

miR-6716-5p promotes metastasis of colorectal cancer through downregulating NAT10 expression

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Background: Human N-acetyltransferase 10 (NAT10) plays pivotal roles in cellular biological processes, such as senescence, autophagy and cytokinesis. The expression of NAT10 is dysregulated in colorectal cancer (CRC) and is associated with the prognosis of patients. However, it remains unclear how NAT10 is regulated in CRC.

Methods: The microRNA(miRNA) regulating NAT10 was predicted by bioinformatics analysis and further validated by real-time quantitative PCR(RT-qPCR), Western blot and dual luciferase reporter assays. The expression of the miRNA regulating NAT10 in CRC tissues was examined using RT-qPCR. Cell proliferation, cell apoptosis, cell migration and cell invasion assays were performed after transfection with miRNA mimic and inhibitor.

Results: Here, we report that miR-6716-5p inhibits the expression of NAT10 in CRC. The NAT10 protein level was downregulated by the miR-6716-5p mimic, and was upregulated by the miR-6716-5p inhibitor in CRC cell lines. In addition, miR-6716-5p bound to the 3'-untranslated region of NAT10 mRNA and decreased NAT10 mRNA levels. Significantly, the miR-6716-5p level was higher in the tumor tissues of the CRC patients with liver metastasis than that in the non-metastatic CRC patients. In addition, the miR-6716-5p level was correlated with poor overall survival of CRC patients with liver metastasis. The miR-6716-5p inhibitor inhibited CRC cell migration and invasion. Consistently, the miR-6716-5p mimic significantly promoted cell migration and invasion, and this effect is dependent on NAT10. However, miR-6716-5p had no effect on CRC cell proliferation and apoptosis. We found that miR-6716-5p negatively regulated E-cadherin protein levels. In addition, E-cadherin was upregulated by NAT10 in CRC cells, confirming that miR-6716-5p downregulated E-cadherin levels by inhibiting NAT10 expression.

Conclusion: We demonstrated that miR-6716-5p acts as a crucial regulator of NAT10 to promote cell migration and invasion in CRC by inhibiting NAT10 expression. Our data suggest that miR-6716-5p/NAT10 might act as a potential therapeutic target for CRC treatment.

Keywords: miR-6716-5p, NAT10, colorectal cancer; CRC, liver metastasis

Introduction

Colorectal cancer (CRC), one of the most common malignancies, is the second leading cause of cancer-related death worldwide. It has been estimated that 1.8 million of CRC cases are diagnosed and approximately 860,000 patients die of CRC annually.¹ The 5-year survival rate of CRC patients with tumors confined to the colorectum is about 90%, while either lymphatic or distant metastasis decreases the 5-year survival rate of the CRC patients to range from 72% to 13%.² Tumor

metastasis is the major cause of mortality in CRC patients.³ Liver is the most common metastatic site of CRC.⁴ Approximately 15% of the CRC patients are diagnosed with synchronous hepatic metastasis, and 50% of CRC patients developed metachronous hepatic metastasis after the primary tumor resection.⁵ The 5-year survival rate of CRC patients with liver metastasis is 25–40% even after resection of the metastatic lesions.⁶ Therefore, identification of the molecules acting as prognostic marker for CRC with liver metastasis and inhibiting cell invasion will contribute to improving the prognosis of patients.

MicroRNAs (miRNAs) are a class of small endogenous noncoding RNAs. Generally, miRNA is composed of about 22 nucleotides, and regulates gene expression by binding to the 3'-untranslated region (UTR) of its target mRNAs.⁷ It has been demonstrated that miRNAs play critical roles in cellular processes, such as cell proliferation, mitosis, apoptosis and differentiation.^{8–10} Numerous evidence have indicated that miRNAs possess tumor-suppressive or oncogenic activities through regulating their downstream targets in human cancers.^{8,11–13} MiRNAs are abnormally altered during the occurrence and development of CRC, and some of them show potential as diagnostic, prognostic or staging markers of CRC.^{14–17} For instance, miR-34a-5p has been identified as an independent prognostic factor for CRC recurrence.¹⁸ Serum miR-203 could be a promising prognostic and predictive biomarker for CRC metastasis.¹⁹ Therefore, the study of the functions of miRNAs in the metastasis or recurrence of CRC would provide more markers for improving the clinical outcome of CRC patients.

NAT10 belongs to the Gcn5-related N-acetyltransferases family and possesses histone acetyltransferase (HAT) activity. It was initially found that NAT10 could upregulate telomerase activity through the transactivation of human telomerase reverse transcriptase promoter.²⁰ NAT10 controls various cellular processes via its acetyltransferase activity. For instance, NAT10 binds to hsSUN1 and regulates the decondensation of chromosomes during mitosis.²¹ NAT10 also controls cytokinesis through acetylating α -tubulin.²² NAT10 activates RNA polymerase I transcription by binding to and acetylating the upstream binding factor in the nucleolus.²³ Recently, it was found that NAT10 functions in the rRNA processing through acetylating rRNA.²⁴ In addition, NAT10 also acetylates tRNA.²⁵ Importantly, NAT10 promotes translation efficiency through acetylating cytidine in mRNA.²⁶ In response to DNA damage, NAT10 activates p53 through the acetylation of p53 K120 to maintain cellular homeostasis.²⁷ Under energy stress, the deacetylation of NAT10 by Sirt1 promotes the transition

from rRNA biogenesis to autophagy.²⁸ Recently, it was reported that chemical inhibition of NAT10 acetyltransferase activity could correct defects of laminopathic cells and treat progeria.²⁹ These findings have identified NAT10 as a key regulator of cellular activities. In addition, an increasing number of studies have reported that dysfunction of NAT10 is involved in tumorigenesis and tumor development. It has been reported that NAT10 is related with the patient's prognosis of CRC and hepatocellular carcinoma.^{30–32} NAT10 mRNA levels were significantly downregulated in CRC tumor tissues in comparison to the levels in normal tissues.²⁷ We previously demonstrated that the function of NAT10 is regulated by its autoacetylation.³³ However, it remains unclear how NAT10 is regulated in CRC.

In this study, we analyzed the miRNAs targeting NAT10 and identified miR-6716-5p as a critical regulator of NAT10 in the liver metastasis of CRC.

Materials and methods

Patients and follow-up

Human CRC tissues were obtained from 80 CRC patients who received surgical resection at Peking University Cancer Hospital and Institute from October 2010 to July 2015. The patients included 54 men and 36 women, with a median age of 57 years. Forty CRC patients only with liver metastasis belonged to stage IV and the remainder belonged to stage (I, II, III) according to the AJCC/UICC (seventh edition) TNM staging system. All patients provided written informed consent for their samples to be used in this study. This study was approved by the ethics committee of Peking University Cancer Hospital and was conducted in accordance with the Declaration of Helsinki. The patients were followed up once every 3 months during the first 2 years, once every 6 months during the third and fourth year and once a year from the fifth year postoperatively. The overall survival time was calculated from the operation date to the death date, and the recurrence or metastasis date or the last censor time were also recorded.

Cell culture and transfections

CRC cell lines (HCT116, LoVo) and HEK293T cell lines were purchased from the National Infrastructure of Cell line Resource (Beijing, People's Republic of China). Cells were cultured in DMEM with 10% fetal bovine serum. Cells were transfected with plasmid DNA or siRNA RNA duplexes (GenePharma, Shanghai, People's Republic of China) by Lipofectamine 2000 (Invitrogen, Carlsbad,

CA, USA) according to the manufacturer's protocols. MiRNA mimics or inhibitors and siRNA sequences are listed in the [Supplemental materials](#).

Luciferase assay

For the luciferase reporter assay, cells were seeded in 24-well plates at a density of 1×10^5 cells per well. The cells were cotransfected with pmirGLO luciferase reporters and either wild type or mutant NAT10 3'UTR plasmid, in combination with miR-6716-5p mimic or control mimic (GenePharma) using Lipofectamine2000 (Invitrogen). After 24 hrs, the firefly and Renilla luciferase activities were measured and analyzed according to the manufacturer's instructions (Promega, Fitchburg, WI, USA).

Cell viability assay

Cells were transfected with miR-6716-5p (mimic or inhibitor) or a negative control (GenePharma) using Lipofectamine 2000 (Invitrogen). After 48 hrs of transfection, a thousand of cells were seeded into 96-well plates and cultured with 10% serum containing media. Cell viability was analyzed using the MTS assay (Promega) according to the manufacturer's instructions. Absorbance at 490 nm was measured using a microplate spectrophotometer once a day for 5 consecutive days.

Colony formation assay

After 48 hrs of transfection, a thousand cells were seeded into 6-well plates and cultured with 10% serum containing media for about 12 days. Cells were fixed and then stained with crystal violet, and visible colonies were counted.

Apoptosis assay

Cells transfected with miR-6716-5p or negative control were harvested. The cells were stained with Annexin V-FITC and Propidium Iodide using an Annexin V-FITC Apoptosis detection kit (KeyGEN, BioTECH, Nanjing, People's Republic of China) according to the manufacturer's instructions. Then, the cells were analyzed using FACSCalibur (BD Bioscience). A total of 10,000 cells were acquired for each sample, and the data were analyzed using BD CellQuest software.

Transwell assay

Migration and invasion assays were performed according to the instructions of the manufacturer (Merck, Millipore). Briefly, 2×10^5 cells were plated in serum-free medium in the upper chamber with a non-coated

membrane (24 well insert, 8 μ m pore size) for the migration assay and with a Matrigel-coated membrane for the invasion assay. The lower chamber was filled with medium containing 10% serum. Then, the cells on the upper surface were removed by cotton swab and the cells on the lower surface were stained with crystal violet and observed under a microscope.

Western blotting analysis

Western blot experiment was carried out as described previously.²⁷ Anti-NAT10 was a gift from Dr B. Zhang. Anti-actin (abclonal, ac004) was purchased from ABclonal Technology. Anti-E-cadherin (BD, 562869) was purchased from BD Bioscience.

Real-time quantitative PCR (RT-qPCR)

RT-qPCR was performed on the ABI 7500 Fast Real-Time PCR systems (Applied Biosystems, Foster City, CA, USA). Total RNA and miRNA were extracted from CRC cells and tissues using MiPure Cell/Tissue miRNA Kit (Vazyme, Nanjing, People's Republic of China). cDNA was synthesized by Universal cDNA Synthesis Kit (Roche, USA). The primer mix contained a specific stem-loop primer of miR-6716-5p and a RNU6A (U6) reverse primer for cDNA synthesis of miRNA. Random hexamer primer was used to synthesize the cDNA of the mRNAs. RT-qPCR was performed using the FastStart Universal SYBR Green Master (Roche) in the ABI 7500 Fast Real-Time PCR system (Applied Biosystems). GAPDH and U6 were used as endogenous controls. A melting curve analysis was performed for the primer sets, and each showed a single peak indicating the specificity of the primers tested. Relative expression levels were computed using the $2^{-\Delta\Delta C_t}$ method. The primer sequences used are listed in the Supplemental Information.

Statistical analysis

Statistical analysis was performed using the SPSS software package (SPSS Standard version 21.0, SPSS Inc, Chicago, IL, USA). Shapiro–Wilk test was performed before using Student's *t* test and Mann–Whitney U test. Levene's test was also performed to evaluate variance homogeneity before using Student's *t* test and Mann–Whitney U test. The statistical significance of differences between groups was assessed by Student's *t* test or Mann–Whitney U test. The relationship between miR-6716-5p expression and the clinical pathological parameters was determined by the χ^2 test. Bonferroni correction was also

performed for determination of relations between miR-6716-5p expression and clinical pathological parameters. Kaplan–Meier analysis was used to evaluate CRC patient survival and a log-rank test was used to compare different survival curves. In cases of comparison of relative expression levels, Non-scaled values were used in the data analysis. The correlation between the expression of NAT10 and miR-6716-5p was analyzed by Spearman's rank correlation coefficient. Data were presented as the mean±SD. *P*-values <0.05 were considered statistically significant.

Results

NAT10 expression was regulated by miR-6716-5p

To uncover the potential miRNAs regulating the expression of NAT10, we analyzed the putative miRNAs which might bind 3'-UTR of NAT10 mRNA using two major prediction software programs, TargetScan (release 7.1, http://www.targetscan.org/vert_71/)³⁴ and DIANA-microT-CDS (5th version, <http://www.microrna.gr/microT-CDS>, the threshold was set to 0.55) (Figure 1A).³⁵ According to the miRNAs predicted by the above two software programs, we identified the common miRNAs between the two and finally obtained 63 putative miRNAs. According to the scores of the 63 putative miRNAs and the binding sequence in the 3'-UTR of NAT10 mRNA, which were obtained from DIANA-microT-CDS software, miR-376a-5p, miR-212-3p, miR-1468-3p and miR-6716-5p were selected as they might potentially bind to the 3'-UTR of NAT10 mRNA (Figure 1B and Figure S1). To determine if these miRNAs regulate NAT10 expression, we firstly evaluated the NAT10 protein level after transfecting different miRNA mimics in HCT116 cells. We showed that the NAT10 protein level was decreased by the miR-6716-5p mimic but not by the mimics of other miRNAs (Figure 1C and D). Similar results were obtained in LoVo cells (Figure 1E and F). These results indicated that NAT10 expression was regulated specifically by miR-6716-5p.

miR-6716-5p bound to the 3'-UTR of NAT10 mRNA to inhibit the expression of NAT10

To further confirm that the expression of NAT10 is regulated by miR-6716-5p, the protein and mRNA levels of NAT10 were evaluated when the miR-6716-5p mimic or inhibitor were transfected into HCT116 cells. NAT10 protein and mRNA levels were significantly decreased

by the introduction of miR-6716-5p mimic in the HCT116 cells (Figure 2A). Consistently, the miR-6716-5p inhibitor increased the NAT10 protein and mRNA levels in HCT116 cells (Figure 2A). Moreover, similar results were obtained in LoVo cells (Figure 2B). Thereafter, we determined whether miR-6716-5p binds to the 3'-UTR of NAT10 mRNA. The 3'-UTR of NAT10 mRNA or its mutant lacking the miR-6716-5p binding site was cloned into the pmirGLO luciferase reporter vector to generate pmirGLO-NAT10 3'-UTR and pmirGLO-NAT10 3'-UTRm (Figure 2C). The constructed plasmid was transfected with miR-6716-5p mimic into HEK293T cells, respectively. Indeed, the relative luciferase activity of the pmirGLO-NAT10 3'-UTR was dramatically reduced when co-transfected with the miR-6716-5p mimic in HEK293T cells (Figure 2D). Similar results were obtained in HCT116 cells (Figure 2E). These results indicated that miR-6716-5p bound to the 3'-UTR of NAT10 mRNA. To confirm this finding, we further analyzed the luciferase activity of pmirGLO-NAT10 3'-UTRm when co-transfected with the miR-6716-5p mimic. We showed that the miR-6716-5p mimic had no effect on the luciferase reporter activity of pmirGLO-NAT10 3'-UTRm (Figure 2D and E), confirming the binding site of miR-6716-5p in the NAT10 3'-UTR. Moreover, the relative luciferase activity of the pmirGLO-NAT10 3'-UTR was reduced by miR-6716-5p in a dose-dependent pattern in the HEK293T cells (Figure 2F). Taken together, these results indicated that miR-6716-5p directly bound to the 3'-UTR of NAT10 mRNA to inhibit NAT10 expression.

The miR-6716-5p level was correlated with liver metastasis and survival in CRC patients

To determine whether miR-6716-5p is related with CRC development, the miR-6716-5p levels were evaluated in human CRC tumor tissues. Firstly, we examined the clinic-pathological significance of miR-6716-5p expression in CRC patients. The median value of the miR-6716-5p levels in 80 CRC tumor tissues was set as the cutoff value. Based on this cutoff value, the CRC patients were divided into a high miR-6716-5p level group and a low miR-6716-5p level group. The characteristics of patients, including gender, age, primary tumor location, vascular invasion, differentiation, TNM stage, and lymph node metastasis, are listed in Table 1. We found that the

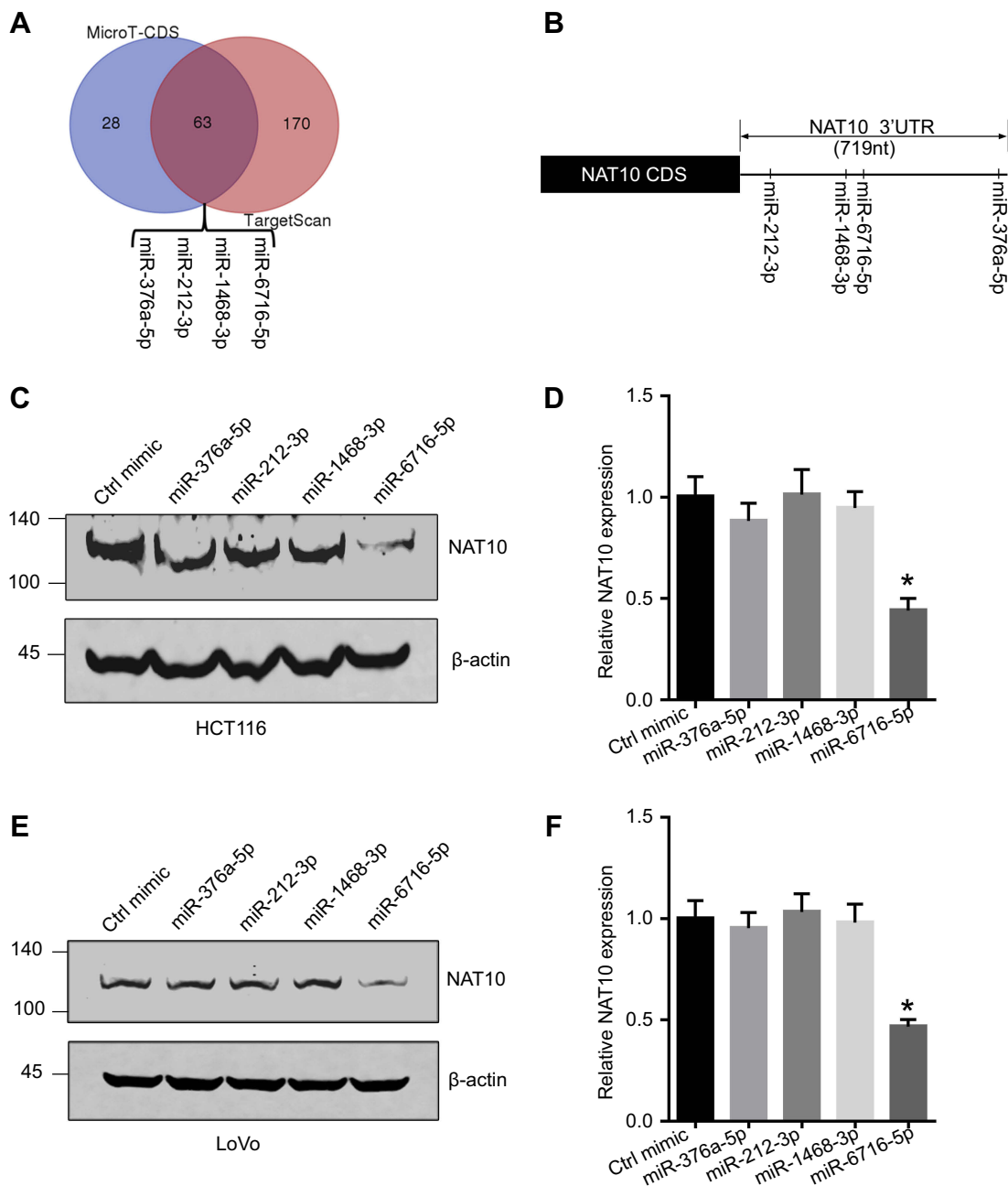


Figure 1 NAT10 expression was regulated by miR-6716-5p. (A) NAT10 might be regulated by miR-376a-5p, miR-212-3p, miR-1468-3p and miR-6716-5p according to two prediction software packages. (B) Illustration of binding site between putative miRNAs and the NAT10 3'-UTR was indicated. (C) HCT116 cells were transfected putative miRNAs mimics and harvested. NAT10 protein level was detected by western blotting and NAT10 protein level was quantified by image J software. (D) LoVo cells were transfected putative miRNAs mimics and harvested. NAT10 protein level was detected by western blotting and NAT10 protein level was quantified by image J software. Error bars represent the SD from three independent experiments. *P*-values were obtained from Student's *t* test. **P*<0.05.

incidence rate of vascular invasion in the high miR-6716-5p level group was significantly higher than that in the low miR-6716-5p level group ($P=0.019$), indicating that miR-6716-5p was correlated with vascular invasion (Table 1). The patients with high miR-6716-5p levels had a poor survival rate tendency though there was no significant difference in the overall survival (OS) rate between the

CRC patients with high miR-6716-5p levels and those with low miR-6716-5p levels ($P=0.0712$) (Figure 3A). Since miR-6716-5p was associated with vascular invasion (Table 1), we wanted to know if miR-6716-5p was related with the survival of patients with metastasis. Indeed, the miR-6716-5p levels in the CRC with liver metastasis (CRLM) patients were significantly higher than those in

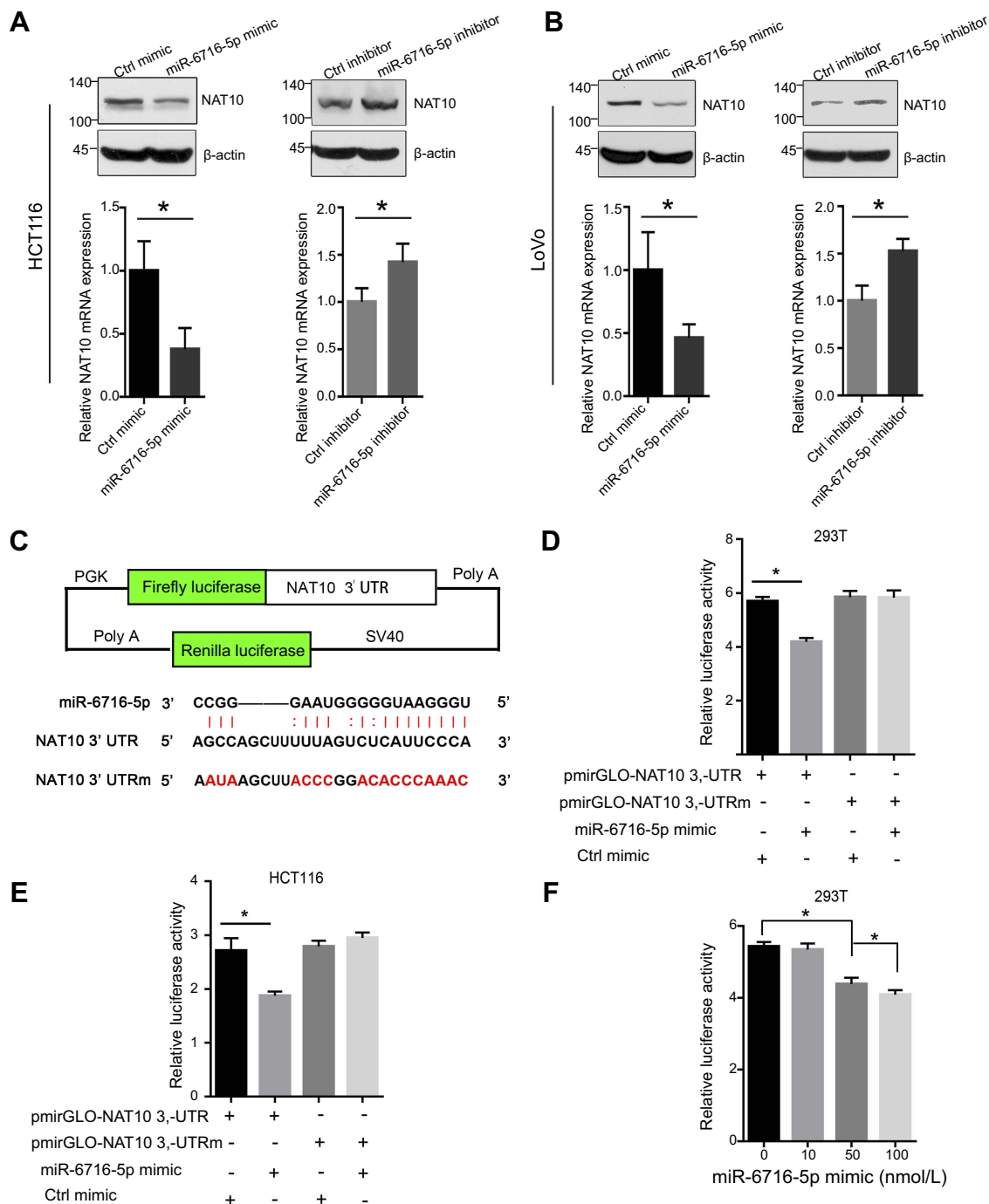


Figure 2 miR-6716-5p bound to the 3'-UTR of NAT10 mRNA to inhibit expression of NAT10. **(A)** HCT116 cells were transfected with either miR-6716-5p mimic or inhibitor. Seventy-two hours later, cells were harvested and lysed. Then, NAT10 protein and mRNA expression levels were detected by western blotting and RT-qPCR analysis, respectively. **(B)** LoVo cells were transfected with either miR-6716-5p mimic or inhibitor. Then, cells were harvested and total proteins were extracted. NAT10 protein and mRNA levels were detected by western blotting and RT-qPCR analysis, respectively. **(C)** Schematic diagram of pmirGLO luciferase reporter vector was illustrated and the predicted miR-6716-5p binding site in 3'-UTR wild-type sequence of NAT10 (pmirGLO-NAT10-3'UTR) and the designed mutant sequence (pmirGLO-NAT10-3'UTRm) were indicated. **(D)** The indicated vectors and miR-6716-5p mimic were transfected into HEK293T cells. Twenty-four hours later, cells were harvested and lysed. Then, the firefly and renilla luciferase activities were measured and analyzed according to the manufacturer's instructions. Luciferase activities were assessed and normalized. **(E)** HCT116 cells were transfected with the indicated vectors. Twenty-four hours later, the firefly and renilla luciferase activities were measured and analyzed according to the manufacturer's instructions. **(F)** HEK293T cells were transfected as indicated and cultured for 24 hrs. Then, the firefly and renilla luciferase activities were measured and analyzed according to the manufacturer's instructions. **(A, B)** Error bars represent the SD from three independent experiments. * $P < 0.05$. **(D-F)** Data were presented as the mean \pm SD ($n=6$, each group). P -values were obtained from Student's t test. * $P < 0.05$.

the non-metastatic CRC patients (Figure 3B). Importantly, the OS rate of the CRLM patients with low miR-6716-5p levels was higher than that of the CRLM patients with

high miR-6716-5p levels ($P=0.0396$) (Figure 3C). The characteristics of the CRLM patients are listed in Table 2. It was also found that the incidence rate of vascular

Table I Relationship between miR-6716-5p expression and clinical characteristics in colorectal cancer patients

Characteristics	No. of patients (%)	miR-6716-5p expression		χ^2	P
		Low n=40 (%)	High n=40 (%)		
Age				0.457	0.499
<60	45 (56.25)	21 (52.5)	24 (60.0)		
≥60	35 (43.75)	19 (47.5)	16 (40.0)		
Gender				0.808	0.369
Female	36 (45.0)	24 (60.0)	20 (50.0)		
Male	44 (55.0)	16 (40.0)	20 (50.0)		
Location				0.889	0.641
Left colon	25 (31.25)	11 (27.5)	14 (35.0)		
Right colon	38 (47.5)	19 (47.5)	19 (47.5)		
Rectum	17 (21.25)	10 (25.0)	7 (17.5)		
TNM stage				1.800	0.180
I II III	40 (50.0)	23 (57.5)	17 (42.5)		
IV	40 (50.0)	17 (42.5)	23 (57.5)		
Vascular invasion				5.495	0.019
Absent	52 (65.0)	31 (77.5)	21 (52.5)		
Present	28 (35.0)	9 (22.5)	19 (47.5)		
Lymph node metastasis				0.202	0.653
N0	36 (45.0)	19 (47.5)	17 (42.5)		
N1-2	44 (55.0)	21 (52.5)	23 (57.5)		
Histology differentiation				0.967	0.617
Poor	11 (13.75)	7 (17.5)	4 (10.0)		
Moderate	65 (81.25)	31 (77.5)	34 (85.0)		
Well	4 (5.0)	2 (5.0)	2 (5.0)		

invasion in the high miR-6716-5p level group was higher than that in the low miR-6716-5p level group in the CRLM patients ($P=0.015$). Moreover, the incidence of multiple liver metastases was significantly higher in the CRLM patients with high miR-6716-5p levels than in the CRLM patients with low miR-6716-5p levels ($P=0.007$). Taken together, these results indicated that high miR-6716-5p levels might be an unfavorable prognostic factor and indicated a poor OS rate in the CRLM patients.

Next, we investigated the relationship between miR-6716-5p levels and the NAT10 mRNA levels. Significantly, the NAT10 mRNA levels in the low miR-6716-5p level group were higher than those in the high miR-6716-5p level group ($P<0.01$) (Figure 3D). We found that there existed negative correlation between NAT10 mRNA and miR-6716-5p ($P<0.01$) and the correlation coefficient was -0.5331 according to Spearman's rank correlation coefficient (Figure 3E).

miR-6716-5p promoted cell migration and invasion by downregulating NAT10 expression

Since miR-6716-5p was correlated with the liver metastasis of CRC, we examined the effect of miR-6716-5p on the migration and invasion of CRC cells. Overexpression of miR-6716-5p significantly increased cell migration and invasion in HCT116 cells (Figure 4A). Meanwhile, inhibitor of miR-6716-5p decreased the cell migration and invasion (Figure 4A, B and C). Similar results were obtained in LoVo cells (Figure 4D, E and F). These results indicated that miR-6716-5p promoted the cell migration and invasion of CRC. To further determine if miR-6716-5p-caused metastasis depends on NAT10 expression, we overexpressed Flag-NAT10 in miR-6716-5p-expressing cells and determined migration and invasion of CRC cells. We showed that

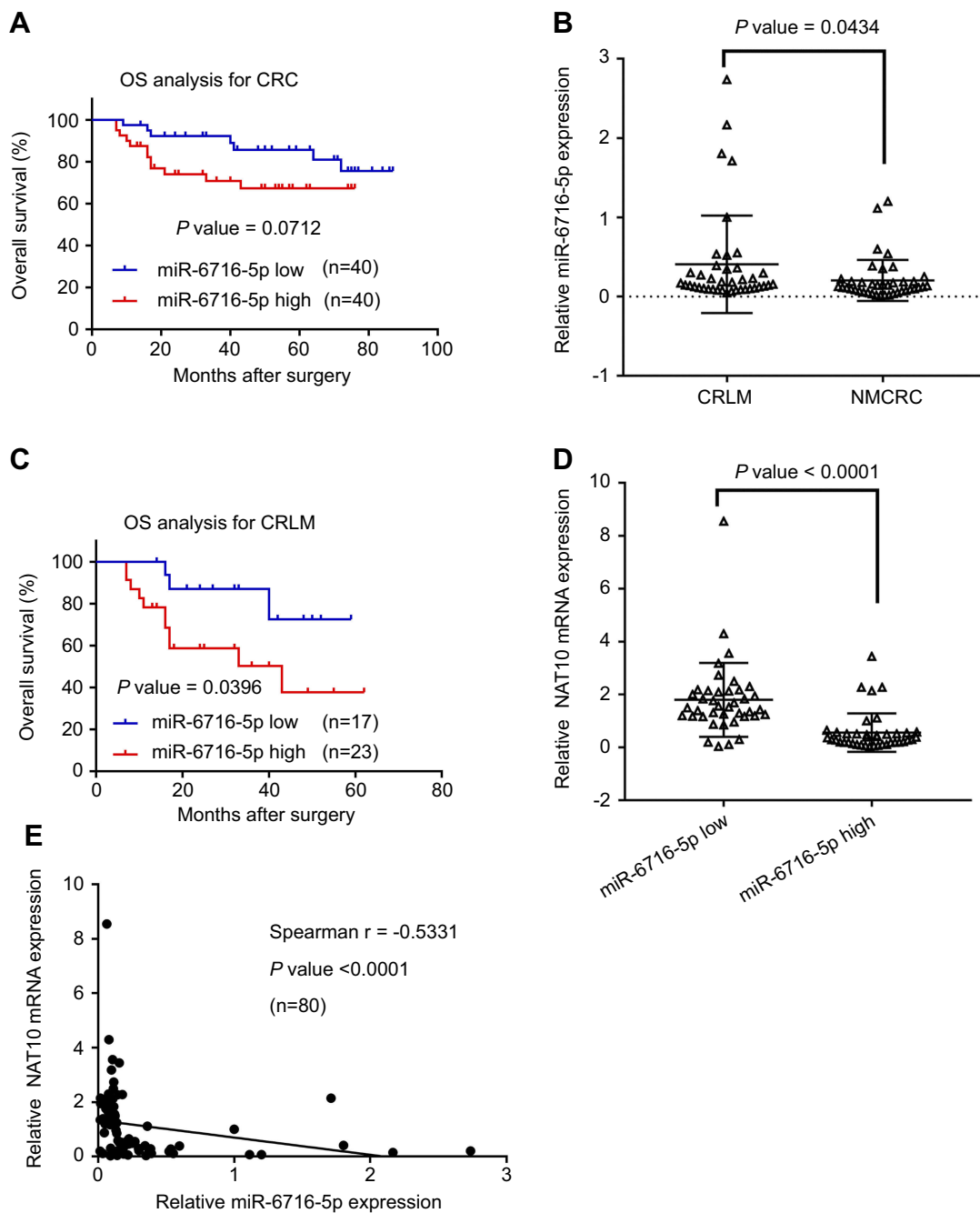


Figure 3 miR-6716-5p level was correlated with liver metastasis and survival of CRC patients. **(A)** Kaplan–Meier analysis for overall survival (OS) rate between miR-6716-5p high-expression group and miR-6716-5p low-expression group in CRC patients. **(B)** Relative miR-6716-5p level was analyzed between non-metastatic CRC (NMCRC) patients and CRC with liver metastasis (CRLM) patients. **(C)** Kaplan–Meier analysis for OS rate between miR-6716-5p high group and miR-6716-5p low group in CRLM patients. **(D)** Relative NAT10 mRNA expression was analyzed between miR-6716-5p high group and miR-6716-5p low group. **(E)** Correlation between miR-6716-5p level and NAT10 mRNA level was calculated according to Spearman's rank correlation coefficient in CRC specimen (n=80). CRLM represents CRC with liver metastasis. NMCRC represents non-metastatic CRC. **(B, D)** The Mann–Whitney U test was used to compare the levels of NAT10 mRNA or miR-6716-5p between groups and horizontal line was the mean value in two groups, respectively.

overexpression of miR-6716-5p significantly increased the migration and invasion of HCT116 cells, which was alleviated by overexpression of Flag-NAT10 (Figure 4G, H and I). These results indicated that the miR-6716-5p-induced increase of CRC migration and

invasion was dependent on NAT10. These results were further confirmed in LoVo cells (Figure 4J, K and L). Together, these findings demonstrated that miR-6716-5p promoted CRC metastasis by downregulating NAT10 expression.

Table 2 Relationship between miR-6716-5p expression and clinical characteristics in colorectal cancer with liver metastasis patients

Characteristics	No. of patients (%)	miR-6716-5p expression		χ^2	P
		Low n=17 (%)	High n=23 (%)		
Age				0.496	0.481
<60	26 (65.0)	10 (58.82)	16 (69.57)		
≥60	14 (35.0)	7 (41.18)	7 (30.43)		
Gender				0.051	0.822
Female	22 (55.0)	9 (52.94)	13 (56.52)		
Male	18 (45.0)	8 (47.06)	10 (43.48)		
Location				0.874	0.646
Left colon	14 (35.0)	7 (41.18)	7 (30.43)		
Right colon	22 (55.0)	9 (52.94)	13 (56.52)		
Rectum	4 (10.0)	1 (5.88)	3 (13.04)		
Vascular invasion				5.966	0.015
Absent	17 (42.5)	11 (64.71)	6 (26.09)		
Present	23 (57.5)	6 (35.29)	17 (73.91)		
Lymph node metastasis				0.349	0.555
N0	11 (45.0)	6 (35.29)	5 (21.74)		
N1-2	29 (55.0)	11 (64.71)	18 (78.26)		
Histology differentiation				0.644	0.725
Poor	4 (13.75)	1 (5.88)	3 (13.04)		
Moderate	32 (81.25)	14 (82.35)	18 (78.26)		
Well	4 (5.0)	2 (11.76)	2 (8.70)		
Liver metastasis size				0.628	0.428
≤3(cm)	23 (57.5)	11 (64.71)	12 (52.17)		
>3(cm)	17 (42.5)	6 (35.29)	11 (47.82)		
Number of liver metastases				7.376	0.007
1	14 (35.0)	10 (58.82)	4 (17.39)		
>1	26 (65.0)	7 (41.18)	19 (82.61)		

miR-6716-5p had no effect on cell proliferation and apoptosis

It was reported that NAT10 regulated cell proliferation and apoptosis.^{27,36–38} Thus, we wanted to know whether miR-6716-5p affects cell proliferation and apoptosis. However, overexpression of miR-6716-5p had no influence on the cell proliferation in HCT116 cells (Figure 5A) ($P>0.05$). Moreover, inhibitor of miR-6716-5p had no effect on cell proliferation (Figure 5C) ($P>0.05$). The similar results were obtained in LoVo cells (Figure 5B and D) ($P>0.05$). In addition, the miR-6716-5p mimic and inhibitor had no effect on cell apoptosis in HCT116 cells (Figure 5E and G) ($P>0.05$). Similar results were observed in LoVo cells (Figure 5F and H) ($P>0.05$). Thus, miR-6716-5p did not affect cell proliferation and apoptosis in CRC cells.

miR-6716-5p might downregulate E-cadherin levels through inhibiting NAT10 expression

It has reported that NAT10 could regulate E-cadherin levels to affect metastasis in HCC and breast cancer (BC) cell lines.^{37,39} Therefore, we wanted to know if miR-6716-5p regulates E-cadherin levels through inhibiting NAT10 expression. We firstly evaluated E-cadherin levels in HCT116 and LoVo cells when cells were treated with either miR-6716-5p mimic or its inhibitor. Both E-cadherin and NAT10 protein levels were significantly decreased when miR-6716-5p was overexpressed, while the NAT10 and E-cadherin protein levels were increased by the miR-6716-5p inhibitor in HCT116 and LoVo cells (Figure 6A and B). These

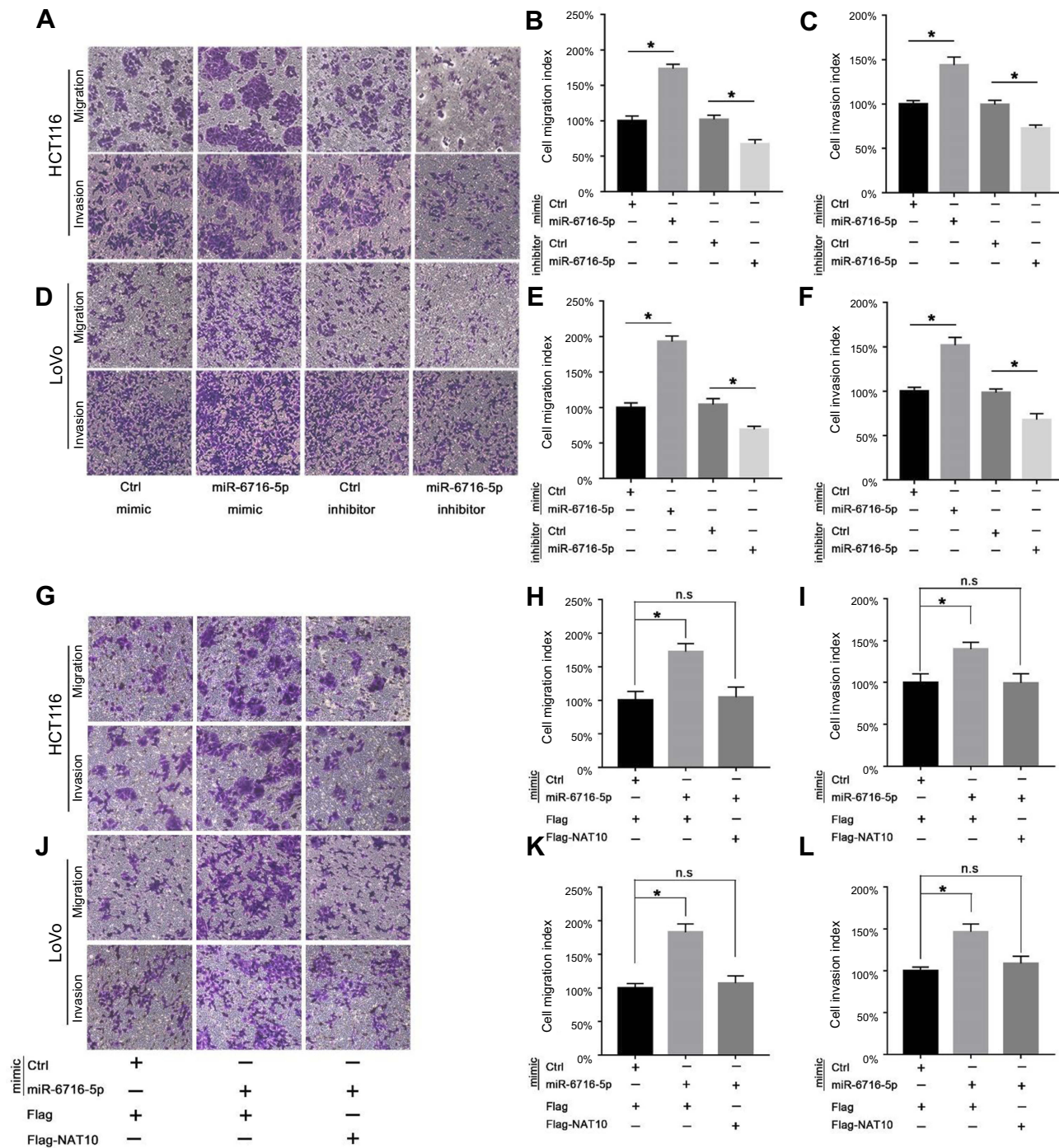


Figure 4 miR-6716-5p promoted cell migration and invasion by downregulating NAT10 expression. (A) HCT116 cells were transfected as indicated. The ability of cell migration and invasion were assessed by transwell assay. (B) The relative migration index of HCT116 cells was calculated. (C) The relative invasion index of HCT116 cells was calculated. (D) LoVo cells were transfected as indicated. Then, the ability of cell migration and invasion were assessed by transwell assay. (E) The relative migration index of LoVo cells was calculated. (F) The relative invasion index of LoVo cells was calculated. (G) HCT116 cells were transfected as indicated. The ability of cell migration and invasion were assessed by transwell assay. (H) The relative migration index of HCT116 cells was calculated. (I) The relative invasion index of HCT116 cells was calculated. (J) LoVo cells were transfected as indicated. Then, the ability of cell migration and invasion were assessed by transwell assay. (K) The relative migration index of LoVo cells was calculated. (L) The relative invasion index of LoVo cells was calculated. Error bars represent the SD from three independent experiments. P-values were obtained from Student's t test. *P< 0.05.

results suggested that miR-6716-5p may downregulate E-cadherin levels through inhibiting NAT10 expression. To confirm this finding, E-cadherin levels were

determined when NAT10 was depleted by siRNA. The depletion of NAT10 by siRNA decreased E-cadherin levels while the ectopic expression of Flag-NAT10

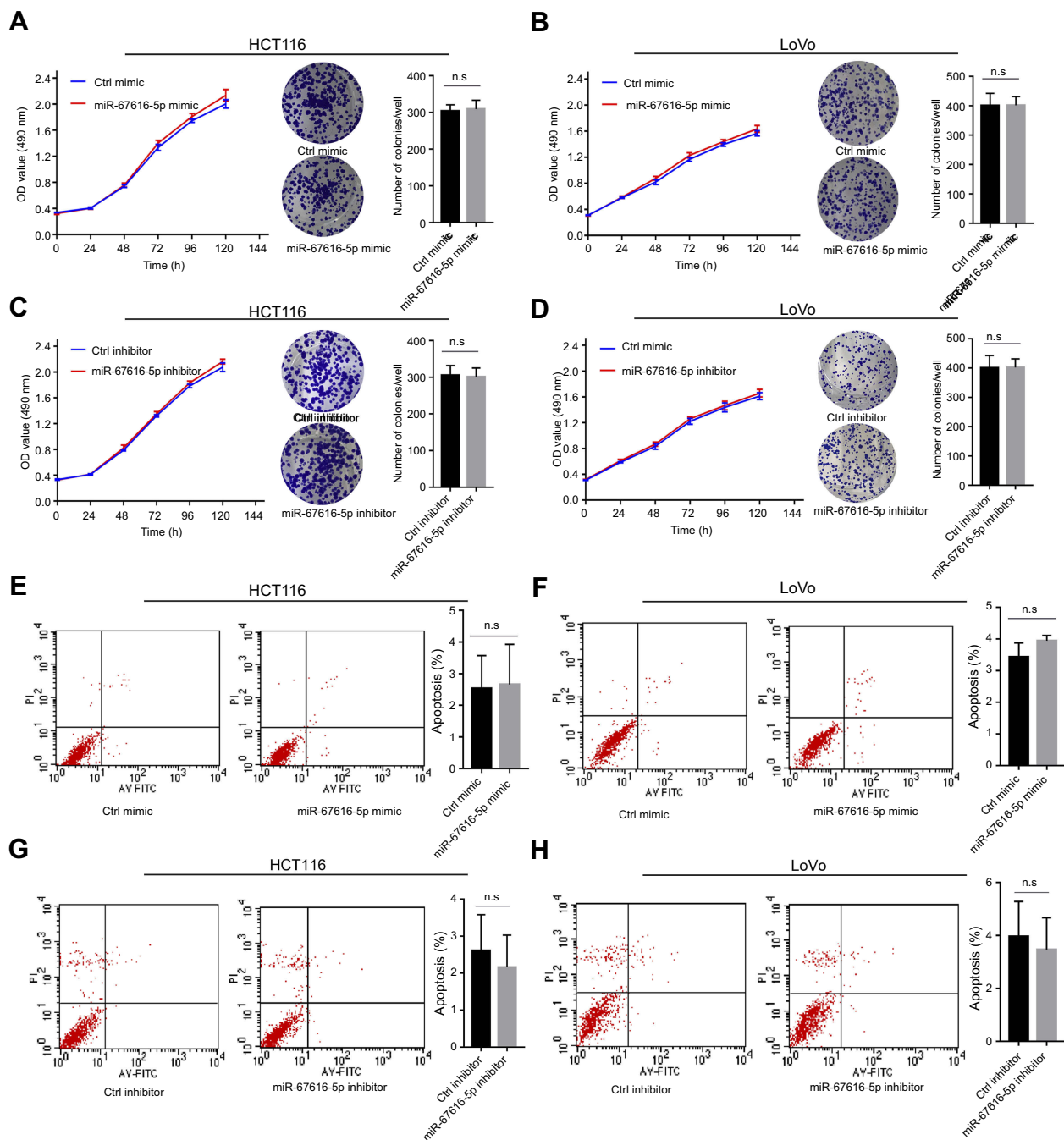


Figure 5 miR-6716-5p had no effect on cell proliferation and apoptosis. (A, B) HCT116 or LoVo cells were transfected with either miR-6716-5p mimic or control mimic. Forty-eight hours later, 1,000 cells were seeded into 96-well or 6-well plates and cultured with 10% serum containing media. The ability of cell proliferation ability was assessed by cell viability assay and colony formation assay. (C, D) HCT116 or LoVo cells were transfected with either miR-6716-5p inhibitor or control inhibitor. Forty-eight hours later, 1,000 cells were seeded into 96-well or 6-well plates and cultured with 10% serum containing media. The ability of cell proliferation was assessed by cell viability assay and colony formation assay. (E, F) HCT116 or LoVo cells were transfected with either miR-6716-5p mimic or control mimic. Then, the apoptosis rate of cells was assessed by flow cytometry. (G, H) HCT116 or LoVo cells were transfected with either miR-6716-5p inhibitor or control inhibitor. Then, the apoptosis rate of cells was assessed by flow cytometry. Error bars represent the SD from three independent experiments. *P*-values were obtained from Student's *t* test. n.s. represents no significance.

increased E-cadherin levels in CRC cells (Figure 6C and D). Taken together, miR-6716-5p downregulated the E-cadherin level to promote CRC metastasis through downregulating NAT10 expression (Figure 7).

Discussion

Since NAT10 plays critical roles in tumor development, uncovering the upstream regulators of NAT10 will provide novel insight into the functions of

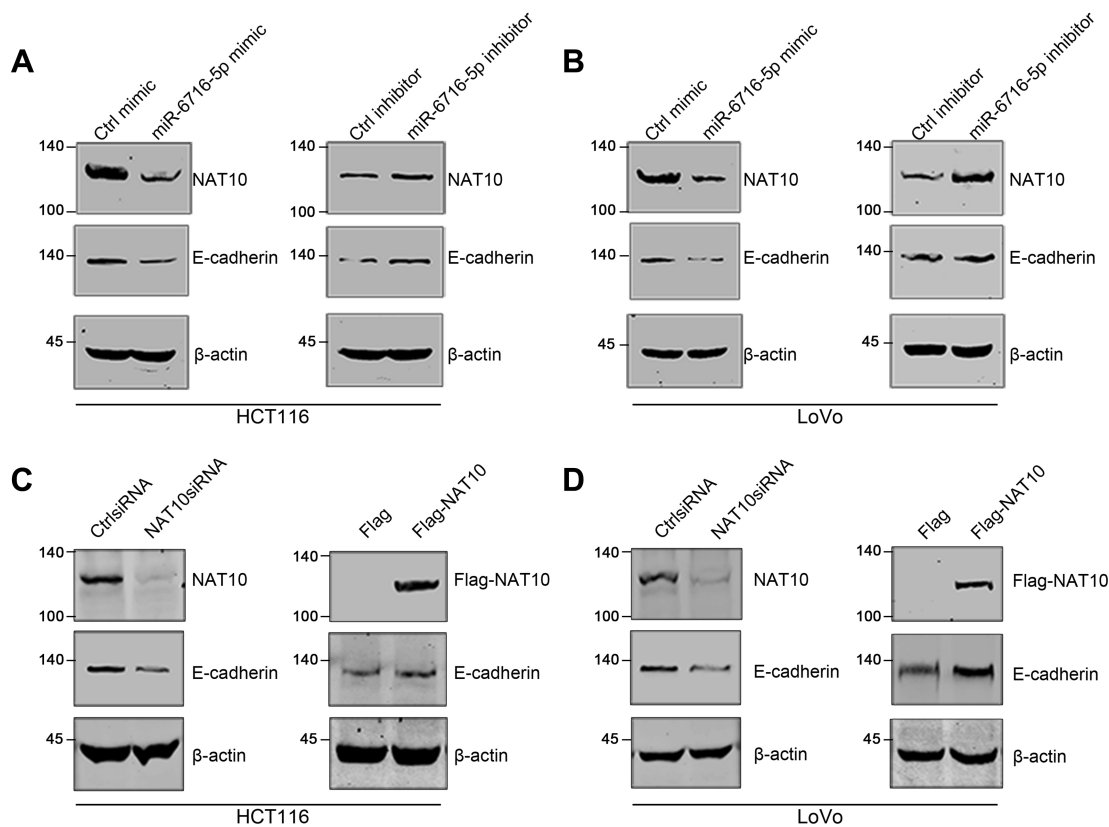


Figure 6 miR-6716-5p may downregulate E-cadherin levels through inhibiting NAT10 expression. (A) HCT116 cells were transfected with the indicated miRNA mimic or inhibitor. Seventy-two hours later, cells were harvested and lysed. NAT10 and E-cadherin protein levels were detected by western blotting. (B) LoVo cells were transfected as indicated. Then, NAT10 and E-cadherin protein levels were detected by western blotting. (C) HCT116 cells were transfected with siRNAs or plasmid as indicated. Then, cells were harvested and total proteins were extracted. NAT10 and E-cadherin protein expression levels were detected by western blotting. (D) LoVo cells were transfected with siRNAs or plasmid as indicated. Then, NAT10 and E-cadherin protein levels were detected by western blotting.

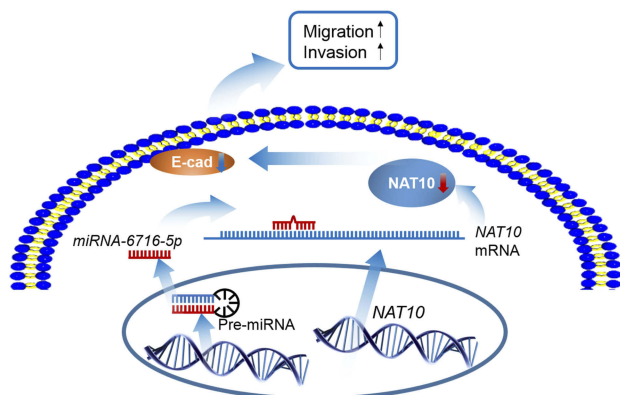


Figure 7 Working model depicting the role of the miR-6716-5p/NAT10 in CRC progression. MiR-6716-5p downregulates the expression of NAT10, which could downregulate E-cadherin level, and in turn promotes CRC cell migration and invasion.

NAT10 in tumor development. In the present study, we identified miR-6716-5p as a critical regulator of NAT10. MiR-6716-5p bound to the 3'-UTR of NAT10 mRNA and inhibited NAT10 expression. Importantly, miR-6716-5p significantly promoted cell migration and

invasion in CRC cells and this function was dependent on NAT10. Significantly, high levels of miR-6716-5p in CRLM patients indicated poor OS, suggesting that miR-6716-5p could be a predictive marker for the poor prognosis of CRLM patients.

Tumor metastasis and recurrence are the main causes of poor prognosis in CRC patients.^{3,40} Therefore, discovering novel regulators of the metastasis or recurrence of CRC would provide a better approach for restraining CRC development. MiRNAs are considered as potential prognostic factors since they play various roles in cellular biological processes by modulating gene expression, and miRNAs are stable and easy to be detected.¹⁰ Some miRNAs could be predictive markers for poor prognosis of CRC patients as they act in the metastasis of CRC.^{41,42} Previous research found that smoking could influence the expression of some mRNAs and miRNAs in CRC patients.^{43,44} It would make sense to include smoking status into the list of analyzed patient characteristics. However, we have no data about the smoking status of

patients in our samples. Thus, miR-6716-5p might be a novel potential clinical biomarker for prognosis of CRC patients.

Previous studies found that the expression stability of some reference genes might be different in different kinds of diseases and under different experimental designs.^{45–49} Levels of NAT10 and miR-6716-5p were assessed using RT-qPCR with reference gene GAPDH and U6, respectively, which might cause experimental errors induced by reference gene instability. U6 is one of the common reference genes for miRNAs RT-qPCR in CRC research.^{50,51} Some researchers found that miR-191-5p could be used as reference gene in CRC research^{48,51} GAPDH and ACTB are commonly used as reference genes for mRNAs RT-qPCR in many research.^{52,53} To determine the stability of U6 and GAPDH, we have evaluated miR-191-5p and ACTB levels in 18 cases of randomly selected samples. The stabilities of U6, GAPDH, miR-191-5p and ACTB were analyzed using the CT values according to the instruction of the NormFinder and geNorm software.^{54,55} The results showed that GAPDH and ACTB had the same stability, U6 had the same stability as miR-191-5p (Table S1). In addition, in order to rule out possible experimental errors caused by using U6 and GAPDH as reference genes, we chose miR-191-5p and ACTB as reference genes to analyze the relative levels of miR-6716-5p and NAT10 mRNA, respectively. We showed the tendency of relative miR-6716-5p levels did not change when U6, miR-191-5p, or combination of U6 with miR-191-5p was used as reference gene, respectively (Figure S2A, C and E). Relative NAT10 mRNA levels had similar tendency when GAPDH, ACTB, or combination GAPDH with ACTB was used as reference gene, respectively (Figure S2B, D and F).

It has been reported that high expression of NAT10 correlated with poor prognosis of HCC patients.^{31,32} Moreover, membranous NAT10 in CRC tissues was associated with a more aggressive CRC phenotype and poor prognosis of CRC patients.³⁰ We demonstrated that simultaneous deletion of residues 68–75 and 989–1018 in NAT10 led to the cytoplasmic and cell membranous localization of NAT10 in HCC cells. We further found that the membranous NAT10 promoted cancer cell invasion and was correlated with poor survival of the HCC patients.⁵⁶ These results suggested that dysregulation of protein level and cellular location of NAT10 might play critical roles in tumor development and metastasis. Recent research found that NAT10 might act in the tumor metastasis through participating in the epithelial-to-mesenchymal transition process.^{37,39} It has been reported that inhibition of NAT10 increased E-cadherin expression and repressed cell invasion

and migration in HCC and BC cell lines.^{37,39} In the present study, we found that both miR-6716-5p and the downregulation of NAT10 decreased E-cadherin levels. Consistent with our findings, the database in the GEPIA website (<http://gepia.cancer-pku.cn>) shows that NAT10 is positively correlated with E-cadherin ($P < 0.01$) in colon adenocarcinoma, stomach adenocarcinoma and pancreas adenocarcinoma (the correlation coefficient was 0.4, 0.4 and 0.32, respectively) (Figure S3A).⁵⁷ There was no correlation between NAT10 and E-cadherin ($P > 0.01$) in esophageal carcinoma, hepatocellular carcinoma or cholangiocarcinoma (Figure S3B). Thus, the regulation of NAT10-E-cadherin might partially explain why NAT10 functions differently in various tumors. Acetylation of lysine residues in histone H3 is associated with relaxed chromatin structure which facilitates gene expression.⁵⁸ Liu et al found that treatment with trichostatin A, a histone deacetylase inhibitor, could enhance the acetylation of histone H3K9 and then induce the expression of E-cadherin mRNA in CRC.⁵⁹ Since NAT10 possesses the HAT activity, we speculate that NAT10 might enhance the acetylation of histones in the promoter region of E-cadherin to activate the transcription of E-cadherin. Chen et al previously reported that there exist three AML1-binding sites, two p300-binding sites and four hepatocyte nuclear factor (HNF3) binding sites in promoter region of E-cadherin.⁶⁰ They also found that HNF3 could enhance the re-expression of E-cadherin by interacting with p300 and AML1.⁶⁰ Both NAT10 and p300 belong to the type-A HATs, which might share similar function in transcription regulation.⁶¹ However, how NAT10 regulates E-cadherin needs further study in the future.

It has been reported that knockdown of NAT10 mildly promotes cell proliferation in CRC cells^{27,28} while inhibition of NAT10 suppresses the growth and proliferation of malignant melanoma cells.³⁶ In normal condition, apoptosis was induced by NAT10 suppression in PA-1 cells.³⁸ However, miR-6716-5p had no effect on cell proliferation and apoptosis in CRC cells in the present study. Given that some miRNAs target multiple mRNAs to regulate cellular processes,⁶² it is likely that miR-6716-5p might target more downstream factors functioning in cell proliferation and apoptosis. The discovery of new miR-6716-5p targets will contribute to completely understanding the functions of miR-6716-5p in cellular processes and tumorigenesis.

A previous study also found that NAT10 was significantly downregulated in CRC tumor samples.²⁷ In this study, we found that decreased level of NAT10 in CRC patients was associated with a poor OS rate (Figure S4A). Consistent with our findings, the Human Protein Atlas

website (<https://www.proteinatlas.org/>) showed that lower NAT10 expression is correlated with a poorer OS rate in rectal cancer patients (Figure S4B).⁶³ These data indicate that NAT10 might act as a prognostic factor in CRC patients.

Conclusion

In the present study, we demonstrated a regulatory mechanism of NAT10 expression by miR-6716-5p in CRC. Disruption of miR-6716-5p-NAT10 regulation may be a potential therapeutic strategy for CRLM patients. Thus, miR-6716-5p inhibitor could be a potential therapeutic drug in the intervention of CRC liver metastasis.

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Disclosure

The authors report no conflicts of interest in this work.

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