

ORIGINAL ARTICLE

MiR-421 inhibited the proliferation and metastasis of colorectal cancer by targeting MTA1

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Summary

Purpose: To investigate the role and molecular mechanism of miR-421 in the development of colorectal cancer (CRC), providing a theoretical basis for the search for new CRC therapeutic targets.

Methods: 30 pairs of human CRC and cancer-adjacent normal tissue samples were collected. The expression of miR-421 was detected in CRC tissues and cells. On-line target gene prediction software was applied to screen metastasis-associated protein 1 (MTA1), the potential downstream target gene of miR-421. The role of miR-421 in regulating MTA1 and its effect on the expression of epithelial-mesenchymal transition (EMT) markers (E-cadherin and Vimentin) were detected.

Results: Compared with that in adjacent normal tissues and normal human intestinal epithelial cells, the miR-421 expres-

sion level in CRC tissues and cells was significantly reduced. The potential target of miR-421 was analyzed by three public databases, in which we found that MTA1 was a direct target of miR-421, and miR-421 inhibited the proliferation, invasion, migration and EMT of CRC cells through the targeted regulation of the expression of target gene MTA1, thus effectively suppressing the ability of CRC cells.

Conclusions: This research demonstrated the suppressive function of miR-421 in CRC. Therefore, the miR-421/MTA1 axis is expected to be one of the targets of CRC targeted therapy.

Key words: colorectal cancer, epithelial-mesenchymal transition, metastasis-associated protein 1 (MTA1), MiR-421

Introduction

Colorectal cancer (CRC) is one of the most common malignant tumors in the digestive tract worldwide. Its morbidity rate ranks 3rd in males and 2nd in females [1]. In recent years, the morbidity and mortality rates of CRC in China had been on the rise due to the residents' bad habits, such as excessive drinking, smoking, high-fat and low-fiber diets and lack of physical activity. In 2015, there were 376,000 new cases and 191,000 deaths, and the morbidity and mortality rates of CRC ranked 5th among cancers in China [2]. At present, the main treatment methods for CRC are dependent on sur-

gery. However, most tumors are in advanced stage when diagnosed. CRC easily recurs after operation, and adjuvant treatments are not satisfactory, thus affecting the patient's survival. Therefore, the occurrence and development mechanisms of CRC need more in-depth research in order to explore more effective treatment approaches.

The occurrence and development of CRC shows a variety of genetic abnormalities and complex pathological changes, including the transformation from normal mucosa to hyperplasia, then to the formation of adenomas, the eventual evolution

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into cancer and complex pathological processes such as cancer invasion and metastasis. Micro RNAs (miRNAs) are a class of non-coding small RNAs that rely on their 5'-end nucleotides, which can bind to 3' untranslated region (3'-UTR) of the target messenger RNA (mRNA) to prevent translation or stability of the mRNA, thereby regulating target gene function [3]. Studies had revealed that miRNAs were closely related to tumors and were hotspots for screening novel tumor markers, prognostic judgments, and therapeutic targets. Acting as oncogenes or tumor suppressor genes, miRNAs were involved in the occurrence and development of various cancers including CRC [4].

As a member of the miRNA family, miR-421 has shown its unique advantages in the diagnosis and treatment of a variety of cancers, such as osteosarcoma [5], prostate cancer [6], gastric cancer [7], breast cancer [8] and cervical cancer [9]. However, there are only few reports on the role of miR-421 in the occurrence and development of CRC and its related molecular mechanism. In this study, the role of miR-421 in the occurrence and development of CRC and its related molecular mechanism were clarified through analyzing the expression of miR-421 and the effects of miR-421 on the biological behaviors of CRC.

Methods

Colorectal cancer cases and cells

This study included 30 CRC patients undergoing surgical operation at the First People's Hospital of Lianyungang. Preoperative chemotherapy or radiotherapy treatment was not allowed. Liquid nitrogen was used to freeze the CRC tissues and corresponding adjacent normal tissues which were kept at -80°C. The adjacent normal tissues were biopsy-examined to make sure that they did not contain CRC cells. The Declaration of Helsinki was followed and the study was approved by the ethics committee of the First People's Hospital of Lianyungang. Signed informed consents were obtained from all participants before study entry.

The human CRC cell line (SW620) together with normal human intestinal epithelial cells (HIECs), were purchased from the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) medium (Invitrogen, Carlsbad, CA, USA), complemented with 10% fetal bovine serum (FBS), 100 mg/mL streptomycin and 100 IU/mL penicillin (Invitrogen, Carlsbad, CA, USA) in 5% CO₂ cell culture incubator. MiR-421 mimics/inhibitors and negative controls (NCs) were purchased from GenePharma (Shanghai, China). Cell transfections were performed according to the instructions of Lipofectamine® 2000 (Thermo Fisher Scientific, Waltham, MA, USA). SW620 cells in the logarithmic growth phase were taken and inoculated into the corresponding 6-well

plates. After 24 hrs, NCs or miR-421 mimics/inhibitors were transfected into SW620 cells with final concentration of 100 nmol/L. Experimental procedures were carried out according to the instructions of lipidosome transfection reagent Lipofectamine® 2000, and 6 hrs after transfection, Dulbecco's modified Eagle Medium (DMEM) was replaced with the complete medium.

Luciferase Reporter Assays

In TargetScan, miRDB and microRNA websites, it was found that MTA1 was a target gene of miR-421. The binding sequence of miR-421 at the 3'-end of MTA1 was mutated using a point mutation kit (Agilent Technologies, Santa Clara, CA, USA), and the mutated MTA1 (Mut-type) and non-mutant MTA1 (WT-type) were connected with the pGL3-Basic luciferase reporter vector (Promega, Madison, WI, USA). PGL3-Basic vector with mutant MTA1 was transfected into SW620 cells after lentivirus intervention on the 24-well plate. The same treatment was performed on the pGL3-Basic vector connected with the non-mutant MTA1 according to steps in the Luciferase Reporter Gene Assay Kit. Then the luciferase activity was detected in a multi-function microplate reader.

Transfection

MiR-421 mimics, control and inhibitor were synthesized and transfected to CRC cell line to analyse the biological function of miR-421. Three groups were established to study the potential relevance between miR-421 and CRC: negative control (NC) group, miR-421 mimics (SW620 cell transfected by miR-421 mimics) and mimics + MTA1 (SW620 cell transfected by miR-421 mimics and si-MTA1). All the stuff was purchased from RiboBio (Guangzhou, China), and was transfected using lipofectamin RNAiMAX (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions.

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis

SW620 cells were detected via qPCR. Total RNA was procured by TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and used in accordance with the manufacturer's protocol. SYBR green qPCR assay was used to measure the level of MTA1, expression and endogenous controlled by glyceraldehyde 3-phosphate dehydrogenase (GAPDH). TaqMan miRNA assays (Applied Biosystems, Foster City, CA, USA) was used to measure the level of miR-488 expression normalized to miRNA U6.

Cell proliferation

When cells grew to the logarithmic growth phase, they were collected, diluted into 1×10⁶ cell suspension, and added into a 96-well cell culture plate (5×10³/100 µL per well). The wells only added with medium were used as blank controls. Cell viability was determined via MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay. 15 µL MTT reagents (500 µg/mL) were added, after which the absorbance was measured using an enzyme-labeled spectrophotometer, followed by zero setting using blank wells.

Cell invasion and migration assays

After 48 hrs of transfection, cell migration and invasion abilities were measured using a Transwell chamber (Corning Incorporated, Corning, NY, USA) with a pore size of 8 μ m, in which Matrigel (BD, Franklin Lakes, NJ, USA) at a concentration of 1:9 was paved in the upper chamber for detection of invasion ability. Subsequently, the upper chamber was added with 250 μ L serum-free medium, while the lower chamber was added with 700 μ L medium containing 10% FBS. Cells (5×10^4 /well) were then added to the upper chamber and placed in an incubator for incubation. After 24 hrs, the chamber was removed, and the remaining cells in the upper chamber were gently wiped off with a cotton swab. The lower chamber cells were then fixed with paraformaldehyde and stained with crystal violet. Finally, 5 fields of view were randomly selected under an inverted microscope ($\times 200$) for counting, and the average was calculated.

Western blot analysis

Radioimmunoprecipitation assay (RIPA) lysate (Santa Cruz, Santa Cruz, CA, USA) was employed to extract the total protein of the cells. The proteins were first separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (Sigma, St. Louis, MO, USA) and then transferred onto polyvinylidene fluoride (PVDF) membranes (Roche, Basel, Switzerland). After that, the membranes were blocked with 5% skimmed milk and incubated at 4°C overnight with antibodies, E-cadherin, vimentin and β -actin [diluted at 1:1000, Cell Signaling Technology (CST) Inc. Danvers, MA, USA]. The next day, the corresponding secondary antibodies (CST, Inc. Danvers, MA, USA) were used for incubation at room temperature, followed by development *via* enhanced chemiluminescence (ECL) (Thermo Fisher Scientific, Waltham, MA, USA), exposure in gel imaging

system, fixation and observation of results. With β -actin as an internal reference, the relative changes in protein expression were detected.

Statistics

Statistical analyses were performed with Student's t-test or F-test. All p values were two-sided and $p < 0.05$ was considered significant and analyzed by Prism 6.02 software (La Jolla, CA, USA).

Results

Expression of miR-421 in CRC tissues and cells

To examine the role of miR-421 in CRC development, we detected the level of miR-421 expression in CRC. MiR-421 expression in CRC tissues and cancer-adjacent tissues was detected using qRT-PCR first. The results showed that the expression of miR-421 was pretty lower in CRC tissues by comparing with the adjacent normal tissues (Figure 1A). Then the expression level of miR-421 in the CRC cell line SW620 and normal HIECs was detected. The same results were got in cellular level (Figure 1B). Taken together, we thought miR-421 might have some regulation effect during the progression of CRC.

Inhibitory effects of miR-421 on the proliferation

To examine the function of miR-421 on the proliferation of CRC cells, we performed MTT assay to detect the cell proliferation rates. The MTT results showed that the cell proliferation rates of SW620 cell were decreased by upregulation of

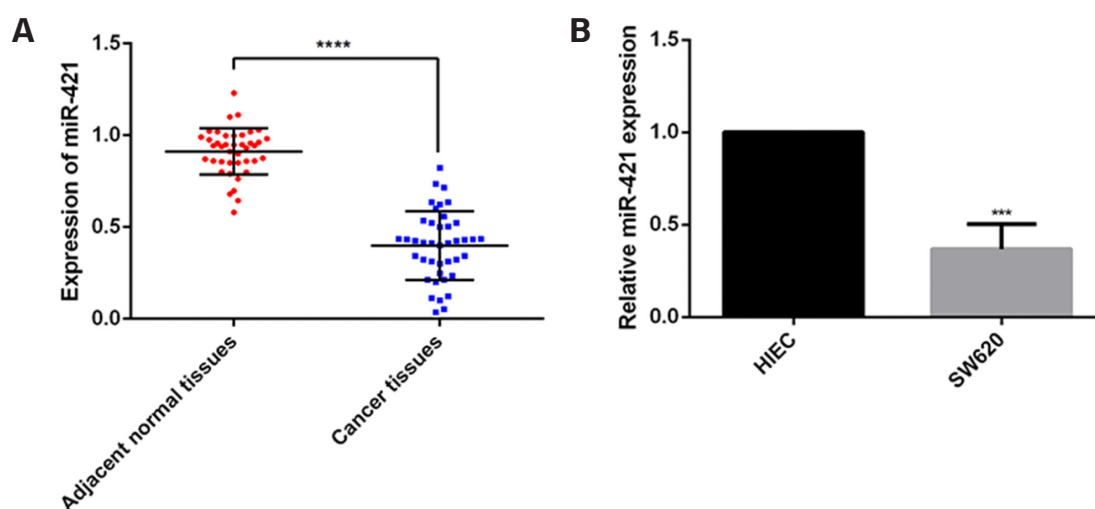


Figure 1. The expressions of miR-421 and MTA1 in colorectal cancer (CRC) tissue samples and cells comparing with corresponding adjacent normal tissues and normal human intestinal epithelial cells. **(A):** Significant difference in the expression of miR-421 between CRC tissues and corresponding adjacent normal tissues (**** $p < 0.0001$) compared with adjacent normal tissue). **(B):** Significant difference in the expression of miR-421 and MTA1 in CRC cells (SW620) and normal human intestinal epithelial cells (HIECs) (*** $p < 0.001$ compared with HIECs).

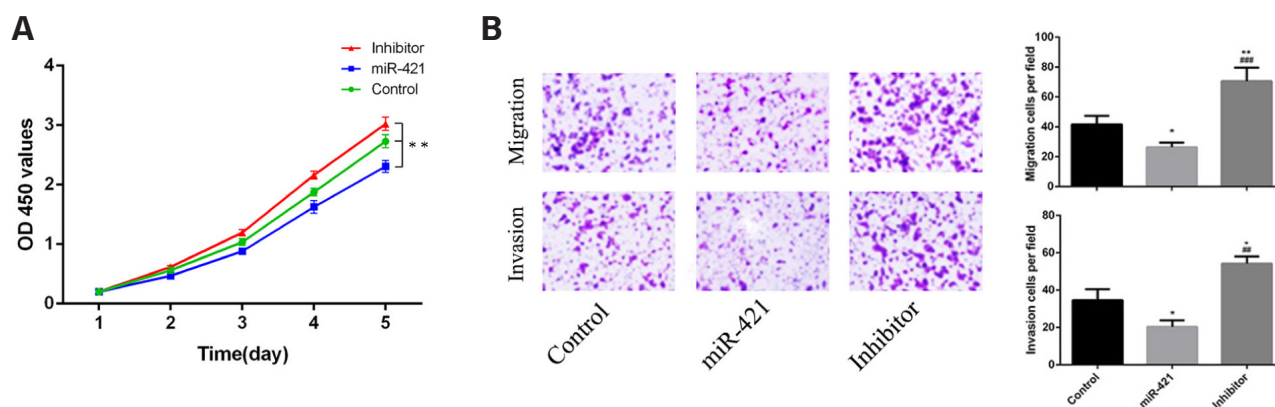


Figure 2. (A): MiR-421 inhibits the proliferation of CRC cells. The proliferation of CRC cells transfected with mimics or inhibitor was analyzed using MTT assay (** $p < 0.01$). MiR-421 inhibited significantly the proliferation of CRC cells. **(B):** The invasion and metastasis of CRC cells post-transfection with mimics or inhibitor were analyzed using Transwell assay and detected by microscope ($\times 200$). MiR-421 inhibited the invasion and migration of CRC cells. (* $p < 0.05$, ** $p < 0.01$ vs. control group; ** $p < 0.01$, *** $p < 0.001$ vs. mimics group).

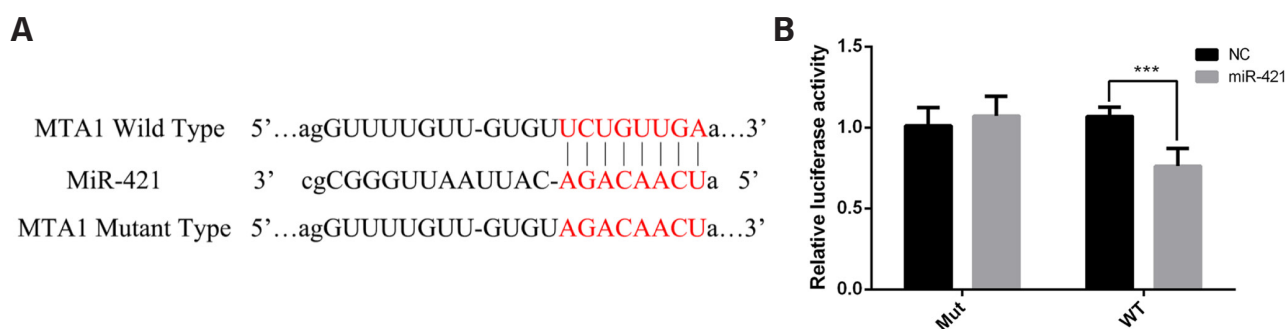


Figure 3. MTA1 is a direct and functional target of miR-421. SW620 cells were transfected with miR-421 mimics and inhibitor. **(A):** Diagram of putative miR-421 binding sites of MTA1. MiR-421 can bind the 3'-UTR of wild type MAT1. **(B):** Relative activities of luciferase reporters. The luciferase activity of wild type MTA1 was significantly decreased after transfection with miR-421 mimics (*** $p < 0.001$).

miR-421 using mimics transfection. In contrast, downregulated miR-421 expedited cell growth of CRC cells ($p < 0.05$) (Figure 2A).

Inhibitory effects of miR-421 on the invasion and migration

To explore whether miR-421 could regulate the invasion and migration ability, miR-421 mimics were applied to upregulate the level of miR-421 in SW620 cells first. Transwell assay was then conducted to detect changes in the migration and invasion abilities of SW620 cells after upregulation or downregulation of miR-421 expression level. The results illustrated that, compared with those in the control group, the migration and invasion abilities of SW620 cells were obviously decreased post transfection of miR-421 mimics, while the migration and invasion abilities were remarkably increased in the inhibitor-treated group (Figure 2B).

Prediction and verification of miR-421 targets

The miR target gene prediction software TargetScan revealed that miR-421 could act on the 3'-UTR of MAT1 (Figure 3A). Dual-Luciferase® Reporter Assay indicated that after the co-transfection of recombinant plasmids, MAT1-3'-UTR-mutant (MUT), MAT1-3'-UTR-wild type (WT) and miRNAs, the luciferase activity of MAT1 was decreased, compared with that in the control group (Figure 3). According to Western blotting, the expression of MAT1 was significantly reduced after miR-421 overexpression (Figure 3B).

Secondly, to explore the correlation between of MAT1 and miR-421 on CRC cells we set up three groups to perform similar experiments (miR-NC group, miR-421 mimics group, and mimics + MAT1 group) in SW620 cells.

As expected, restoration of MAT1 has the reverse force on the depressing effect induced by miR-421 on the proliferation of CRC cells, pro-

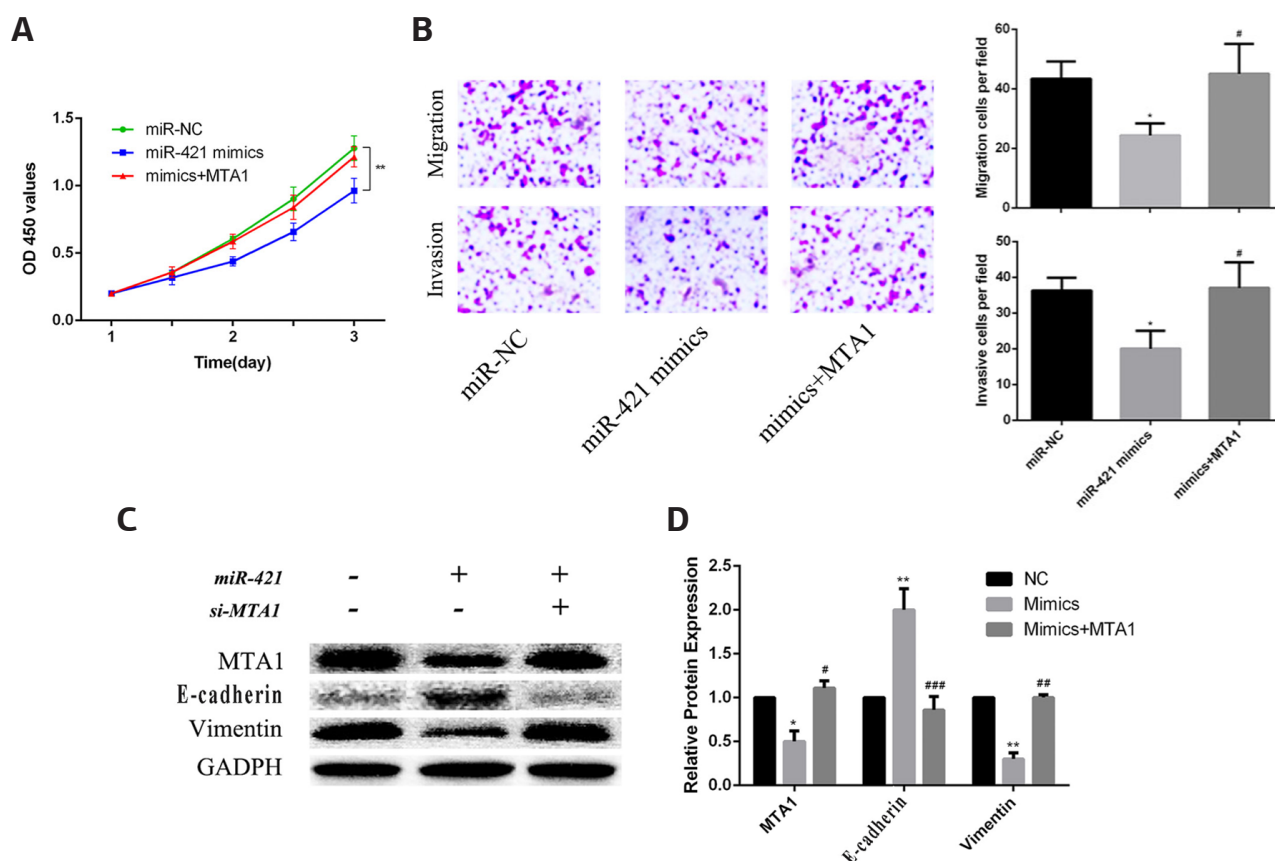


Figure 4. MTA1 overexpression attenuates the suppressive effect of miR-421 on CRC cells. **(A):** cell proliferation test by MTT assay. Restoration of MAT1 could partially reverse miR-421-induced inhibition of cell proliferation. **(B):** the invasion and metastasis test by Transwell assay. Restoration of MAT1 reversed miR-421-induced inhibition of tumor invasion and metastasis. **(C-D):** The epithelial-mesenchymal transition (EMT) of CRC cells. The expression of MTA1 and epithelial-mesenchymal transition (EMT) markers post-transfection with mimics or si-MTA1 were detected by western blot. E-cadherin was significantly decreased after transfection with si-MTA1, and vimentin was significantly increased after restoration of MAT1. Data were presented as means $\pm$ standard deviations. (* $p < 0.05$, ** $p < 0.01$ vs. NC group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. mimics group).

tein expression, migration, and invasion (Figure 4A,B). Taken it all, the results indicated that the upregulation of miR-421 has a negative relationship with MAT1 in CRC cells, miR-421 suppressed the metastasis and proliferation of CRC cells and MAT1 restoration partially weaken the inhibition of miR-421.

Inhibitory effect of miR-421 on the epithelial-mesenchymal transition

EMT plays important roles in the metastasis processes of tumor cells, but whether miR-421 exerted an effect on the EMT of CRC cells has not yet been reported. Therefore, Western blotting was employed to detect the effects of miR-421 on the expressions of EMT markers (E-cadherin and vimentin) in CRC cells. The results manifested that after overexpression of miR-421 in SW620 cells, the expression of the epithelial marker E-cadherin was notably increased, while the expres-

sion of the mesenchymal marker Vimentin was obviously decreased. The above results suggest that miR-421 could inhibit the EMT of CRC cells (Figure 4C,D).

Discussion

CRC is a common malignant tumor of the digestive system. In recent years, the diagnosis and treatment of CRC have been improved to some extent, but the surgical operation is still the radical treatment for CRC at present [10]. However, as CRC is characterized by high metastasis rate and easy recurrence, the prognosis of patients with CRC is still poor [10]. In recent years, targeted therapies have provided new ideas and hopes for the diagnosis and treatment of tumors including CRC. Correlations of miRs with the occurrence and development of tumors, as well as intrinsic mechanisms, were the research fields frequently explored

in depth [11]. A variety of studies had consistently demonstrated that miR-421 was lowly expressed in various tumor cells and can exerted its antitumor effects by participating in the regulation of tumor cell proliferation, apoptosis, invasion, migration and other biological functions [6-9]. However, the expression and role of miR-421 in CRC are not yet clear. This results of this study illustrated that miR-421 was lowly expressed in CRC tissues and cell lines, suggesting that miR-421 may be an antioncogene in CRC. Transwell assay revealed that miR-421 was closely associated with the invasion and migration of CRC cells.

Metastasis-associated protein (MTA) is the product of a family of tumor-related genes discovered in recent years, and has important relationships with the occurrence, development and metastasis of tumors [12]. In this family, MTA1 was the earliest one to be found with the largest number of relevant studies. MTA was first discovered in breast cancer, and its encoded proteins were highly expressed in normal testicular tissues but not expressed or weakly expressed in other normal tissues. However, MTA1 expression was up-regulated in different degrees in various human malignant tumors [13-15], and the role of MTA1 was to promote the migration of tumor cells and infiltration into the surrounding and distant tissues [16]. Besides, MTA1 can inactivate the tumor suppressor gene p53, thereby reducing cell growth inhibition and natural apoptosis [15]. In addition, MTA1 can also promote the development of tumor blood vessels by enabling hypoxia-inducible factor 1- α (HIF1- α) to be stable [17]. At the same time, studies had also manifested that the transcription

of MTA1 is positively correlated with grade of malignancy [18]. More importantly, the role of MTA1 gene-mediated EMT in tumor cell dissemination has been confirmed [19,20].

EMT played an important role in the invasion and migration processes of tumor cells [21]. When EMT occurs in tumor cells, the expression of the epithelial marker E-cadherin is downregulated, while the expression of the mesenchymal marker vimentin is upregulated [22]. Abnormally expressed miRs can participate in the regulation of EMT in tumor cells [23-25]. This study revealed that the expression of the epithelial marker E-cadherin was increased whereas the expression of the mesenchymal marker vimentin was decreased with miR-421 overexpression. However, after upregulating the expression of MAT1, the inhibitory effect of miR-421 was counteracted. The above results indicated that miR-421 can inhibit the EMT progression of CRC cells by targeting the regulation of MAT1 expression.

Conclusions

In summary, this study demonstrated that the expression of miR-421 was downregulated in CRC. In addition, miR-421 could inhibit CRC cell proliferation, invasion, migration and EMT by regulating MAT1 to exert antitumor effect. Therefore, the miR-421/MAT1 axis is expected to be one of the targets of CRC targeted therapy.

Conflict of interests

The authors declare no conflict of interests.

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