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MicroRNA-30a Mediates Cell Migration and Invasion by Targeting Metadherin in Colorectal Cancer

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Abstract

MicroRNAs play critical roles in the occurrence and progression in various cancers including colorectal cancer. Here, we found that microRNA-30a expression was significantly downregulated in colorectal cancer tissues compared to adjacent noncancerous tissues, and the suppression levels of microRNA-30a were significantly associated with tumor differentiation and lymph node metastasis. We also discovered that the expression level of microRNA-30a was inversely proportional to the invasive potential of several colorectal cancer cell lines. Moreover, overexpression of microRNA-30a in colorectal cancer cells inhibited activity of cell migration and invasion. Luciferase reporter assay confirmed metadherin could be a direct target of microRNA-30a, as the overexpression of microRNA-30a decreased metadherin expression at both the protein and messenger RNA levels. Furthermore, the knockdown of metadherin expression in SW620 significantly decreased cell metastasis and invasion. The upregulation of metadherin at the protein level negatively correlated with the expression of microRNA-30a in colorectal cancer tissues, and this upregulation could partially attenuate the effect induced by microRNA-30a. These findings indicate that microRNA-30a may act as a tumor suppressor in colorectal cancer and that microRNA-30a represses cell migration and invasion by decreasing metadherin, highlighting the therapeutic potential of microRNA-30a and metadherin in colorectal cancer treatment.

Keywords

miR-30a, metadherin, invasion, migration, colorectal cancer

Abbreviations

AEG-1, astrocyte elevated gene-1; CRC, colorectal cancer; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ITGB3, integrin β3; miRNAs, microRNAs; mRNA, messenger RNA; MTDH, metadherin; NC, negative control; PCR, polymerase chain reaction; PIK3CD, phosphoinositide 3-kinase catalytic subunit delta; qRT-PCR, quantitative reverse transcription–polymerase chain reaction; siRNA, small interfering RNA; TNM, tumor node metastasis; UTR, untranslated region.

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Introduction

Colorectal cancer (CRC) is one of the most common gastrointestinal cancers worldwide, and invasion and metastasis are considered to be the leading causes of the recurrence and poor prognosis of CRC.[1] In recent years, with the rapid development of molecular biology techniques, many novel oncogenes have been discovered. However, the basic mechanisms underlying CRC initiation, progression, and chemoresistance remain largely unknown. Better understanding of the molecular mechanisms underlying tumor development in CRC would be helpful in improving diagnosis, therapy, and prevention.

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MicroRNAs (miRNAs) are 21 to 25 nucleotides in length and evolutionarily conserved RNA molecules. MicroRNAs repress gene expression by a combination of degrading messenger RNA (mRNA) and inhibiting translation.[2] To date, studies have shown that several miRNAs are concerned with the dysregulation of metadherin (MTDH) signaling in CRC, such as miR-217,[3] miR-181a-2, and miR-9.[4] In recent years, many studies have indicated that microRNA-30a (miR-30a) is involved in the metastasis and invasion of different types of cancers, such as breast cancer, non-small cell lung cancer, hepatocellular carcinoma, gastric cancer, and ovarian serous adenocarcinoma.[5-9] Researchers have also found that miR-30a is significantly decreased in CRC tissue and cell lines[10,11]; however, the detailed molecular mechanism has not yet been clearly elucidated.

Su *et al* first cloned the HIV-1-inducible astrocyte elevated gene-1 (AEG-1), also known as MTDH.[12] Metadherin has an active role in the formation, progression, invasion, and metastasis of multiple malignant tumors through the regulation of NF-κB, Wnt/β-catenin, PI3K/AKT and other signaling pathways.[13-15] Additionally, Emdad *et al* demonstrated that aberrant MTDH expression plays a vital role in regulating oncogenic transformation and angiogenesis.[16] Many reports show that MTDH is an important oncogene that is overexpressed in various human cancers, such as hepatocellular carcinoma, glioma, gastric cancer, non-small cell lung cancer, breast cancer, and colorectal carcinoma. Additionally, MTDH overexpression predicts tumor progression and poor prognosis.[17-22] Taken together, these findings indicate that the inhibition of MTDH may be a potential treatment for multiple malignant tumors, including CRC.

In the present study, we found that miR-30a expression was reduced in CRC tissue and cell lines and that the overexpression of miR-30a could inhibit the invasion and migration of CRC cell lines. In addition, we identified MTDH as a novel target of miR-30a in CRC cell lines. Finally, we showed that the overexpression of MTDH restored the suppressive effect of miR-30a in CRC cell lines. Thus, our findings will help elucidate the functions of miR-30a and its roles in CRC progression as well as provide further evidence of the targeting of MTDH by miRNAs.

Materials and Methods

Patients and Clinical Specimens

Sixty-four pairs of patients with CRC who underwent surgery at The First Affiliated Hospital of Zhengzhou University (Zhengzhou, Henan) between 2015 and 2016 were recruited. No preoperative chemotherapy or radiotherapy was administered to our recruited patients. The histopathology of the disease was confirmed by 2 pathologists according to the criteria of the World Health Organization. Clinical staging was performed according to the Union for International Cancer Control classification. Participants with a family history of cancer were excluded. Written informed consent procedures, approved by the ethics committee of The First Affiliated Hospital of Zhengzhou University, were obtained from all patients. Detailed clinical information on these patients, including age, gender, tumor size, tumor differentiation, tumor node metastasis (TNM) stage, lymph node metastasis, and distant metastasis, is summarized in Table 1. The tissue samples were snap-frozen and stored at −80°C for RNA and protein extraction.

Table 1. Association Between miR-30a Expression and Clinicopathological Features.

Clinicopathological Features	N	miR-30a Expression	*P* Value
Age, years			.614
≤60	21	0.51 (0.23)	
>60	43	0.49 (0.20)	
Gender			.631
Female	29	0.51 (0.28)	
Male	35	0.49 (0.21)	
Tumor size (cm)			.183
≤2	24	0.54 (0.21)	
>2	40	0.47 (0.20)	
Tumor differentiation			.019[a]
Well/moderate	26	0.57 (0.24)	
Poor	38	0.44 (0.16)	
TNM stage			.442
I + II	40	0.51 (0.17)	
III + IV	24	0.47 (0.26)	
Lymph node metastasis			.015[a]
Positive	37	0.44 (0.19)	
Negative	27	0.56 (0.21)	
Distant metastasis			.123
Positive	15	0.57 (0.24)	
Negative	49	0.47 (0.19)	

Abbreviations: miR-30a, MicroRNA-30a; TNM, tumor node metastasis.
[a]Statistically significant ($P < .05$).

Cell Culture

Colorectal cancer cell lines SW480, $CaCO_2$, HT29, HCT116, and SW620 were kindly provided by Prof Yi Zhang (Department of Oncology, The First Affiliated Hospital of Zhengzhou University). SW480 and SW620 cells were maintained in RPMI-1640 medium, HT29 and HCT116 cells were cultured in McCoy's 5a medium, and $CaCO_2$ cells were preserved in Dulbecco modified Eagle Medium. All media (HyClone, Logan, Utah) were supplemented with 10% fetal bovine serum (FBS; HyClone) and 1× antibiotic/antimycotic (100 units/mL streptomycin, 100 units/mL penicillin, and 0.25 mg/mL amphotericin B). All cell lines were cultivated in a humidified atmosphere at 37°C with 5% CO_2.

miRNA and siRNA Transfection

miR-30a mimics, miR-30a inhibitor, negative control (NC), MTDH small interfering RNA (MTDH-siRNA), and scrambled small interfering NC were all designed and purchased from GenePharma (Shanghai, China). The miRNA mimics were used at a final concentration of 80 nM. The miRNA inhibitors

were used at a final concentration of 150 nM. Cells were plated in 24- or 6-well plates 1 day prior to transfection. Analyses were performed after 12 to 72 hours of transfection. The coding sequences for MTDH were amplified and cloned into pcDNA3.1(+) to generate the MTDH expression vector pcDNA3-MTDH (Austin, Texas). The full-length wild-type MTDH 3′-untranslated region (3′-UTR) and mutant MTDH 3′-UTR were cloned into a pGL3 luciferase vector (TaKaRa, Dalian, China). All transfections were performed using Lipofectamine 2000 (Invitrogen; Life Technologies, Carlsbad, California) according to the manufacturer's protocol.

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from tissue samples and cell lines using TRIzol reagent (TaKaRa) according to the manufacturer's protocol. RNA concentration was measured using NanoDrop 2000c (NanoDrop, Wilmington, Delaware). Detection of the mature form of miR-30a was performed using the TaqMan stem-loop RT-PCR method. The small nuclear RNA U6 was used as an internal control. To detect MTDH, complementary DNA was synthesized from 1 μg of total RNA using the PrimeScript RT reagent kit (TaKaRa). Expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), the internal control. Real-time PCR was performed using SYBR Premix Ex Taq II (TaKaRa). The relative expression levels of each gene were calculated and normalized using the $2^{-\Delta\Delta Ct}$ method relative to GAPDH or U6 snRNA. All reactions were run in triplicate using an ABI 7300HT instrument (Applied Biosystems). The amplification program was as follows: 5 minutes at 94°C, followed by 30 cycles of 30 seconds at 94°C, 45 seconds at 60°C, and finally, 10 minutes at 72°C.

The primer sequences are as follows:

MTDH: 5′-CGCGGATCCCCTGAATTGGACAT-GTGTTTGC-3′ (forward); and 5′- CCCAAGCTTCTGTGCATAAGATCCAAGGAATTGC-3′ (reverse); *GAPDH*: 5′-AAGGTGAAGGTCGGAGTCAAC-3′ (forward); and 5′-GGGGTCATTGATGGCAACAATA-3′ (reverse).

Cell Migration and Invasion Assays

For the migration assay, 5×10^4 HCT116/SW620 cells in 200 μL of serum-free medium were seeded into the upper chamber of each insert (BD Biosciences, Franklin Lakes, New Jersey), and 600 μL of medium supplemented with 10% FBS was added into the lower chamber. The samples were then incubated for 24 hours at 37°C and then fixed and stained. For the cell invasion assay, the polycarbonate membranes of the upper surface of the chambers were precoated with a matrix gel (BD Biosciences). Cells that migrated and invaded through the membrane were fixed using 10% methanol and stained with 0.1% crystal violet. These cells were quantified using a 10 × 20 inverted microscope (Olympus, Japan). Cells were counted from photographs of 5 randomly selected fields of the fixed cells. Each experiment was independently repeated 3 times.

Western Blotting

Protein was extracted from cells using RIPA lysis buffer with proteinase inhibitor. Concentrations of total cellular protein were determined using a BCA assay kit (Beyotime, Jiangsu, China). Then, 20 μg of protein mixed with 2× SDS loading buffer was loaded per lane, separated by 10% SDS-PAGE, and electrophoretically transferred onto a nitrocellulose membrane (Bio-Rad, Munich, Germany). Antibodies to MTDH (1:1000; Abcam, Cambridge, United Kingdom) and GAPDH (1:2500; Abcam) were incubated with the membranes overnight at 4°C. The membranes were washed and incubated with horseradish peroxidase–conjugated secondary antibodies (1:5000; Santa Cruz Biotechnology, California). The results were visualized with a chemiluminescent detection system (Beyotime) and exposed with an autoradiography film (Kodak, Shanghai, China). Protein levels were quantified by density analysis using Quantity One software (Bio-Rad).

Luciferase Reporter Assay

Cells were transfected with miR-30a mimics or NC and were then cotransfected with wild-type or mutant vectors. After 48 hours of transfection, the cells were lysed for luciferase assays using the dual-luciferase assay system (Promega, Madison, Wisconsin). Renilla luciferase was used for normalization. The experiments were independently performed in triplicate.

Statistical Analyses

Statistical analyses were performed using SPSS 19.0 with the 2-tailed Student *t* test. The correlation between 2 groups was analyzed by the Spearman correlation test. All results are presented as means (SD). *P* values of <.05 were considered statistically significant.

Results

MiR-30a Expression in CRC Tissues and Cell Lines and Its Correlation With the Clinicopathological Features of Patients With CRC

Based on data from 64 patients with CRC, miR-30a expression was significantly downregulated ($P < .01$) in tumor tissues compared to corresponding normal tissues (Figure 1A). Furthermore, the association between miR-30a expression in tissues and clinicopathological features was analyzed. As shown in Table 1, the level of miR-30a expression in tissues was significantly decreased in patients with poor differentiation and positive lymph node metastasis ($P < .05$). There was no association between miR-30a expression and characteristics such as age, gender, tumor size, TNM stage, and distant metastasis. To better understand the relationship between miR-30a and metastasis, we measured the miR-30a expression levels in

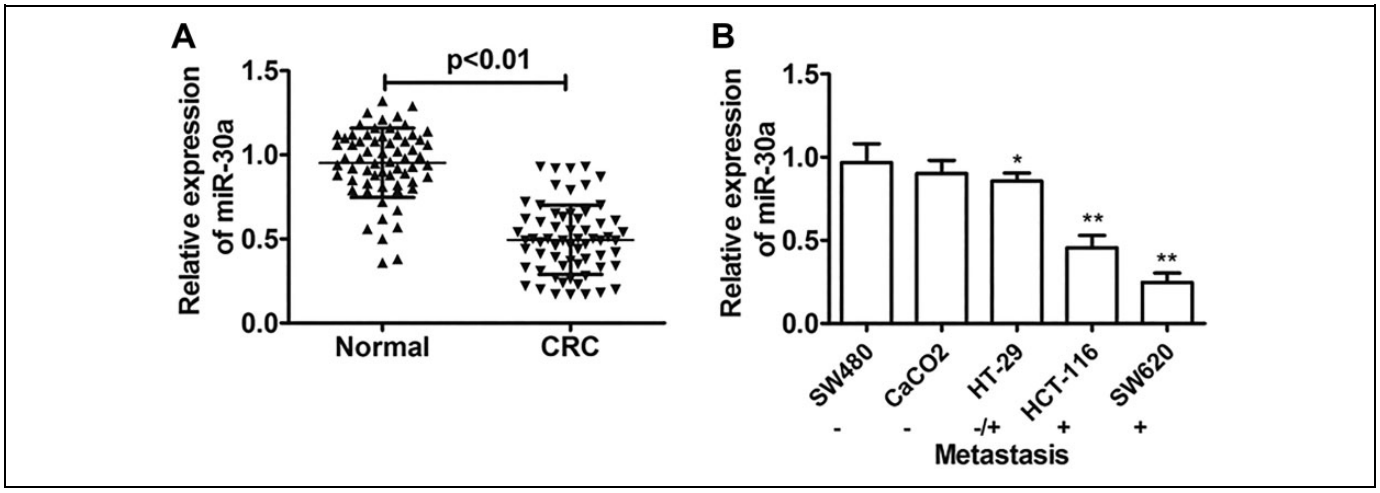

Figure 1. Relative expression of miR-30a in CRC tissues and cell lines. A, Relative expression levels of miR-30a in 64 CRC tissues and matched noncancerous tissues were examined by qRT-PCR. B, Relative expression levels of miR-30a among 5 CRC cell lines with different degrees of metastasis. Experiments were independently performed in triplicate. *$P < .05$ and **$P < .01$ versus the control. CRC indicates colorectal cancer; miR-30a, MicroRNA-30a.

multiple CRC cell lines with different metastatic potential. Compared to SW480, miR-30a expression levels were lower in HT29 and HCT116 cells and lowest in SW620 cells that were derived from the lymph nodes of a patient (Figure 1B). However, the effect of miR-30a in CRC progression remains unknown.

MiR-30a Inhibits the Migration and Invasion of CRC Cell Lines

The effect of miR-30a was further investigated with a series of trials *in vitro*, which played a role in human CRC cell lines. The relative expression levels of miR-30a in HCT116 and SW620 was verified by qRT-PCR analysis ($P < .01$; Figure 2A and B). Both the 2 cell lines with high expression of miR-30a showed significantly less activity of migration and invasion compared with control group (Figure 2C and D); at the same time, down-regulation of miR-30a in HCT116 could increase activity of migration and invasion compared with the control (Figure 2C). We have studied the effect of miR-30a-inhibitor on cell proliferation and migration in SW480 cells, which showed that the inhibition of miR-30a promoted activity of cell proliferation and migration (Supplemental Figure 1A and C). These results indicated that the overexpression of miR-30a substantially inhibited the migration and invasion of CRC cell lines. However, the underlying molecular mechanism requires further analysis.

MTDH is a Direct Target of miR-30a

To investigate the mechanism of miR-30a in the development of CRC, a computational approach was adopted using several available prediction algorithms, which are based on evolutionary conservation of target sites across species, including miRDB, PicTar, and TargetScan. One limitation of this approach is that each of these algorithms predicts hundreds of possible targets for miR-30a. To address this problem, we focused on the targets important for cell migration or invasion that were predicted by all of these programs. Among the predicted candidate targets, the 3′-UTRs of human MTDH that contained regions matching the seed sequences of miR-30a had higher scores (Figure 3A). Therefore, MTDH was selected for further investigation of its role in the repression of miR-30a expression. As shown in Figure 3A, MTDH 3′-UTR$_{WT}$ was shown to bind with miR-30a, but MTDH$_{MUT}$ had no binding sites with miR-30a. Luciferase activity assay showed that miR-30a significantly suppressed the luciferase activity of the wild type but not the mutant 3′-UTR of MTDH (Figure 3B). Although MTDH was identified as a target gene for miR-30a, it was unknown whether miR-30a can regulate the endogenous MTDH expression. Thus, SW620 cells that were transfected with miR-30a mimics and HCT116 cells that were transfected with miR-30a mimics and inhibitor, respectively, were conducted to investigate whether the dysregulation of miR-30a expression can affect endogenous MTDH expression. Moreover, inhibition of miR-30a increased the expression of MTDH in SW480 cells (Supplemental Figure 1B). Compared with the control, the MTDH level was significantly suppressed by miR-30a at both the mRNA and protein levels in SW620 and HCT116 cell lines (Figure 3C and D). These findings demonstrate that MTDH is a target of miR-30a in CRC cells.

MTDH Promotes the Migration and Invasion of CRC Cells

To investigate the role of MTDH in CRC development, we examined the effect of MTDH on cell migration and invasion. We knocked down MTDH in SW620 using siRNA, which reducing the expression to less than 65% of the scrambled control (Figure 4A). As shown in Figure 4B-D, knockdown

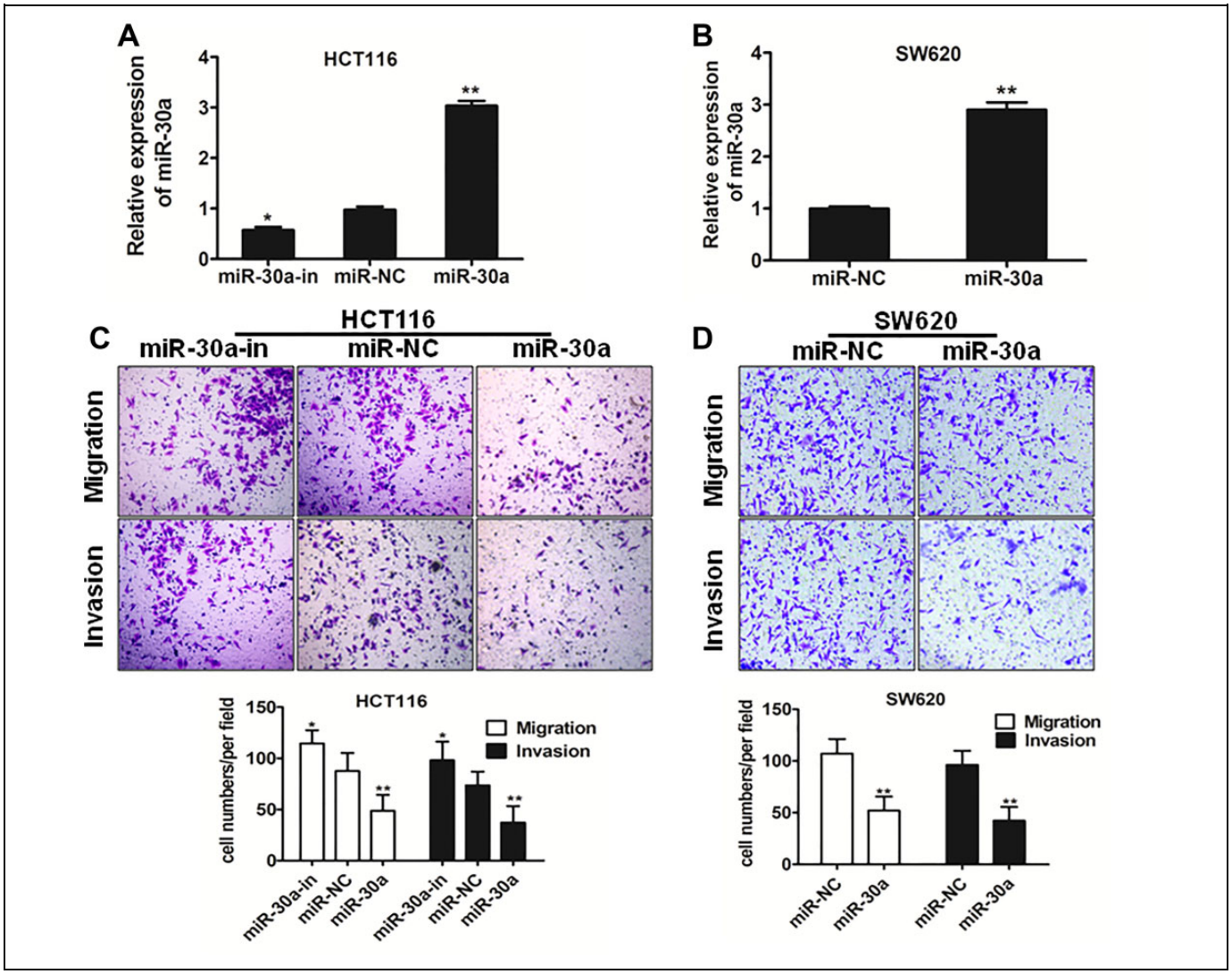

Figure 2. miR-30a suppresses the migration and invasion of CRC cells *in vitro*. A, Relative expression levels of miR-30a in HCT116 transfected with miR-30a inhibitor (miR-30a-in), miR-30a mimics (miR-30a) compared with normal control cells (miR-NC), as determined by qRT-PCR. B, The effect of miR-30a mimics on the expression of miR-30a in SW620 cells. C, Migration and invasion assays were performed in HCT116 that were transfected with miR-30a inhibitor, mimics, and negative control. D, Migration and invasion assays were performed in SW620 that were transfected with miR-30a mimics and negative control. Experiments were independently performed in triplicate. $*P < .05$ and $**P < .01$ versus the control. CRC indicates colorectal cancer; miR-30a, microRNA-30a.

of MTDH expression significantly suppressed cell migration and invasion, which was consistent with the effect of miR-30a overexpression.

MiR-30a Inhibits CRC Migration and Invasion in Part by Downregulating MTDH Expression

To assess the expression pattern of MTDH in CRC, we examined MTDH expression in 64 CRC tissues and their matched paracancerous tissues using qRT-PCR. The expression levels of MTDH were found to be significantly upregulated in CRC tissues compared with levels from paired adjacent nontumor tissues (Figure 5A). MicroRNA-30a and MTDH expression levels in CRC tissues showed a significant inverse correlation according to Spearman correlation test (Figure 5B; $r = -.6053$, $P < .01$), which further indicates that MTDH is a potential target of miR-30a in CRC cells. To examine whether MTDH is the primary target suppressed by miR-30a in CRC, the MTDH expression vector or scramble vector was individually transfected into SW620. As expected, the overexpression of MTDH partially reverses the inhibitory effect of miR-30a in CRC cells. Taken together, these results indicate that miR-30a inhibits cell migration and invasion in part by downregulating the expression of MTDH (Figure 5C and D).

Discussion

MicroRNAs are considered to play critical roles in tumorigenesis as a result of their involvement in many cellular processes,

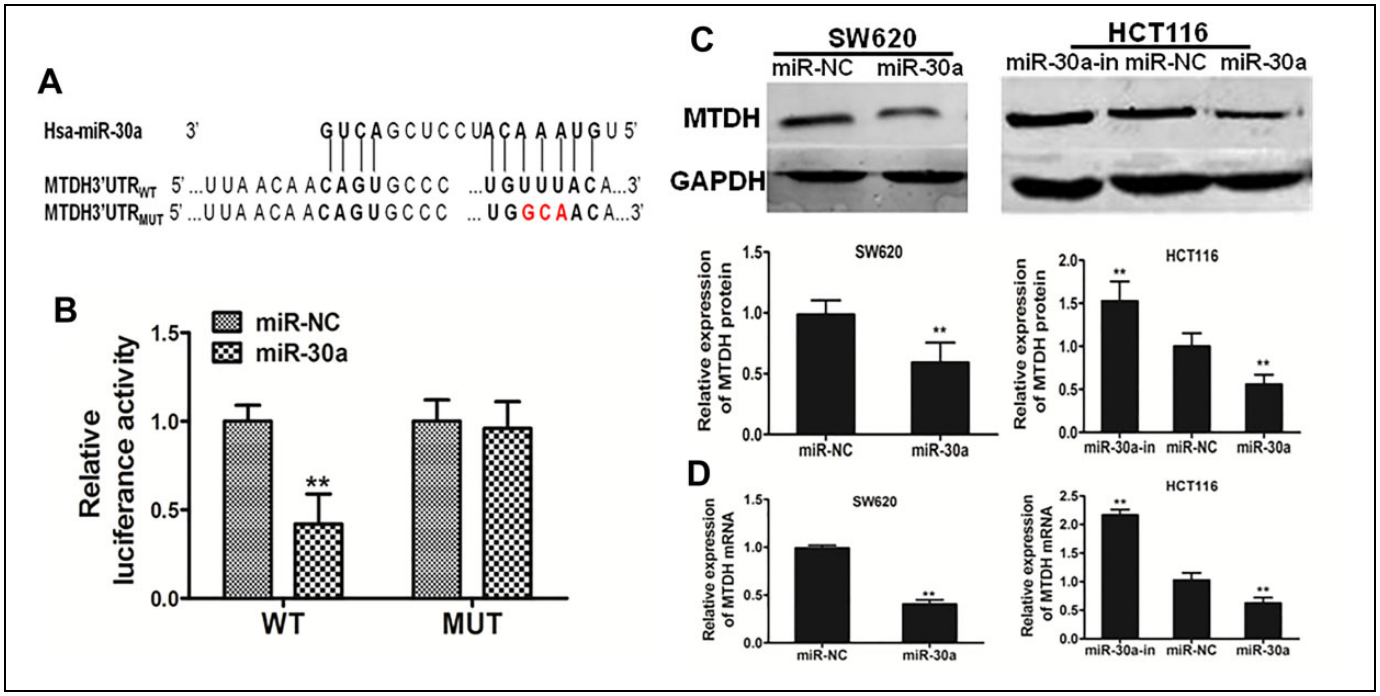

Figure 3. MTDH is a target of miR-30a. A, Schematic representation of the miR-30a targeting sequences with the 3′-UTR of MTDH. Mutant 3′-UTR of MTDH were showed in red. B, A dual-luciferase reporter assay was performed in SW620 cells. Experiments were performed in triplicate. C, The protein level of MTDH was detected by Western blot in SW620 cells that were transfected with miR-30a mimics or negative control. GAPDH was included as an internal control. D, Expression of MTDH mRNA was detected by qRT-PCR. $^{**}P < .01$ compared with the control. GAPDH indicates glyceraldehyde 3-phosphate dehydrogenase; miR-30a, MicroRNA-30a; mRNA, messenger RNA; MTDH, metadherin; UTR, untranslated region.

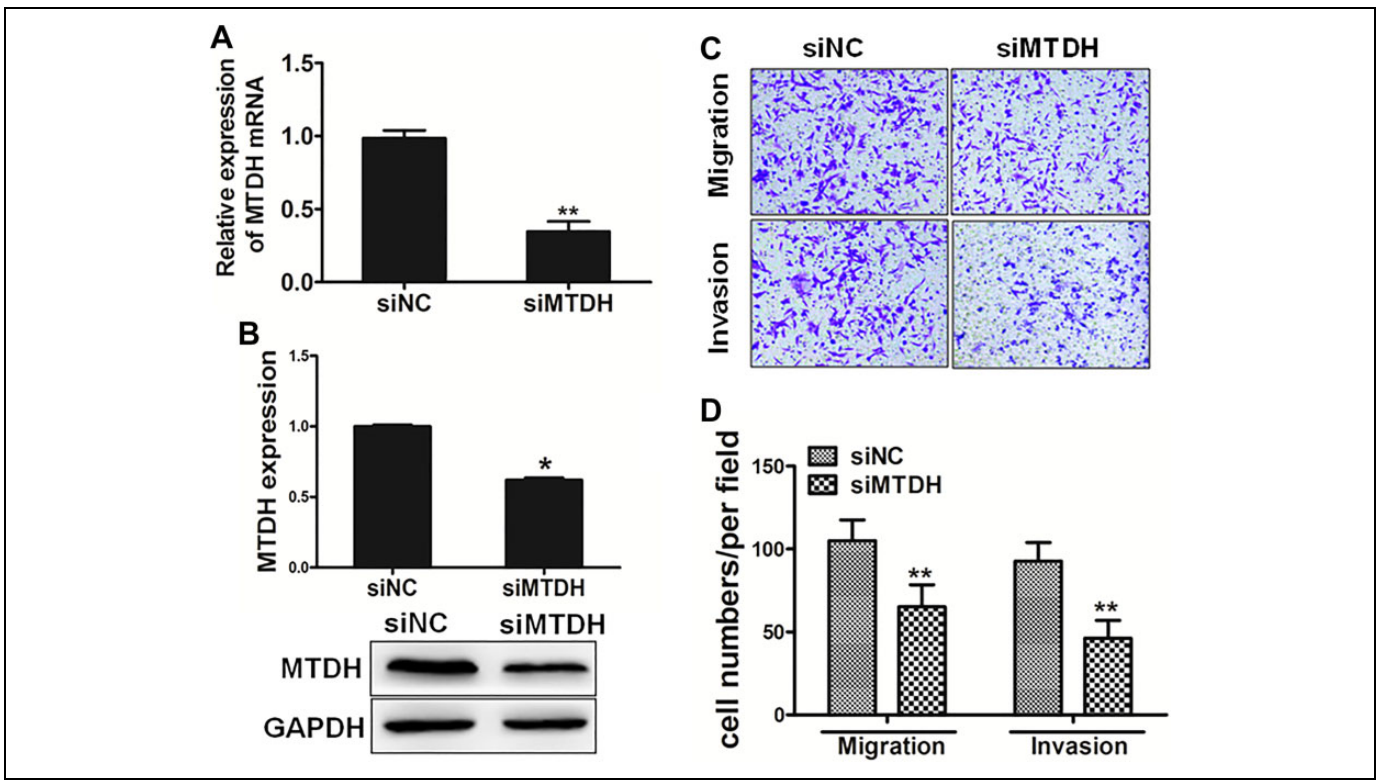

Figure 4. MTDH promotes the migration and invasion in SW620 cells. A, The expression of MTDH mRNA in SW620 cells transfected with MTDH siRNA or siNC vector was assayed by qRT-PCR. B, The expression of MTDH protein in SW620 cells transfected with MTDH siRNA or siNC. C and D, The migration and invasion of SW620 cells following siRNA knockdown. Experiments were performed in triplicate. $^{*}P < .05$ and $^{**}P < .01$ compared with the control. mRNA indicates messenger RNA; MTDH, metadherin; siNC, small interfering negative control; siRNA, small interfering RNA.

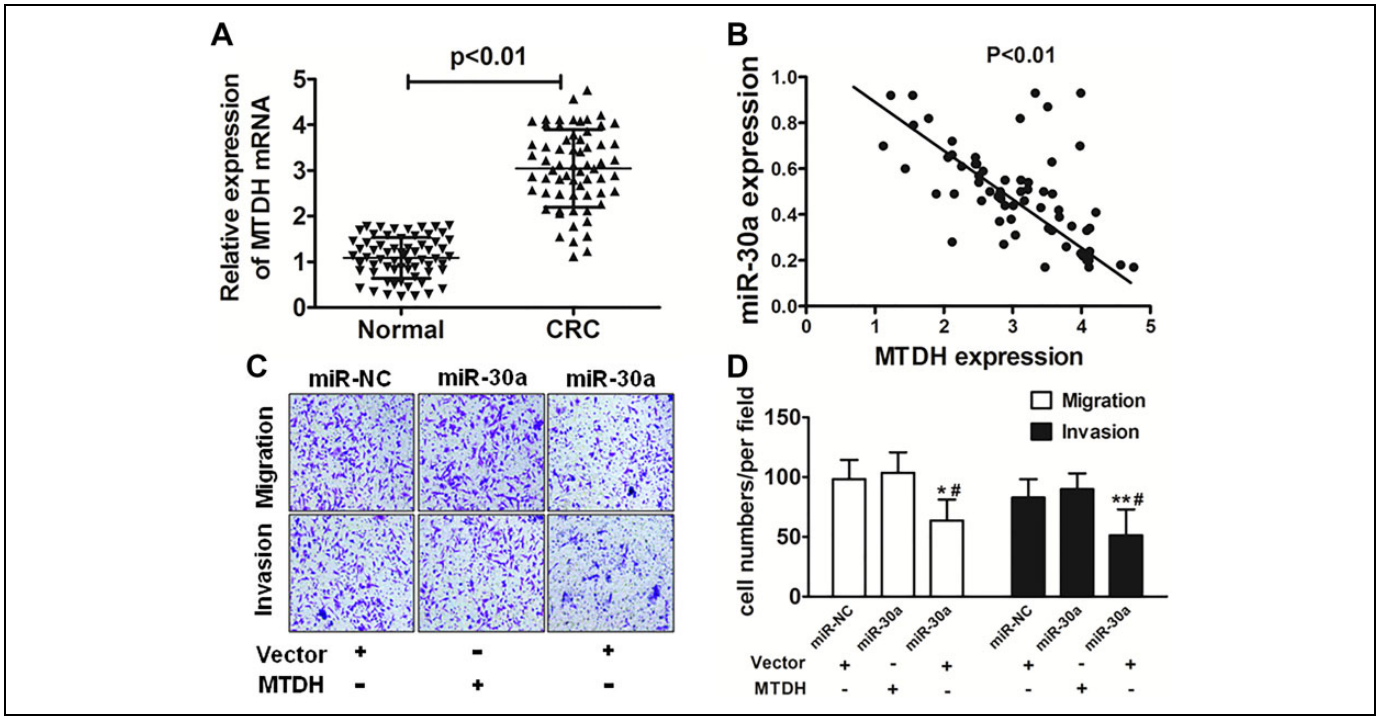

Figure 5. MTDH is involved in miR-30a-mediated migration and invasion of SW620 cells. A, MTDH expression in CRC tissues and matched normal tissues was assayed by qRT-PCR. B, The correlation between the expression of miR-30a and MTDH was analyzed by Spearman correlation test. C and D, The migration and invasion of SW620 cells cotransfected with miR-30a and MTDH or control vector. *$P < .05$ and **$P < .01$ versus the control, #$P < .05$ versus miR-30a plus MTDH expression. CRC indicates colorectal cancer; miR-30a, MicroRNA-30a; MTDH, metadherin.

including cell proliferation, differentiation, apoptosis, and invasion.[2] In the present study, we focused on miR-30a, which is located on chromosome 6q13 and has been reported to be abnormally expressed in a variety of cancer types,[5-9,11,23-25] including CRC.[10,11] In our study, qRT-PCR analysis demonstrated that miR-30a was significantly downregulated in CRC tissue samples and cell lines. Additionally, the miR-30a level in CRC tissues was associated with tumor differentiation and lymph node metastasis. We also discovered that the expression level of miR-30a was inversely proportional to the invasive potential of multiple CRC cell lines. Together, these indicated that miR-30a may participate in the invasion and migration of CRC. However, the underlying molecular mechanism requires further analysis.

In our study, we had identified the role of miR-30a in tumorigenesis by targeting MTDH. With the aid of computational algorithms, MTDH was screened as a putative target gene of miR-30a in CRC cell lines. To confirm this conclusion, several lines of experimental evidence in this study were evaluated. First, a complementary sequence of miR-30a was identified in the 3′-UTR of MTDH mRNA. Second, the overexpression of miR-30a significantly decreased the expression of MTDH at both the mRNA and protein levels and downregulation of miR-30a exerted the opposite effects. Third, the luciferase reporter activity revealed that the overexpression of miR-30a decreased the reporter activity within the MTDH 3′-UTR and that this effect could be abolished by mutating the miR-30a seed-binding site of the MTDH 3′-UTR. In addition, MTDH expression was naturally upregulated in CRC tissues, which was inversely correlated with miR-30a level, which further supports that MTDH is a target of miR-30a. Coincidentally, Zhang *et al* confirmed that miR-30a suppressed breast tumor cell proliferation and metastasis by downregulating MTDH.[24] Additionally, in non-small cell lung cancer, miR-30a inhibited the metastasis of A549 cells *in vitro* via the targeted suppression of MTDH, Snail, and Vimentin.[26] Interestingly, Zhong *et al* showed that miR-30a inhibited cell migration and invasion by downregulating phosphoinositide 3-kinase catalytic subunit delta (PIK3CD) in colorectal carcinoma.[23] Furthermore, Wei *et al* suggested that miR-30a-5p suppresses tumor metastasis of human CRC by targeting integrin β3 (ITGB3).[25] However, in our study, MTDH was confirmed to be a target of miR-30a during invasion and migration of CRC. Because each miRNA may have the capacity to regulate the expression of hundreds of target genes, affecting a diverse range of biological processes, there may be competitive endogenous RNA regulatory networks among MTDH, PIK3CD, and ITGB3 that are mediated via competitive binding to miR-30a, which should be investigated in the future.

To determine the role of MTDH in CRC, we knocked down MTDH, which resulted in the inhibition of cell migration and invasion, similar to the effect of miR-30a overexpression. In addition, MTDH overexpression could partially attenuate the suppressive function of miR-30a in CRC cells. Metadherin,

which is also known as lysine-rich carcinoembryonic antigen-related cell adhesion molecule (CEACAM)-1-coisolated (LYRIC) protein and AEG-1 protein, has been noted in recent years as a key contributor to the carcinogenic process in diverse organs and tissues, including CRC.[12,13,15-18,20,22,26-28] To date, MTDH has been reported to be a promising therapeutic target for CRC.[22,29] Song *et al* found that the elevated expression of MTDH correlates with poor overall survival in patients with colorectal carcinoma.[29] Gnosa *et al* showed that MTDH is upregulated during CRC development and aggressiveness at the mRNA and protein level, and this upregulation is relative to the tumor location and TNM stage.[30] Furthermore, there have been some reports on the contribution of MTDH in CRC migration and invasion. In CRC cells, MTDH overexpression increased nuclear β-catenin accumulation, promoting migration and invasion.[22] Afterward, Song *et al* established that the ectopic expression of MTDH could enhance the invasion and metastasis by enhancing MMP9 activity.[28] Knockdown of MTDH could significantly inhibit colon cancer cell proliferation, colony formation, and invasion as well as promote apoptosis. Additionally, downregulation of MTDH may also lead to the modification of key elemental characteristics such as miRNAs, which may be a potential therapeutic strategy for CRC.[4] Moreover, other studies have demonstrated that patients with high MTDH expression are more likely to be identified early in the course of liver metastasis and lung relapse than those with a low expression level.[27,31]

In conclusion, our study demonstrates that miR-30a inhibits CRC cell migration and invasion. MTDH, an important oncogene, was identified as a target of miR-30a. Our findings suggest that miR-30a and MTDH may have an important role in gene therapy for treating CRC in the future.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental Material

Supplementary material for this article is available online.

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