miR-375 in CRC tissues and its clinical significance

It was known that miR-375 was decreased in CRC [16]. To validate this, we firstly applied qRT-PCR to determine its expression in 100 pairs of CRC tissues. It came out a consistent significant downregulation of miR-375 expression in tumor tissues than that of adjancent non-can-

Table 4. The relationship of miRNA-375 gene methylation with clinicopathological features of CRC patients

Clinicopathological characteristics	No.	MiRNA-375 gene methylation		Positive Rate	χ^2	P
		Positive	Negative			
Gender						
Male	50	34	16	68.0%	0.1783	0.6729
Female	50	32	18	64.0%		
Age (years)						
<50	26	17	9	65.4%	0.0059	0.9386
≥50	74	49	25	66.2%		
Differentiation extent						
High-Medium	37	18	19	48.6%	7.8796	0.0050
Low	63	48	15	76.2%		
TNM stage						
I+II	62	43	19	69.4%	0.8183	0.3657
III+IV	38	23	15	60.5%		
Lymphnode involvement						
Positive	41	27	14	65.9%	0.0007	0.9795
Negative	59	39	20	66.1%		
Organ metastasis						
Positive	16	8	8	50.0%	2.1730	0.1405
Negative	84	58	26	69.0%		
Tumor location						
Colon	42	25	17	59.5%	1.3534	0.2447
Rectum	58	41	17	70.7%		
CEA						
<3.5 μg/L	41	28	13	68.3%	0.1628	0.6866
≥3.5 μg/L	59	38	21	64.4%		

cerous tissues (P<0.001). Pair-wise comparison revealed that the expression level of miR-375 in CRC tumors was 0.197 fold of that in matching controls (**Figure 1A**). To value its clinical significance, we analyzed the association of miR-375 levels with clinicopathological features of these patients. As shown in **Table 2**, miR-375 expression was significantly correlated with differentiation extent of tumor (P= 0.002), in which lower miR-375 levels correlated with lower differentiation extent. However, miR-375 expression was not associated with gender, TNM stage, lymphnode involvement, organ metastasis, tumor location and CEA levels (P>0.05). Taken together, these results supports a decreased expression of miR-375 and its vital clinical value in CRC as had been referred to previously.

Methylation status of miR-375 gene promoter in CRC tissues

Methylation plays an important role in regulating gene expression, including the expression of miRNA. Recently, some studies have shown that the promoter methylation is closely related to the miRNA expression [21, 22]. In addition, we used the CpG island online prediction tool (http://www.ebi.ac.uk/Tools/emboss/cpgplot/) and found that there are several CpG islands in the miR-375 gene promoter region. Since CpG islands are the structural basis for regulation by methylation, thus, in the current study, we hypothesized that DNA methylation was a major cause of the reduced expression of endogenous miR-375. To verify this hypothesis, we assessed the methylation level of the miR-375 gene promoter by MSP. The result showed that the methylation proportion of miR-375 was significantly higher in CRC tissues than those of corresponding normal controls (66/100 vs 29/100, P<0.0001) (**Figure 1B**; **Table 3**). And expected MSP result (no product) was obtained in the negative control (NE, blank control) (**Figure 1B**). The high methylation status was negatively correlated with miR-375 gene expression. Therefore, we think that the

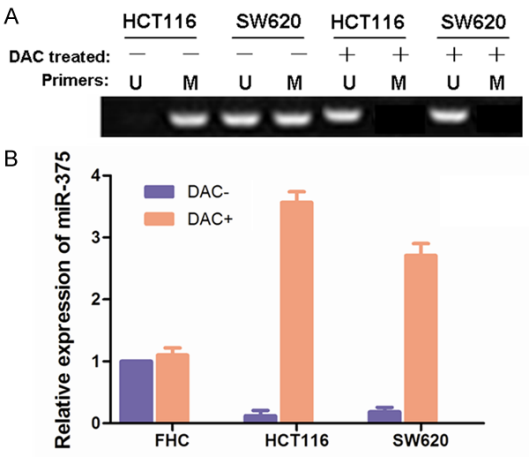

Figure 2. miR-375 is highly methylated and re-expressed by DAC treatment in CRC cell lines. A. The methylation status of miRNA375 in HCT116 and SW620 cell lines was detected using MSP method. Indicated cells were harvested and DNA was extracted for further MSP analysis. (-: Samples untreated by DAC; +: Samples treated by DAC; M: result of MSP of methylated status; U: result of MSP unmethylated status). B. MiR-375 was significantly increased in HCT116 and SW620 cells after treated with DAC compared with untreated cells. After treated by 1 mM DAC, cells were harvested and miR-375 expression was determined by qRT-PCR. The miR-375 level in FHC cells untreated by DAC (DAC-) was used for normalization.

hypermethylation of the miR-375 gene promoter may be responsible for the decreased expression of miR-375 in CRC tissues.

Association of miR-375 methylation with clinicopathological features

We next set out to judge the clinical significance of miR-375 gene promoter methylation in CRC patients. As described above in **Table 2**, we also analyzed the association of miR-375 DNA methylation with the clinicopathological features of these 60 CRC patients. As shown in **Table 4**, a significant correlation was observed between miR-375 methylation frequency with differentiation extent of tumors (P=0.005). However, we did not observe remarkable associations of methylation frequency of miR-375 gene promoter with other features of patients, including gender, age, TNM stage, lymphnode involvement, organ metastasis, tumor location and CEA levels (P>0.05). This was in line with the results in **Table 2**. Hence, our findings suggest that, hypermethylation in miR-375 gene promoter may contribute to its downregulation in CRC, both of which are correlated with tumor differentiation extent in CRC.

DAC treatment of CRC cell lines reveals a methylation regulation of miR-375

Then we want to further examine whether miR-375 gene was functionally methylated in CRC cells, and we treated the CRC cell lines with DAC, a specific inhibitor of DNA methylation. As shown by MSP method in **Figure 2A**, after treated with DAC, the methylation level of miR-375 gene promoter was significantly elevated. Specifically, endogenous completely methylation was shifted to totally unmethylation in HCT116 cells. To the contrast, partial methylation was altered to become entirely unmethylation. Therefore, DAC treatment resulted in demethylation of miR-375 gene promoter. We then quantified the expression of miR-375 before and after DAC treatment in HCT116, SW620 CRC cell lines and the FHC normal colon epithelial cell line. As shown in **Figure 2B**, in untreated normal groups, the expression of miR-375 was decreased in HCT116 and SW620 cells compared with that in FHC cells. However, DAC treatment significantly restored and even markedly up-regulated its expression in HCT-116 and SW620 cells. All of these results demonstrates that the DNA hypermethylation suppresses miR-375 expression and this downregulation can be significantly reversed by DAC treatment.

Cell growth inhibition by miR-375 overexpression in CRC cells

To investigate the role of miR-375 in CRC cells, we increased the endogenous levels of miR-375 in HCT116 and SW620 cells by mimics transfection (**Figure 3A**). Relative to miRNA control mimic, the miR-375 mimic-transfected cells showed reduced cell proliferation activity, as determined by MTT assay (**Figure 3B**). In addition, we analyzed the colony formation ability of these transfected cells. As shown in **Figure 3C**, forced expression of miR-375 resulted in a marked decrease of colony number. Thus, our data indicates that miR-375 inhibits cell growth, which has been discussed in a previous report [16].

YAP1 is a direct target of miR-375 in CRC cells

To elucidate the underlying mechanisms by which miR-375 executes its function, we ana-

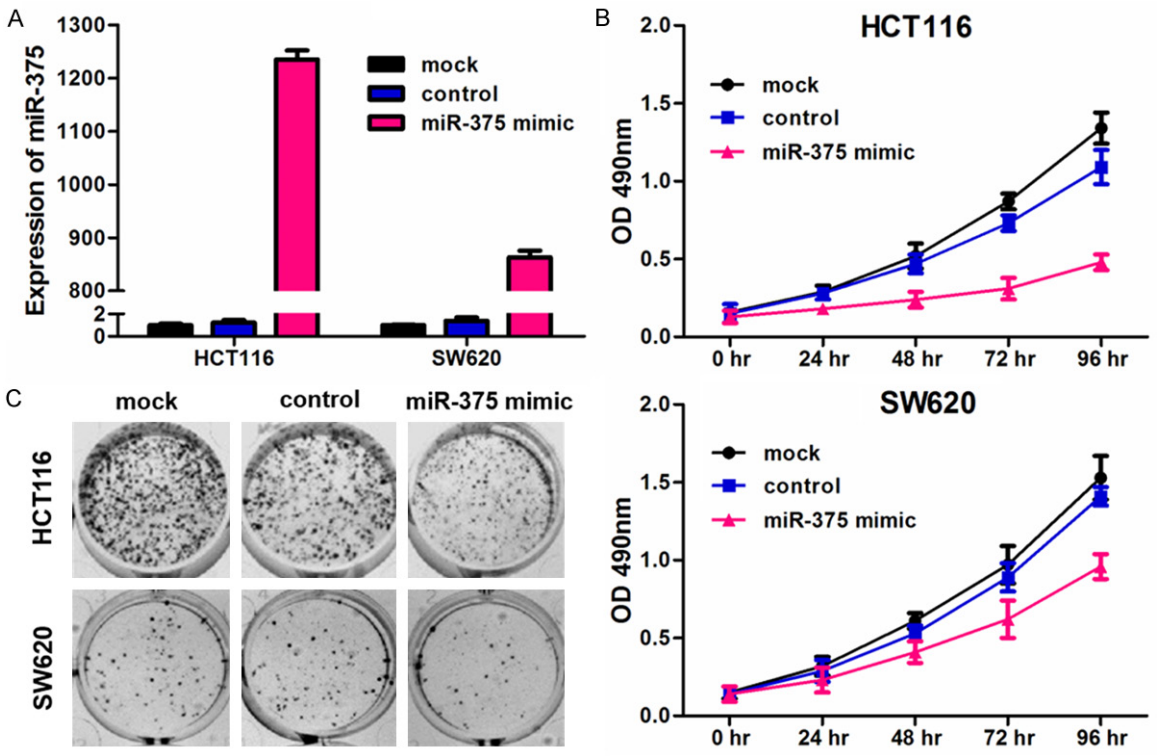

Figure 3. The growth inhibiting effect of miR-375 in CRC cells. A. HCT116 and SW620 cells were transfected with miRNA control or miR-375 mimic. The miR-375 expression levels were detected by qRT-PCR. B. MTT assay was used to measure the cell proliferation activity after miR-375 was forced expressed in the two cell lines. Data represented the mean ± SD from three independent measurements. C. Cell colony formation was performed in the indicated cells.

lyzed changes in the expression profiles of target genes for miR-375 predicted with TargetScan (http://www.targetscan.org/). In particular, we focused on growth related genes. We found that there were two putative binding sites in the 3'-UTR of YAP1 (**Figure 4A**). YAP1 was selected also because it is a growth inhibitor that has previously been shown to be negatively regulated by miR-375 in small cell carcinoma of the lung [23]. To test whether miR-375 directly targeted YAP1 in CRC cells, we constructed luciferase reporter genes containing wild type (wt1, wt2) or mutated (mu1, mu2) miR-375 binding sites into the pmirGLO luciferase vector. Nucleotide changes for binding site mutants were indicated in **Figure 4A**. As shown in **Figure 4B**, a decrease in relative luciferase activity was noted when the YAP1 3'-UTR was co-transfected with the miR-375 mimic. However, no decrease in relative luciferase activity was observed in CRC cells cotransfected with mutant 3'-UTR. Furthermore, overexpression of miR-375 led to a significant decrease in YAP1 expression at the protein level (**Figure 4C**) in both HCT116 and SW620 cells. These results suggests that 3'-UTR of YAP1 is a functional target of miR-375 in CRC cells.

Discussion

The present study showed that miR-375 gene promoter was hypermethylated in CRC tissues and cells. Corresponding to these, the expression level of miR-375 was significantly decreased in CRC. Statistical analysis revealed that both methylation frequency and the expression level of miR-375 were significantly correlated with differentiation extent of CRC tumors. Furthermore, by treating with DAC in CRC cells, the methylation levels of the miR-375 gene was decreased significantly, and the expression level of miR-375 was upregulated. In summary, our results demonstrated that the DNA hypermethylation of the miR-375 gene was a major cause for the down-expression of endogenous miR-375, which may provide a novel therapeutic strategy for human CRC treatment.

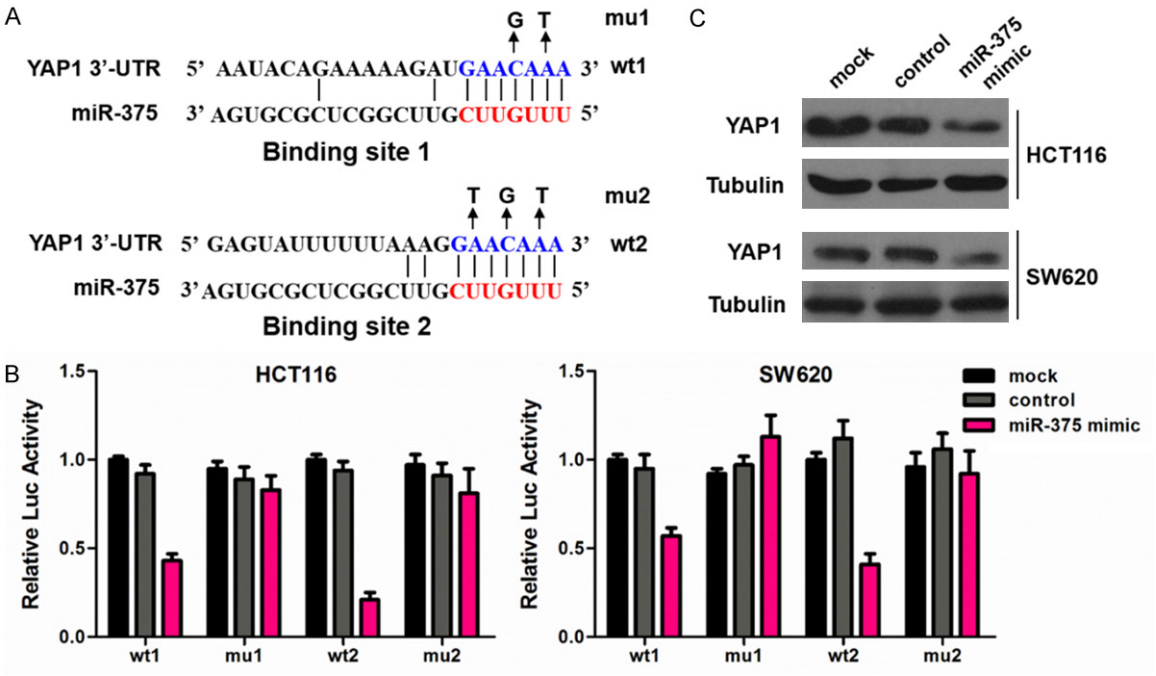

Figure 4. Validation of YAP1 as a target of miR-375 in CRC cells. A. Putative two binding sites for miR-375 in 3'-UTR of YAP1 gene. Two wild-type 3'-UTRs containing miR-375 binding sites were represented as wt1 and wt2. Mutations for binding site are indicated (mu1 and mu2). B. Relative activity of the luciferase reporter fused with the wt1/2 or mu1/2 3'-UTR of YAP1 gene. HCT116 and SW620 cells were cotransfected with miRNA control or miR-375 mimic. The luciferase activities were measured 24 h later. C. YAP1 protein expression levels in miR-375 mimic-transfected HCT116 and SW620 cells, as detected by western blotting. Tubulin was used as the internal loading control.

The occurrence, development, diagnosis and prognosis have become hot topics in the current clinical cancer research. Much effort has been paid to reveal the biological pathogenesis of CRC and to discover specific biomarkers for better diagnosis and prognosis of CRC. Nowadays, numerous studies have revealed that aberrant expression of miRNAs can function as oncogenes or tumor suppressors and play important roles in the development and progression of CRC [24, 25]. Nevertheless, the mechanism leading to aberrant expression of miRNAs has not been fully characterized. DNA methylation of the promoter region represented as an epigenetic mechanism that could repress the transcription of downstream genes [26]. Meanwhile, related studies have reported that DNA methylation in the promoter regions of miRNA genes is a possible mechanism responsible for its downregulation in cancer [27]. In addition, in the previous studies, epigenetically silencing of miR-375 have been proved in breast cancer, esophageal cancer and prostate cancer [28-30]. However, the molecular regulation mechanisms by which endogenous miR-375 expression is down-regulated in CRC cells remain to be clearly elucidated.

Interestingly, we noted a significant association of miR-375 methylation with differentiation extent of CRC tumors (hypermethylation frequency in low differentiated tumors), which is a poor risk factor for survival. Similarly, miR-375 expression was also shown to be associated with low differentiation extent. These findings also indicated that hypermethylation induced deregulation of miR-375 contributed to lower CRC cell differentiation. Therefore, the higher methylation level of miR-375 was an important reason and mechanism for its decreased expression in CRC cells. At cellular levels, we verified that miR-375 could suppress cell growth. Furthermore, we validated in CRC cells that miR-375 directly targeted YAP1. Hence, our findings support a tumor suppressor role of miR-375 in CRC cells by targeting YAP1, although the evidence we had provided was still a bit shortened.

In conclusion, this study highlights that the hypermethylation status of miR-375 in CRC tis-

sues contributes to its downregulation in CRC. Although there are some limitations, the study still provides useful information on promoter methylation pattern of miR-375 in CRC. This epigenetic dysregulation of miR-375 also constitutes a vital mechanism implicated in the development of CRC and will provide new strategies for prevention and treatment of CRC through developing new DNA demethylating agents. Further study is needed to fully elucidate the mechanism of the hypermethylation of this miRNA, as well as the molecular mechanisms that governing its growth inhibiting activity in CRC cells.

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Disclosure of conflict of interest

None.

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