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MiR-182-5p inhibited proliferation and metastasis of colorectal cancer by targeting MTDH

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Abstract. – OBJECTIVE: To investigate the role and molecular mechanism of miR-182-5p in the development of colorectal cancer (CRC), thereby providing a theoretical basis for new CRC therapeutic targets.

PATIENTS AND METHODS: The expression of miR-182-5p in CRC clinical cases and biological cell lines was detected. On-line target gene prediction and Luciferase reporter gene assay were performed to screen and verify the target of miR-182-5p, respectively. The role of miR-182-5p in CRC cell function was further analyzed.

RESULTS: MiR-182-5p expression was significantly decreased in both CRC tissues and cell lines. Metadherin (MTDH) was screened and verified as a functional target of miR-182-5p. The cell proliferation, invasion and migration ability of CRC cells were significantly inhibited after the up-regulation of miR-182-5p. However, MTDH limited the anti-cancer effects of miR-182-5p in CRC cells.

CONCLUSIONS: Our research demonstrated the inhibitory function of miR-182-5p in CRC. Therefore, the miR-182-5p/MTDH axis was expected to be one of the targets of CRC targeted therapy.

Key Words:
MicroRNA-182-5p (MiR-182-5p), Colorectal cancer (CRC), Metadherin (MTDH), Epithelial-mesenchymal transition (EMT).

Introduction

In the world, colorectal cancer (CRC) has become the third and second most important malignant tumor in males and females, respectively[1]. Due to universal implementation of early screening and improvement of treatment methods, the mortality rate of CRC has exhibited a declining trend. However, a large number of patients still die of CRC every year[2]. Moreover, the incidence and mortality rates of CRC remain persistently high in China[3]. No evident symptoms are manifested in patients with early-stage CRC. Meanwhile, most of the patients have already been in the advanced stage when diagnosed. Rapid development has been achieved in targeted therapies for tumor, which provides a glimmer of hope for the treatment of CRC patients. However, there are still limited efficacious targets in the clinical treatment of CRC. Furthermore, the effective rate of treatment is far from being satisfactory[4,5]. Therefore, it is urgent to further explore the pathogenesis of CRC and actively seek for novel potential therapeutic targets, eventually providing new theoretical bases for the treatment of CRC.

Micro-ribonucleic acids (miRNAs) are a category of small non-coding single-stranded RNAs with about 22 nt in length. MiRNAs are processed from single-stranded RNA precursors by nuclease and Dicer cleavage. They can degrade messenger RNA (mRNA) or inhibit mRNA translation at the post-transcriptional level mainly by reverse complementary pairing with the 3'-untranslated region (UTR) of the target gene, thereby controlling gene expression[6-8]. A large number of studies have demonstrated that miRNA participates in the regulation of 30% human genes. Meanwhile, it involves multiple physiological processes, such as cell differentiation, proliferation, invasion, apoptosis and angiogenesis[9,10]. In 2002, Calin et al[11] discovered that miR-15 and miR-16 are related to the regulation of chronic lymphoma. Moreover, they have also revealed that miRNA is closely correlated with the occurrence and development of malignant tumor for the first time. These findings lead to the upsurge of research on miRNA in tumors.

As a member of the miRNA family, the role of miR-182-5p in various diseases has been reported, especially in the tumor. However, miR-182-5p

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appears to have different roles in different malignancies. In hepatocellular carcinoma, miR-182-5p enhances motility and invasive ability of HCC cells[12]. Similarly, miR-182-5p also serves as a cancer-promoting factor in gastric cancer[13] and breast cancer[14]. However, the tumor suppressor effect of miR-182-5p has been discovered in renal cell carcinoma[15]. In this work, the role of miR-182-5p in the occurrence and development of CRC was clarified. In addition, we explored its related molecular mechanism, hoping to provide some experimental basis for the treatment of CRC.

Patients and Methods

CRC Cases and Cells

CRC tissues and adjacent normal tissues (>5 cm from the edge of cancer tissue) were harvested from 40 CRC patients who were diagnosed and surgically treated in our hospital from July 2015 to July 2017. Preoperative chemotherapy or radiotherapy treatment were forbidden. The collected specimens were kept in -80ºC refrigerator immediately. The Declaration of Helsinki should be mentioned and respected. This study has been approved by the Ethics Committee of Zhongnan Hospital of Wuhan University. Signed informed consents were obtained from all participants before the study.

Human CRC cell line (SW620) together with normal human intestinal epithelial cell line (HIECs) was purchased from the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) complemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 μg/mL streptomycin and 100 IU/ml penicillin (Gibco, Grand Island, NY, USA) in a 5% CO_2 cell culture incubator.

Target Prediction

The possible targets of miR-182-5p were predicted using target gene prediction software, including miRBase (http://www.mirbase.org/), TargetScan (http://www.targetscan.org/) and PicTar (http://pictar.mdc-berlin.de/).

Luciferase Reporter Gene Assay

The binding sequence of miR-182-5p at the 3'-UTR of metadherin (MTDH) was mutated using a point mutation kit (Agilent Technologies, Santa Clara, CA, USA). Both mutated MTDH (Mut-type) and non-mutant MTDH (WT-type) were connected to the pGL3-Basic Luciferase reporter vector (Promega, Madison, WI, USA). Subsequently, Mut- and WT-type were transfected into SW620 cells after lentivirus intervention on 24-well plates. Four groups were established: miR-182-5p/MTDH-Wt-type group, NC/MTDH-Wt-type group, miR-182-5p/MTDH-Mut-type group and NC/MTDH-Mut-type group. 48 h after transfection, Luciferase activity was detected by a multi-function microplate reader.

Cell Transfection

Cells in logarithmic growth phase were seeded into 6-well plates at a density of $4 * 10^5$ cells/well. Cell transfection was performed according to instructions of Lipofectamine™2000 (Invitrogen, Carlsbad, CA, USA) after cell attachment. 6 h after transfection, the culture medium was replaced and cultured for another 24 h. The transfection efficiency was detected by qRT-PCR.

Three groups, including NC group (negative control), miR-182-5p mimics (CRC cells transfected with miR-182-5p mimics) and mimics + MTDH (CRC cell transfected with miR-182-5p mimics and LV-MTDH) were established *in vitro*.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) Analysis

Total RNA was extracted by TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. SYBR green qPCR assay was used to measure the expression level of MTDH. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. TaqMan miRNA assay (Applied Biosystems, Foster City, CA, USA) was used to measure the expression level of miR-182-5p normalized to miRNA U6. Primer sequences used in this study were as follows: MTDH, F: 5'-CCCTCCTTACTCAGGAACCC-3', R: 5'-CGAAGGCTAGGGATGTGTCA-3'; miR-182-5p, F: 5'-CTCTGTGTAAACGGGTCCTCGACTG-3', R: 5'-TCCGGGTGTCGTGGAGTCG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blot (WB) Analysis

Total protein in cells of each group was extracted by radio-immunoprecipitation assay (RIPA) lysate (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The concentration of extracted protein was determined by bicinchoninic acid

(BCA) method (Pierce, Rockford, IL, USA). A total of 20 μg protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). After blocking with 5% milk, the membranes were incubated with the following primary antibodies: MTDH, E-cadherin, Vimentin and β-actin diluted at 1:1000 (Cell Signaling Technology (CST) Inc., Danvers, MA, USA) at 4°C overnight. Then, the membranes were washed with TBST three times, followed by incubation with the corresponding secondary antibodies (CST, Inc., Danvers, MA, USA) at room temperature for 2 h. Finally, the membranes were exposed in a gel imaging system. β-actin was used as an internal reference, and the relative expression level of protein was calculated.

Cell Proliferation

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Sigma-Aldrich, St. Louis, MO, USA) was used to detect the proliferation of the transfected cells. After transfection, CRC cells were seed into 96-well plates at a concentration of 3×10^4/well. After 24 h of culture, 50 μL of MTT solution (concentration of 5 mg/mL) was added to each well, followed by incubation for another 4 h. Then, the supernatant was discarded and mixed with 150 μL of dimethyl sulfoxide (DMSO) solution (Sigma-Aldrich, St. Louis, MO, USA). The absorbance at 24, 48, 72, and 96 h after transfection was detected by a microplate reader, respectively. 8 replicate wells were set in each group.

Cell Invasion and Migration Assays

Cell migration and invasion abilities were measured using a transwell chamber (Corning Inc., Corning, NY, USA).

Three groups of cells were collected 48 h after transfection, and cell density was adjusted to 1×10^5/mL. 200 μL of cell solution was inoculated into the upper chamber, and the serum-free medium was added. Meanwhile, the lower chamber was added with the culture medium containing 10% FBS. After 36 h of incubation, the chamber was taken, and un-migrated cells in the upper chamber were removed. Subsequently, the cells were washed with phosphate-buffered saline (PBS) twice and fixed with 4% paraformaldehyde for 10 min, followed by staining with 0.1% crystal violet for 5 min. Five fields were randomly selected for each sample under an inverted microscope (×200). The number of migrating cells was counted, and the average was calculated. For cell invasion, the transwell chamber was pre-coated with Matrigel gel, and the remaining steps were are the same as the cell migration assay.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 16.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. The experimental results were expressed by ($\bar{x}\pm s$). The *t*-test was used to compare the differences between the two groups. $p<0.05$ was considered statistically significant.

Results

Expression of MiR-182-5p in CRC Tissues and Cells

Abnormal expression of miRNA is the basis of its role in biological function. The expression of miR-182-5p in CRC tissues was markedly lower than that of the adjacent normal tissues (Figure 1A). Consistent with the results, we found that the miR-182-5p expression was also significantly decreased in CRC cell lines (SW620) (Figure 1B). To further explore the role of miR-182-5p in CRC, we altered the expression of miR-182-5p in SW620 cells *in vitro* and verified by a series of related experiments.

Prediction and Verification of MiR-182-5p Targets

MiRNA online databases, including TargetScanHuman, miRBase and PicTar, predicted that MTDH had a binding site with miR-182-5p at 3'-UTR (Figure 2A). This meant that miR-182-5p might be able to targeted regulate MTDH expression. Luciferase reporter gene assay was used to further verify our assumption. Surprisingly, the inhibitory effects of miR-182-5p on the fluorescence expression of psiCHECK-MTDH (MTDH-3'UTR-wild) was found. However, no inhibition was observed in the fluorescence expression of psiCHECK-MTDH-mut (MTDH-3'UTR-mut). This result proved that miR-182-5p could inhibit the MTDH expression by targeted binding to the 3'-UTR of MTDH (Figure 2B-2C).

In vitro results confirmed the targeted regulation of miR-182-5p on MTDH. However, whether this effect could be reflected in CRC organization and cells needed to be further investigated.

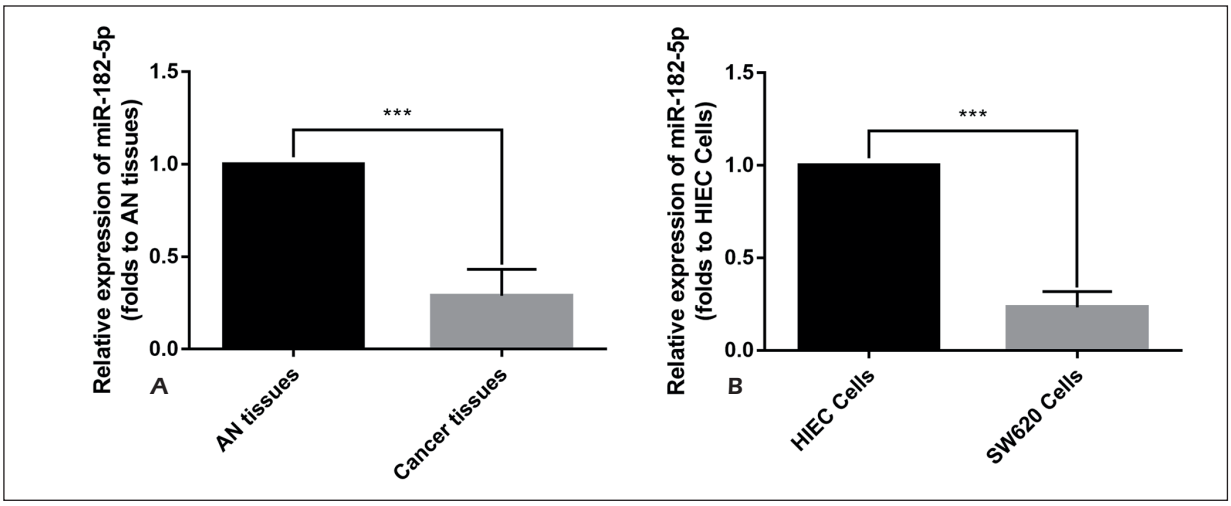

Figure 1. The expressions of miR-182-5p in CRC tissue samples and cells compared with corresponding adjacent normal tissues and normal human intestinal epithelial cells. **A**, Difference in the expression of miR-182-5p between CRC tissues and corresponding adjacent normal tissues (***p<0.001). **B**, Difference in the expression of miR-182-5p between CRC cells (SW620) and normal human intestinal epithelial cells (HIECs) (***p<0.001).

Subsequently, we detected the expression of MTDH in CRC tissues and adjusted normal tissues by qRT-PCR and WB. The results indicated that the protein expression of MTDH in CRC tissues was significantly higher than that of normal tissues. However, no significant differences between the two kinds of tissues were found in mRNA level (Figure 3A). It became evident that the regulation of MTDH expression was mediated by post-transcriptional regulation.

Further cell experiments found that miR-182-5p could change the protein expression of MTDH in SW620 cells. These results indicated that we could regulate MTDH expression by interfering with miR-182-5p in SW620 cells (Figure 3B).

Role of MiR-182-5p in Cell Function

MTT results showed that the growth inhibition was significantly improved in the miR-182-5p mimics group when compared with the NC group

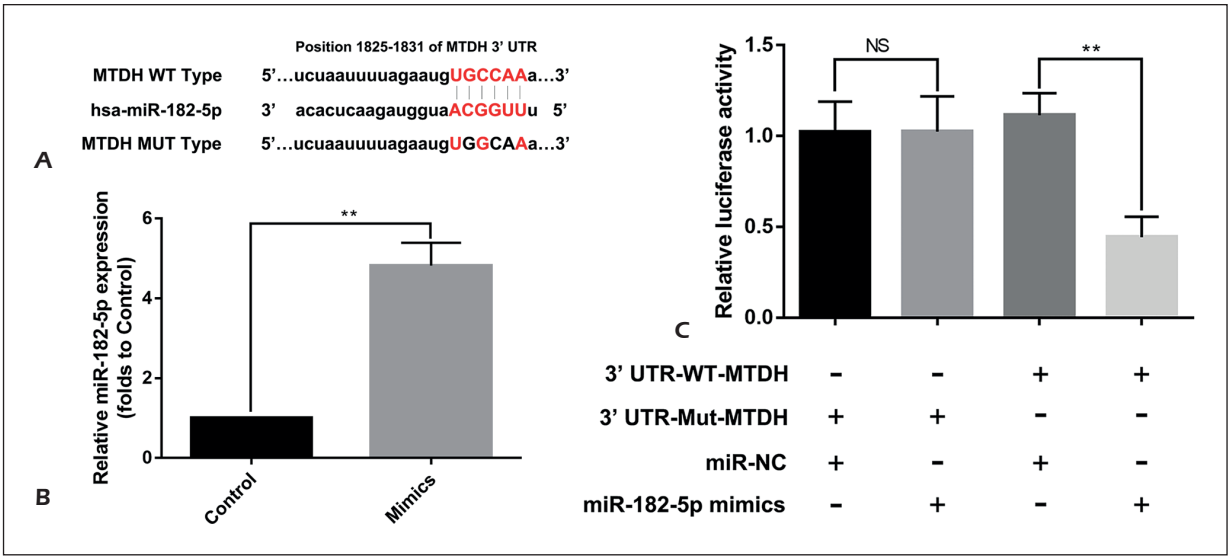

Figure 2. MTDH was a direct and functional target of miR-182-5p. **A**, Diagram of putative miR-182-5p binding sites of MTDH. **B**, Transfection efficiency detected by qRT-PCR (**p<0.01). **C**, Relative activities of Luciferase reporters (**p<0.01).

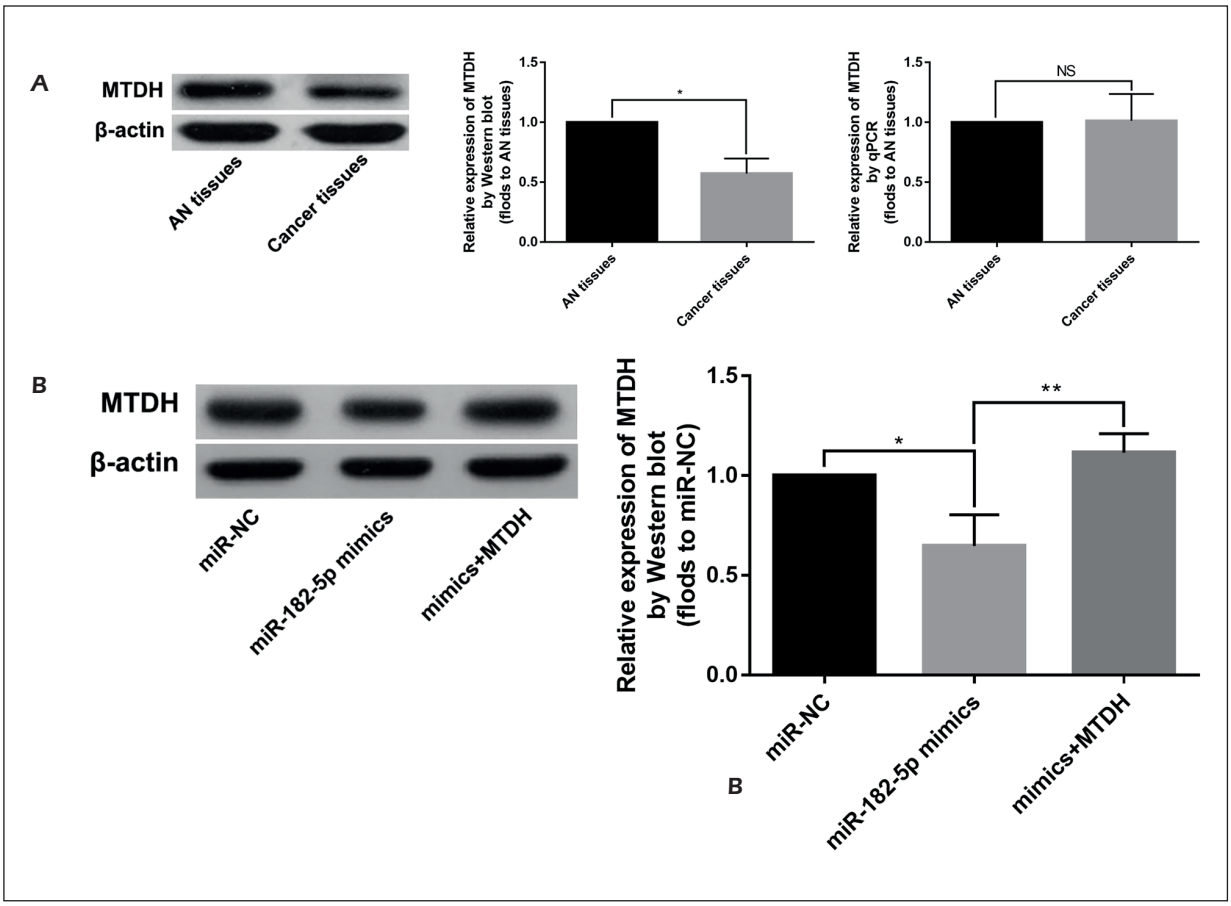

Figure 3. A, The expressions of MTDH in CRC tissues and adjacent normal tissues were detected by WB and qRT-PCR assay (*p<0.05). **B**, MiR-16-5p decreased the expression level of MTDH after transfection (*p<0.05, **p<0.01).

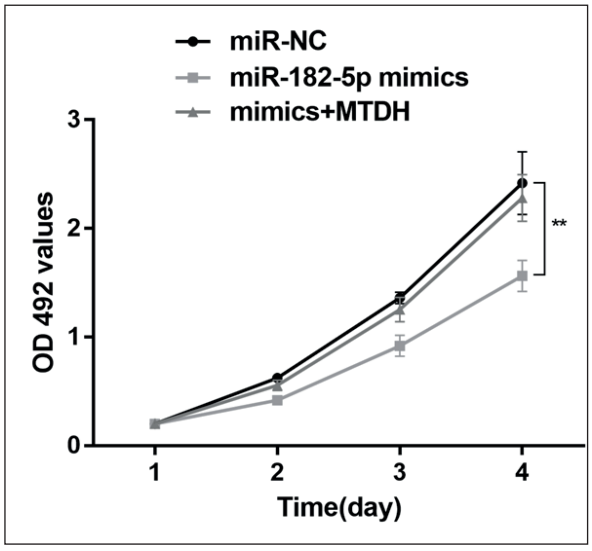

Figure 4. MiR-182-5p inhibited the proliferation of CRC cells. The proliferation of CRC cells transfected with mimics or inhibitor was analyzed using MTT assay (**p<0.01).

after transfection (p<0.05). Cells in the miR-182-5p mimics group showed a significant growth inhibition (Figure 4).

The invasion and migration ability is the basic ability of tumor cells to metastasize. In transwell assay, miR-182-5p could significantly reduce the transferability of SW620 cells. The number of migration and invasion cells in the miR-182-5p mimics group was remarkably decreased (Figure 5A, 5B). The epithelial-mesenchymal transition (EMT) is another important indicator reflecting the ability of cell metastasis. In our work, the expressions of EMT associated markers were detected by WB assay. The results showed that after overexpression of miR-182-5p in SW620 cells, the expression of epithelial marker E-cadherin was notably increased. However, the protein expression of mesenchymal marker Vimentin was markedly decreased (Figure 5C, 5D).

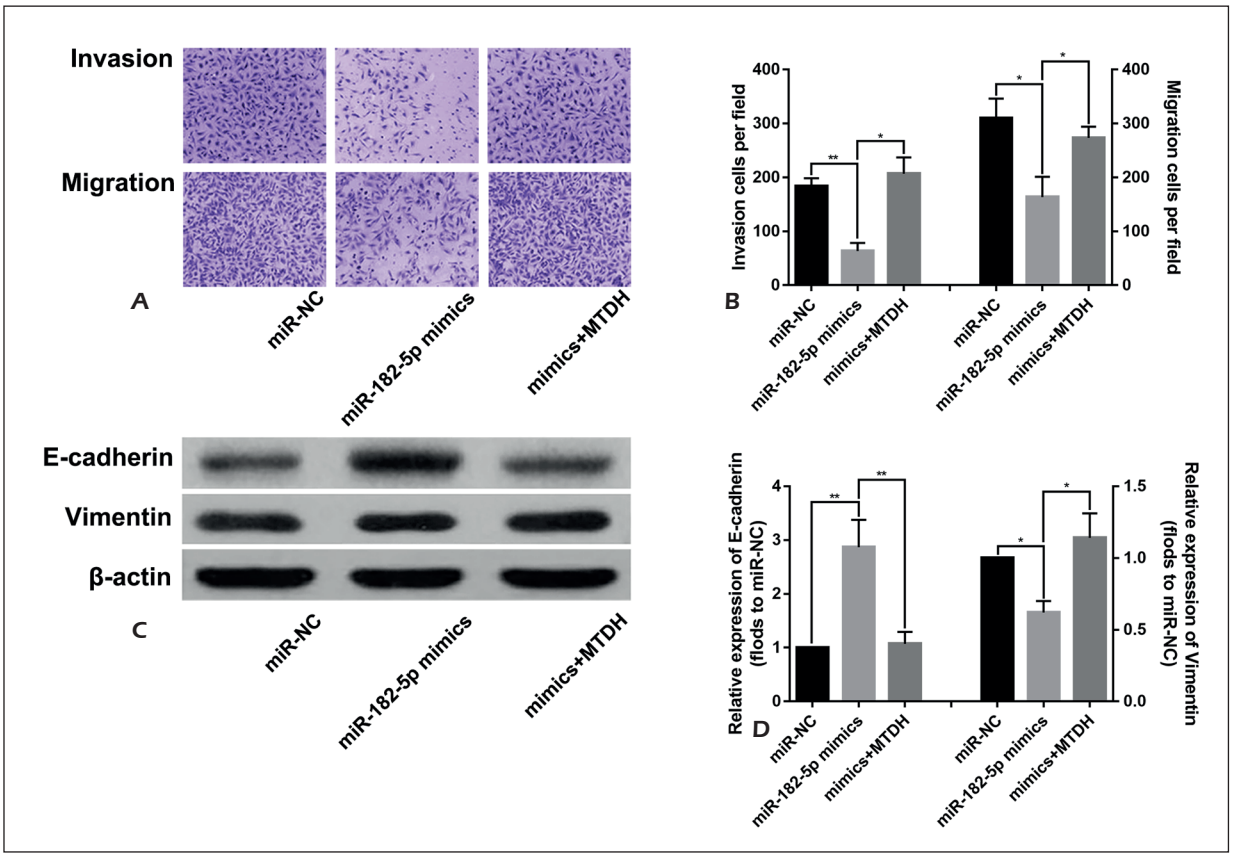

Figure 5. A-B, MiR-182-5p inhibited the invasion and migration of CRC cells. The invasion and metastasis of CRC cell post-transfection were analyzed using transwell assay. The number of migrating and invasive cells was detected by microscope (×200) (*p<0.05, **p<0.01). **C-D**, Epithelial-mesenchymal transition (EMT) of CRC cells. EMT associated markers post-transfection were detected by Western blot. Data were presented as means ± standard deviations (*p<0.05, **p<0.01).

The above results demonstrated that miR-182-5p could effectively limit SW620 cell function, including proliferation, migration and invasion.

Next, we increased the expression of MTDH exogenously in miR-182-5p overexpressed SW620 cells. In addition, the malignant capacity of SW620 cells was apparently improved.

Discussion

The occurrence and development of CRC involve a variety of abnormal gene changes and complex pathological changes. This process includes the transformation from normal mucosa to excessive hyperplasia, the formation of adenoma, the progression to cancer, and even cancer infiltration and metastasis. Currently, a great number of studies have manifested that miRNAs are closely correlated with malignant tumors. As non-coding RNAs with short sequences, miRNAs can regulate the expression of approximately 1/3 human genes at post-transcriptional level through reverse complementary pairing with the 3'-UTR of target genes. In addition, different miRNAs play varying roles in different diseases. Some have pro-oncogenic effects, while others exert anti-oncogenic effects. Furthermore, the same miRNA may exert completely opposite effects on the biological behaviors of different tumor cells. Studies have indicated that abnormal expressions of many miRNAs exist in CRC. For example, highly expressed miR-21 in CRC patients is associated with lymph node positivity and distant metastases[16]. MiR-148a triggers the apoptotic pathway by silencing Bcl-2 in CRC[17]. MiR-144 is a meaningful prognostic marker. The downregulation of miR-144 leads to the poor prognosis of CRC patients *via* the activation of the mTOR signaling pathway[18]. Although considerable studies have verified that miRNAs play important roles in tumor, the specific mech-

anism needs to be further explored. In this work, it was found that the expression level of miR-182-5p in CRC tissues and cells was remarkably declined. Hence, three on-line bioinformatics softwares were applied to screen miRNAs. The results showed that miR-182-5p was capable of binding to the complementary sites within the 3'-UTR of MTDH, and the seed sequences were conservative among species.

Metadherin (MTDH), also known as Lyric or AEG-1, is located on human chromosome 8th (8q22). Due to its function to help tumor cells close to long-distance blood vessels, it is of great significance for the spread and metastasis of cancer cells. The overexpression of MTDH usually indicates poor prognosis and drug resistance in breast cancer[19,20]. Besides, MTDH has been reported in other malignant tumors by affecting various malignant biological behaviors, such as cell proliferation, invasion and metastasis[21-24]. Therefore, we believed that MTDH could be a potential therapeutic target gene. Further detection in our study indicated that the protein expression level of MTDH in CRC tissues was significantly decreased, which was negatively correlated with the miR-182-5p level. However, no change in mRNA expression level was observed. This was exactly in line with the regulatory mechanism of mRNA. The Luciferase reporter gene assay also proved that overexpressed miR-182-5p could suppress the Luciferase activity of wild-type MTDH rather than mutant-type MTDH.

Carcinogenesis is the process in which normal cells are freed from the intrinsic regulatory mechanisms of cells, eventually forming uncontrolled and persistent cells. Obtaining abnormal proliferation ability is a key step in the process of carcinogenesis. On the other hand, some metastasis capacity is necessary when tumor cells break away from the original tissue and invade the bloodstream and surrounding tissues. In general, the strong mobility of tumor cells indicates higher malignancy. Therefore, proliferation, invasion and migration abilities have become a positive aspect of the current cancer research. In our study, the up-regulation of miR-182-5p significantly inhibited the proliferation, invasion and migration abilities of CRC cells. However, when we artificially up-regulate the expression of MTDH in miR-182-5p overexpressed CRC cells, the malignant proliferation and metastasis ability of CRC cells recovered.

EMT is a vital mechanism of tumor metastasis, which can alter cell phenotypes and accelerate tumor metastasis by inhibiting cell adhesion molecules. It can also restore the epithelial specificity of CRC, and promote the transmission of metastatic cells to distant organs. E-cadherin, as a transmembrane glycoprotein, is generally located at the adhering junction of epithelial cells. It plays a crucial role in maintaining the integrity of epithelial structure[25]. The loss of E-cadherin expression has been reported in several malignant tumors, including CRC[26]. Reduced adhesion between cells can stimulate tumor cells proliferation and accelerate tumor progression[27,28]. Vimentin is expressed in almost all normal interstitial cells. Meanwhile, it is an important member of the intermediate filament protein family. The property of vimentin is of positive significance for maintaining cell integrity and defending against extracellular stress injury. A broad consensus has been reached on vimentin as a marker of EMT standard so far. In our transwell experiments, we did not unexpectedly found that miR-182-5p could significantly inhibit EMT of CRC cells, while MTDH also influenced the effects of miR-182-5p on EMT.

Conclusions

We showed that the expression of miR-182-5p was down-regulated in CRC tissues and cells. The anti-cancer effects of miR-182-5p were reflected in CRC cells *in vitro*. Unfortunately, the role of miR-182-5p *in vivo* was not investigated, which should be elucidated in the future.

Conflict of Interests

The authors declare that they have no conflict of interest.

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