miR-205 suppresses cell migration, invasion and EMT of colon cancer by targeting mouse double minute 4

YUJING FAN\(^1\) and KUANYU WANG\(^2\)

\(^1\)Department of Gastroenterology, Beijing Jishuitan Hospital, Beijing 100036; \(^2\)Department of The Second Surgery, First Affiliated Hospital, Heilongjiang University of Chinese Medicine, Harbin, Heilongjiang 150040, P.R. China

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Abstract. Colon cancer is one of the most frequent malignant tumors, and microRNA (miR)-205 is involved in the tumor progression. The present study aimed to explore the effects of miR-205 on human colon cancer and its targeting mechanism. The levels of miR-205 and mouse double minute 4 (MDM4) were determined via reverse transcription-quantitative (RT-q) PCR and western blot analysis. A luciferase activity assay was performed to analyze the association between miR-205 and MDM4. Cell viability, migration and invasion were determined via Cell Counting Kit-8, wound healing and Transwell assays, respectively. The levels of epithelial-mesenchymal transition (EMT)-associated factors were determined by RT-qPCR and western blot analysis. It was identified that MDM4 was overexpressed in colon cancer tissues and cells, and that there was a negative correlation between miR-205 and MDM4 expression in colon cancer. Similarly, miR-205 inhibited MDM4 expression by binding to its 3'untranslated region. In addition, miR-205 directly targeted MDM4, accompanied by suppressed proliferation, migration and invasion of HCT116 cells. EMT processes were suppressed in miR-205-overexpressed cells; upregulation of E-cadherin, and downregulation of N-cadherin, vimentin, matrix metalloproteinase (MMP)2 and MMP9 were observed. Collectively, miR-205 conspicuously depressed the viability, migration, invasion and EMT process of human colon cancer cells via targeting MDM4. miR-205 could be potentially used in the treatment of human colon cancer.

Introduction

Colon cancer is one of the most frequent malignant tumors and the second leading cause of tumor-related mortality in the United States (1,2). The incidence of colon cancer is increasing annually, and the disease seriously threatens the physical and mental health of patients (3). In recent years, a large number of clinical studies have reported that metastatic recurrence is the primary cause of the prognosis of colon cancer (4-6). In the early stages, the 5-year survival rate of colon cancer is >90%; however, when colon cancer is diagnosed with local lymph node metastasis, the 5-year survival rate decreases to 65% (7). Hence, it is of great clinical significance to explore the inhibition of tumor metastasis.

Tumor metastasis is a sequential process of interaction among tumor cells, host cells and the tissue microenvironment (8). Epithelial-mesenchymal transition (EMT), which plays a key role in tumor metastasis (9,10), is characterized by the deficiency of epithelial phenotypes, loss of cell polarity, reduced contact with surrounding cells and matrix, and enhanced cell migration and invasion in the presence of interstitial phenotype (11). Furthermore, EMT participates in nearly all physiological and pathological processes, such as the differentiation of various tissues and organs, repair of tissue damage, tissue fibrosis, tumor occurrence and metastasis (12-14).

MicroRNAs (miRNAs/miRs), a class of small, 18- to 28-nucleotide-long, noncoding RNA molecules, are involved in the progression of tumors (15,16). To date, the human genome contains ~1,000 miRNAs, and each miRNA is expected to interact with dozens or even hundreds of genes via matching 5' sequences and 3' untranslated regions (3'UTRs) of target mRNAs (17-19). miR-205 is a highly conserved miRNA, and its homologous chromosomes can be found in different species (20,21). Homo sapiens (hsa)-miR-205 is located in the second intron of the LOC642587 site of the first chromosome (22). Whether miR-205 is an oncogene or a tumor suppressor still remains controversial (23), though studies have reported that miR-205 participates in the EMT process of tumor cells (24-26).

Mouse double minute 4 (MDM4), which was isolated and identified in 1996, is also known as MDMX, and is an important upstream regulator of p53 (27). Following MDM4 phosphorylation, the p53 binding domain of MDM4 can...
combine with the transcriptional activation domain of the wild
and mutant p53 proteins to form a MDM4/p53 complex to
inhibit the transcriptional activity of p53 (28,29). Increasing
evidence has shown that MDM4 is abnormally expressed in
a number of tumor tissues such as breast cancer, retinoblas-
toma, lung cancer, colon cancer and gastric cancer (30-33).
Accordingly, the inhibition of abnormal expression of MDM4
has attracted increasing attention of researchers in the field of
anti-tumor mechanisms.

The current study determined the expression of miR-205
and MDM4 in colon cancer tissues, adjacent normal tissues,
and colon and colorectal cancer cell lines. In addition, the
correlation between the expression of miR-205 and MDM4
in colon cancer tissues, adjacent normal tissues,
and colon cancer are presented in Table I. Patients were divided
between mir-205 expression and clinical characteristics of
tissues would be used for clinical research. The relationship
between miR-205 expression and clinical characteristics of
colon cancer are presented in Table I. Patients were divided
into high- and low-expression groups for analysis of associa-
tions with clinical characteristics based on the mean miR-205
expression value in tumor tissues. The study was reviewed
and approved by the Ethics Committee of Beijing Jishuitan
Hospital (permit no. J20110104015).

Materials and methods

Tissue source. The colon cancer tissues and adjacent normal tissues (distance from tumor margin, 2 cm) were obtained from
47 patients with colon cancer who were diagnosed at Beijing
Jishuitian Hospital between March 2011 and March 2016. All
patients signed informed consent and agreed that their
tissues would be used for clinical research. The relationship
between miR-205 expression and clinical characteristics of
colon cancer are presented in Table I. Patients were divided
into high- and low-expression groups for analysis of associa-
tions with clinical characteristics based on the mean miR-205
expression value in tumor tissues. The study was reviewed
and approved by the Ethics Committee of Beijing Jishuitan
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Cell culture and transfection. The human colorectal cancer cell
line (HT29), colon cancer cell lines (HCT116, HCT8, LS174T
and SW480) and 293T cells were purchased from Shanghai
Gaining Biotechnology Co., Ltd. HT29, HCT116, HCT8,
LS174T and SW480 cells were maintained in RPMI-1640
Medium (Thermo Fisher Scientific, Inc.) for 24 h at 37˚C. The transfection efficiency was assessed
by western blotting analysis. Subsequent experiments were
conducted at 24 h post-transfection.

Reverse transcription-quantitative (RT-q)PCR. Total RNA
was extracted from tissues and cells (1.3x10^6 cells/well) using
RNAiso Plus (Thermo Fisher Scientific, Inc.), according
to the manufacturer’s protocols. A total of 1 µg of RNA
was used to synthesize cDNA using a ReverTra Aid™
cDNA Synthesis kit (Takara Biotechnology Co., Ltd.), according
to the manufacturer’s protocol. Subsequently, qPCR
was performed using the SYBR Premix Ex Taq™ II kit (Thermo
Fisher Scientific, Inc.), according to the manufacturer’s
protocol. The following thermocycling conditions were used
for qPCR: Initial denaturation at 95˚C for 10 min; followed by
40 cycles of 94˚C for 2 min, 60˚C for 50 sec; a final extension
at 60˚C for 1 min; and storage at 4˚C. The primer sequences
are listed in Table II. U6 and GAPDH were used as internal
references. The formula 2^(-ΔΔCq) was used to calculate relative
gene expression (34).

Western blot analysis. Total protein in tissues and cells
were extracted using RIPA lysis buffer (Beyotime Institute
of Biotechnology). Protein quantification was performed
using a BCA protein assay kit (Bio-Rad Laboratories, Inc.).
Subsequently, proteins (40 µg) were separated via 10%
SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked in 5% non-fat milk at room temperature
for 2 h. Subsequently, the membrane was incubated overnight
at 4˚C with the following primary antibodies: Anti- MDM4
(1:1,200; cat. no. ab154324; Abcam), anti-E-cadherin (1,800; cat.
no. MAB1838; R&D Systems, Inc.), anti-N-cadherin (1:2,200;
cat. no. ab18203; Abcam), anti-vimentin (1:700; cat. no. AF2105;
R&D Systems, Inc.), anti-matix metalloproteinase (MMP)2
(1:1,000; cat. no. MA1-772; Invitrogen; Thermo Fisher Scientific,
Inc.), anti-MMP9 (1:800; cat. no. AF911; R&D Systems, Inc.)
and anti-GAPDH (1:800; cat. no. AF5718; R&D Systems, Inc.).
Following primary incubation, the membranes were incu-
bated with corresponding horseradish peroxidase-conjugated
secondary antibodies for 90 min at room temperature [rabbit
anti-mouse IgG (1:5,000; cat. no. 58802; Cell Signaling
Technology, Inc.); goat anti-mouse IgG (1:8,000; cat. no. 31430;
Invitrogen; Thermo Fisher Scientific, Inc.); mouse anti-rabbit
IgG (1:10,000; cat. no. 31464; Invitrogen; Thermo Fisher
Scientific, Inc.)]. Finally, the protein was exposed using an ECL
chemiluminescence kit [Yeasen Biotechnology (Shanghai)
Co., Ltd.]. Protein expression was quantified using ImageJ software
(version 5.0; Bio-Rad Laboratories, Inc.) with GAPDH as the
loading control.

Bioinformatics prediction. According to the computational
analysis performed using TargetScan software (version 7.2;
www.targetscan.org/vert_72) (35), the 3’UTR of MDM4
contained a predicted binding site for miR-205.
Luciferase activity analysis. At 24 h post-transfection, the 293T cells were lysed using RIPA lysis buffer. The cell suspension was centrifuged at 500 x g for 5 min at room temperature, the supernatant was placed in 96-well plates and luciferase detection reagent was added (Promega Corporation). Luciferase activity was detected with a Nano-Glo® dual-luciferase Reporter Assay System (Promega Corporation). Firefly luciferase activity was normalized to Renilla luciferase activity.

Cell Counting Kit-8 (CCK-8) analysis. CCK-8 (Beyotime Institute of Biotechnology) was carried out to determine the cell viability of HCT116 cells following the manufacturer’s protocol. Cells were transfected for 24 h, re-seeded into 96-well plates (6x10^3 cells/well) and incubated for 0, 24, 48 and 72 h at 37°C. Subsequently, CCK-8 reagent (10 µl) was added to the cells and incubated for 4 h at 37°C. The absorbance was then analyzed at 450 nm using a microplate reader (FilterMax F3/F5; Molecular Devices, LLC).

Transwell analysis. BD Matrigel (Qcbio Science & Technologies Co., Ltd.) was added into the upper chambers of Transwell inserts (96-well inserts; pore size, 0.4 µm; diameter, 4.26 mm) at room temperature for 25 min, and RPMI-1640 medium was added into the upper chambers. Subsequently,

Table I. Relationship between miR-205 expression and clinical characteristics of colon cancer.

| Clinicopathological variable | n   | Low | High | χ² | P-value |
|------------------------------|-----|-----|------|----|---------|
| All cases                    | 47  | 24  | 23   |    |         |
| Age ≤65                      | 21  | 10  | 11   | 0.180 | 0.671   |
| Age >65                      | 26  | 14  | 12   |    |         |
| Sex Female                   | 20  | 11  | 9    | 0.216 | 0.642   |
| Sex Male                     | 27  | 13  | 14   |    |         |
| Pathological grade I-II      | 35  | 21  | 14   | 4.381 | 0.036   |
| Pathological grade III       | 12  | 3   | 9    | 7.817 | 0.005   |
| Stage I-II                   | 28  | 19  | 9    |    |         |
| Stage III-IV                 | 19  | 5   | 14   |    |         |
| Lymph node metastasis Positive | 19  | 6   | 13   | 4.846 | 0.028   |
| Lymph node metastasis Negative | 28  | 18  | 10   |    |         |

miR, microRNA.

Table II. Primer sequences.

| Primer    | Sequence (5’→3’) | Primer    | Sequence (5’→3’) |
|-----------|------------------|-----------|------------------|
| MDM4      | GAAAGACCCAAGCCCTCTCT | mir-205   | CTCGACAGTGCCAGAGGCTGTTG |
| E-cadherin| AGCATTTGCACTACACTAC | N-cadherin| CTTGCCAGAAACTCCAGG |
| MMP2      | CAGGCCCTGAAATTTCCATT | MMP9      | GAGACTCTACACCAGAGCACG |
| Vimentin  | AATAAGATCTTGCAGCCGGA | U6        | ACACCAAGCAGTCCAGAGAG |
| GAPDH     | CCATCTTCCAGGAGCGAGAT |           |                  |

MDM4, mouse double minute 4; miR, microRNA; MMP, matrix metalloproteinase.

Luciferase activity analysis. At 24 h post-transfection, the 293T cells were lysed using RIPA lysis buffer. The cell suspension was centrifuged at 500 x g for 5 min at room temperature, the supernatant was placed in 96-well plates and luciferase detection reagent was added (Promega Corporation). Luciferase activity was detected with a Nano-Glo® Dual-Luciferase Reporter Assay System (Promega Corporation). Firefly luciferase activity was normalized to Renilla luciferase activity.
the Transwell inserts were placed in the culture plate. RPMI 1640 medium with 15% FBS was placed in the lower chamber to attract cells. HCT116 cell suspensions (4x10^6 cells/well) were cultured in RPMI-1640 medium in the upper chambers at 37°C for 24 h. The cells were fixed with 4% paraformaldehyde for 15 min at room temperature, stained with 0.05% crystal violet (Beijing Solarbio Science & Technology Co., Ltd.) for 20 min at room temperature, and washed with PBS three times. Finally, the cells were observed and photographed under a fluorescence microscope (magnification, x200; MF53; Guangzhou Micro-shot Technology Co., Ltd.).

Wound healing assay. Cells were transfected with 50 nM PBS, miR-205 mimic, mimic nc, sinc or siMdM4 for 0 and 12 h. During the wound healing assay, cells were serum-starved (0.2% FBS). Following transfection, HCT116 cells were seeded in 6-well plates (2x10^4 cell/well) and cultured in an incubator for 24 h at 37°C. Following culturing, a 6-µm width scratch was created in the cells using a pipette tip, and the cells were washed by the medium 3 times. Cells were observed and photographed under an inverted microscope (magnification, x200).

Statistical analysis. All experiments were conducted in triplicate. The data were shown as the mean ± standard deviation using SPSS software (version 20; IBM, Corp.). Associations between miR-205 expression and clinicopathological characteristics were analyzed using χ² tests. One-way analysis of variance and Bonferroni’s post hoc test were used to evaluate the differences among groups. The correlation between the miR-205 and MDM4 mRNA expression was analyzed by Pearson correlation analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Negative correlation between miR-205 and MDM4 expression in colon cancer tissue. In order to determine the association between miR-205 and MDM4 in colon cancer tissues and cells, RT-qPCR and western blot analyses were performed. The results demonstrated that the expression level of miR-205 in normal tissue was higher compared with tumor tissue. In addition, miR-205 expression was determined in different colon tumor cells, and it was found that miR-205 had the lowest expression in HCT116 cells. However, mRNA and protein levels of MDM4 in normal tissue were lower compared with tumor tissue, and mRNA expression of MDM4 was the highest in HCT116 cells (P<0.01; Fig. 1A-E). Thus, HCT116 cells were selected for subsequent experiments. In addition, the data revealed a negative correlation between miR-205 and MDM4 mRNA expression in colon cancer tissue (r=-0.4614, P<0.0001; Fig. 1F). As Table I demonstrates, miR-205 expression was closely associated with pathological grade, stage and lymph node metastasis (P<0.05), and age and sex had no significant effect.

miR-205 silences MDM4 by binding with its 3’UTR. TargetScan prediction software was used to examine whether MDM4 is a potential target gene for miR-205, and the results indicated that there was a single eight-nucleotide complementary sequence at position 570-577 of the MDM4 3’UTR (Fig. 2A). Subsequently, luciferase activity assays demonstrated that when 293T cells were co-transfected with miR-205 and WT MDM4 3’UTR, the luciferase activity was significantly reduced. In addition, MT MDM4 3’UTR had no effect on luciferase activity (P<0.05; Fig. 2B). In addition, RT-qPCR analysis demonstrated that miR-205 was significantly upregulated in 293T cells transfected with miR-205 mimic compared with mimic NC (P<0.001; Fig. 2C). Thus, it was demonstrated that miR-205 inhibited MDM4 expression by interacting with its 3’UTR.

miR-205 suppresses cell viability. RT-qPCR, western blot and CCK-8 analyses were performed to evaluate the expression levels of miR-205 and MDM4, and the viability of HCT116 cells exposed to PBS, miR-205 mimic, mimic NC, siNC and siMDM4. miR-205 was markedly upregulated in the miR-205 mimic group compared with the mimic NC group (P<0.001; Fig. 3A). As RT-qPCR and western blot assay results revealed, siMDM4 significantly downregulated the mRNA and protein level of MDM4 compared with the siNC group. In comparison with the mimic NC group, miR-205 mimic transfection also significantly reduced the expression of MDM4 (P<0.01; Fig. 3B-D). In addition, the CCK-8 data demonstrated that compared with the mimic NC group, the OD value significantly decreased in the miR-205 mimic group at 48 and 72 h, and MDM4 silencing in cells reduced the value of OD compared with the siNC group at 24, 48 and 72 h (P<0.05; Fig. 3E).

miR-205 suppresses cell invasion and migration. The invasive and migratory abilities of cells were explored via Transwell and wound healing assays. The data from Transwell assays demonstrated that the invasion of cells was inhibited by siMDM4 compared with siNC group; similarly, miR-205 overexpression significantly decreased cell invasion compared with the mimic NC group (P<0.05; Fig. 4A). In addition, the wound healing assay results revealed that the wound width was increased in the miR-205 mimic and siMDM4 groups compared with the mimic NC and siNC groups, respectively. The wound healing results demonstrated that miR-205 suppressed cell migration (P<0.05; Fig. 4B).

miR-205 mediates the expression of EMT-associated factors. In order to further study the molecular mechanism of miR-205 inhibiting cell invasion and migration, the expression levels of E-cadherin, N-cadherin, vimentin, MMP-2 and MMP-9 were measured by RT-qPCR and western blotting. As RT-qPCR and western blot assays revealed, siMDM4 downregulated the mRNA and protein levels of MDM4, N-cadherin, vimentin, MMP2 and MMP9 compared with the siNC group. In comparison with the mimic NC group, miR-205 overexpression also significantly reduced the expression of N-cadherin, vimentin, MMP2 and MMP9. However, the expression levels of E-cadherin were upregulated in the siMDM4 and miR-205 mimic groups compared with the siNC and mimic NC groups (P<0.05; Fig. 5).

Silencing MDM4 partially reverses the regulatory effects of miR-205 inhibition on invasion, migration and EMT. RT-qPCR analysis demonstrated that miR-205 was significantly down-regulated in cells transfected with miR-205 inhibitor compared with the inhibitor control (P<0.001; Fig. 6A). In order to further verify the effects of miR-205 through MDM4, rescue experiments were performed. It was identified that miR-205 inhibitor...
significantly promoted cell invasion (P<0.01; Fig. 6B) and migration (P<0.01; Fig. 6C). In addition, silencing MDM4 could partially reversed the increase effects of miR-205 inhibition on invasion and migration (P<0.01). Furthermore, the expression of EMT-related proteins was also observed. miR-205 inhibition significantly inhibited E-cadherin expression, while increasing N-cadherin and vimentin levels. Silencing MDM4 could also partially reversed the regulatory effects of miR-205 inhibition on EMT (P<0.01; Fig. 7A and B).

**Discussion**

MDM4, which mediates p53-independent activities, is abnormally expressed in various cancer cells and contributes to the development of cancer (36-38). Gilkes *et al* (39) noted that MDM4 is overexpressed in human colon tumors; consistent with these findings, the present results also revealed overexpressed MDM4 in human colon cancer tissues and cells. In addition, it has been found that inhibition of the expression of MDM4 can impede the proliferation and metastasis of tumor cells (40). To some extent, the present study demonstrated that MDM4 silencing in human colon tumor HCT116 cells significantly suppressed proliferation, migration and invasion.

A number of miRNAs can regulate cancer cell progress by targeting MDM4. For example, Jiang *et al* (41) reported that overexpressed miR-33a can suppress renal cell cancer growth by inhibiting the expression of MDM4. miR-766 can increase human colon cancer cell apoptosis through MDM4 (42).
Previous studies suggested that miR-205 possibly has distinct functions in different cancers. It is reported that miR-205 is downregulated in colon, breast and prostate cancers (43-45), but upregulated in lung, bladder and ovarian cancers (46,47). The present study also demonstrated the downregulation of miR-205 in human colon tumor tissues and cells. miR-205 also
can interact with the 3'UTR of certain genes, and then mediate the translation of genes and regulate tumor processes (48-50). Zhuang et al (51) demonstrated that miR-205 suppresses human pancreatic cancer progression by targeting runt-related transcription factor 2. A previous study indicated that miR-205 downregulates Prospero homeobox 1 by binding to its 3'UTR, thus further suppressing the viability and metastasis of human colon cancer cells (44). Thus, the present study investigated the relationship between miR-205 and MDM4 in colon cancer, and the data demonstrated that the expression levels of miR-205 and MDM4 were negatively correlated. In addition, the prediction results indicated that there was a single 8-nucleotide complementary sequence between hsa-miR-205 and the position 570-577 of MDM4 3'UTR.

miR-205 plays a vital role in the growth, migration and invasion of tumors (52,53). Previous studies have confirmed that miR-205 has anti-proliferation and anti-invasion effects on gastric and cervical tumors (54,55). As expected, the
upregulation of miR-205 in HCT116 cells notably attenuated cell proliferation, migration and invasion by silencing the MDM4 gene. Furthermore, EMT-related proteins, including E-cadherin, N-cadherin, vimentin, MMP2 and MMP9, were detected. E-cadherin is an important adhesion molecule for maintaining epithelial cell characteristics. N-cadherin, which plays a key role in promoting cell movement, is considered as one of the characteristic molecular markers of mesenchymal
A recent study observed that the overexpression of miR-205 in anaplastic thyroid carcinoma predominantly blocks the process of EMT by targeting zinc finger E-box-binding homeobox 1 gene, which upregulates E-cadherin expression, and downregulates N-cadherin, vimentin, MMP2 and MMP9 expression levels (26). Similarly, the results of the present study demonstrated that miR-205 targeted the MDM4 gene to suppress EMT, followed by downregulation of N-cadherin, vimentin, MMP2 and MMP9, and upregulation of E-cadherin.

In conclusion, the present study characterized the miR-205-MDM4 mechanism in human colon cancer. It found that miR-205 and MDM4 expressions are negatively correlated in human colon cancer. In addition, miR-205 significantly suppressed proliferation, migration, invasion and EMT of human colon cancer cells by silencing MDM4 gene. Thus, miR-205 could be employed in the treatment of human colon cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YF made substantial contributions to the study conception and design. KW performed data acquisition, data analysis and interpretation. YF drafted the manuscript and critically revised it for important intellectual content. Both authors gave final approval to the published version of the study and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved. Both authors read and approved the final manuscript.

Ethics approval and consent to participate

The current study was reviewed and approved by the Ethics Committee of Beijing Jishuitan Hospital (approval no. J20110104015). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and the Declaration of Helsinki. All patients signed informed consent and agreed that their tissues would be used for clinical research.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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