

## Original Article

# MicroRNA-212-5p down-regulation suppresses colorectal cancer migration and invasion by up-regulating SMAD4

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**Abstract:** The expression and exact roles of miR-212-5p in CRC and the underlying molecular mechanism is still unclear. This study we aimed to investigate the expression and the role of miR-212-5p in colorectal cancer and further explore the underlying molecular mechanism. We first detected the expression level of miR-212-5p in colorectal cancer tissues and cells and we found that miR-212-5p was up-regulated in colorectal cancer. We performed TargetScan and Miranda databases to predict the putative targets of miR-212-5p, and the prediction was verified by dual-luciferase reporter assay. To investigate the role of miR-212-5p in colorectal cancer, a stable miR-212-5p-low-expression cell line was established by using miR-212-5p inhibitor. MTT, cell migration and invasion assay were used to investigate the proliferation, cell migration and invasion ability of colorectal cancer HCT116 cells. Moreover, gene mRNA and protein expression were detected by qRT-PCR and western blotting. We found that miR-212-5p directly targets Smad4, and Smad4 was significantly increased after miR-212-5p down-regulation. After down-regulating miR-212-5p, the proliferation of HCT116 cells was significantly decreased, and the migration and invasion ability of HCT116 cells were markedly declined. In addition, the findings suggested that compared with the control, miR212-5p low-expression significantly decreased the protein expression level of N-cadherin in HCT116 cells, while the E-cadherin expression level remarkably increased. In conclusion, microRNA-212-5p down-regulation suppresses colorectal cancer migration and invasion by up-regulating SMAD4.

**Keywords:** MiR-212-5p, Smad4, target, proliferation, migration, invasion

## Introduction

Colorectal cancer (CRC), which accounting for about 10% of the cancer mortalities every year, is one of the most common cancer in both men and women and a major cause of cancer-related deaths in the world [1-3]. About 1.4 million cases colorectal cancer are newly diagnosed and nearly 50% of the patients died from this disease every year [4]. Currently, the mainstay of anti-colorectal cancer treatment is surgery, chemotherapy and radiotherapy [5]. Although a lot of improvement has been made for surgical and medical treatments of colorectal cancer, there are still limitations of these treatments [6, 7]. Because of the recurrence and metastasis of tumor, the long-term survival and prognosis of patients with colorectal cancer is still very

poor [8, 9]. Therefore, it is significantly and urgently to investigate and identify new and effective therapy targets for the treatment of colorectal cancer.

MicroRNAs (miRNAs) are small (19-25 nucleotides in length) non coding RNAs, widely exists in eukaryotic cells, completely or partially base bind to the 3' untranslated regions (UTR) of their multiple target mRNAs, leading to the degradation or translation inhibition of their target mRNAs, and post-transcriptionally regulate gene expression [10, 11]. Studies have revealed that miRNAs involved in the development of various cancers, including the colorectal cancer [12-15], and a large amount of evidence indicated that the abnormal expression of miRNAs is associated with the occurrence and develop-

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**Table 1.** Primer sequence for PCR

Sequence (5'-3')
Smad4-Forward: 5'GACAGTGTCTGTGTAATCC3'
Smad4-Reverse: 5'TACTTGGCGGGTGTGGATG3'
MiR-212-5p-Forward: 5'CGCTAACAGTCTCCAGTC3'
MiR-212-5p-Reverse: 5'GTGCAGGGTCCGAGGT3'
U6-Forward: 5'GCTTCGGCAGCACATATACTAAAAT3'
U6-Reverse: 5'CGCTTACGAATTTGCGTGTCA3'
$\beta$ -actin-Forward: 5'GGAGTCTGTGGCATCCACG3'
$\beta$ -actin-Reverse: 5'CTAGAAGCATTGCGGTGGA3'

ment of colorectal cancer by regulating the expression of their target gene including oncogenes and tumor inhibitor genes [14-18].

To the best of our knowledge, the expression and exact roles of miR-212-5p in CRC and the underlying molecular mechanism is still unclear. Therefore, in the present study, we will investigate the expression and the role of miR-212-5p in colorectal cancer and further explore the underlying molecular mechanism.

## Materials and methods

### Tissue samples

The study was approved by the Human Ethics Committees Review Board at Nanjing Drum Tower Hospital, The Affiliated Hospital of Nanjing University Medical School, Nanjing, China and informed consent was obtained from all patients. 20 tumor tissues from patients diagnosed with colorectal cancer at Nanjing Drum Tower Hospital between 2014 and 2015 and 20 paired adjacent non-tumoural tissues were collected and used for miRNA-212-5p expression detection. Native tumor and paired non-tumoural colon tissues were obtained after surgical resection and immediately stored in -80°C.

### Cell culture

Human colorectal cancer cell line HCT116, human normal colon epithelial cell line FHC and human embryonic kidney cell line 293T were purchased from American type culture collection (ATCC, USA). HCT116 cells were grown in RPMI-1640 medium and 293T cells were grown in high glucose DMEM (Gibco, USA) medium supplemented with 10% fetal bovine serum (FBS) (Gibco, USA), 1% streptomycin-penicillin solution, and cells were incubated in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C. Cells were passaged every 2-3 days. FHC cells were grown in DMEM/F12 medium (Gibco, USA) containing 10% FBS and 1% streptomycin-penicillin solution at 37°C with 5% CO<sub>2</sub>.

ated atmosphere with 5% CO<sub>2</sub> at 37°C. Cells were passaged every 2-3 days. FHC cells were grown in DMEM/F12 medium (Gibco, USA) containing 10% FBS and 1% streptomycin-penicillin solution at 37°C with 5% CO<sub>2</sub>.

### Dual luciferase reporter assay

To determine whether miR-212-5p directly targets the 3'UTRs of Smad4, the vectors named SMAD4-3'UTR-WT and SMAD4-3'UTR-MUT with wild-type and mutated 3'UTR of SMAD4 mRNA were established as previously described [19]. 293T cells were seeded in a 24-well plate and then co-transfected with SMAD4-3'UTR-WT or SMAD4-3'UTR-MUT and miR-212-5p or its negative control (hsamiR-NC) vector using Lipofectamine 2000 transfection reagent in accordance with the manufacturer's instructions. 48 h after transfection, the Dual-Luciferase Reporter Assay Kit (Promega, USA) was performed to measure the luciferase activity according to the manufacturer's instructions.

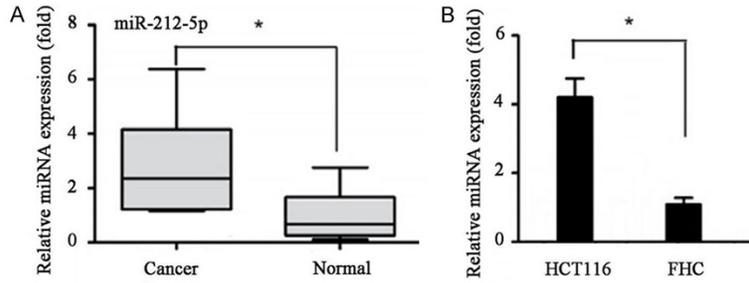
### Cell transfection

The day before transfection, human colorectal cancer HCT116 cells were seeded in 6-well plates at a density of 3×10<sup>5</sup> per well. When cells reached 50%-60% confluence, miR-212-5p inhibitor and its negative control were transfected into HCT116 cells with 50  $\mu$ l Lipofectamine 2000 transfection reagent (Invitrogen, USA) following the manufacturer's instructions. Cells without treatments act as the control. After incubating for 4 h, fresh culture medium was replaced. The transfected cells were used for following experiments analysis after incubating for another 24 h.

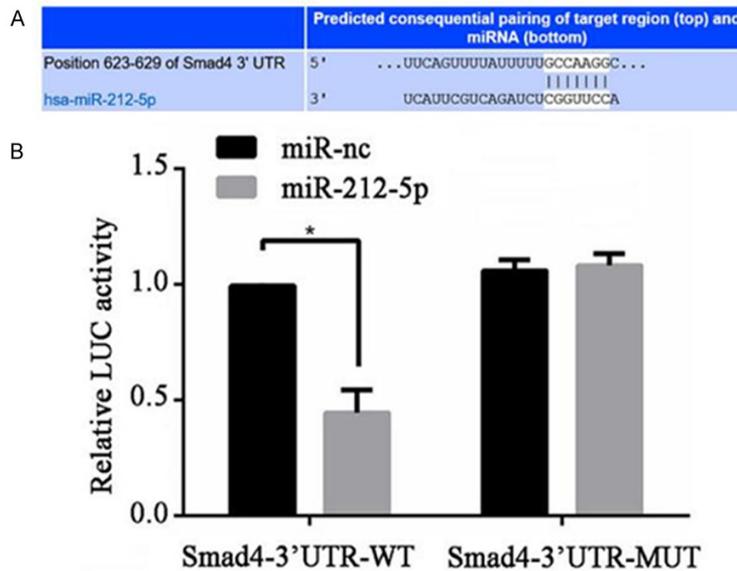
### Cell proliferation assay

Cell proliferation ability of HCT116 cells was determined by performing MTT assay. 24 h after transfection, Log-phase human colorectal cancer HCT116 cells were collected using 0.25% trypsin, then the cells were seeded in a 96-well plate with 1.5×10<sup>4</sup> cells per well. 20  $\mu$ L MTT (5 g/L) was added into each well and then incubating for 4 h. After the culture medium was discarded, 200  $\mu$ L DMSO was added into each well. The OD value at 490 nm was measured using a spectrophotometer. Experiments repeated in triplicate.

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**Figure 1.** MiR-212-5p is up-regulated in CRC. QRT-PCR was performed to determine the miR-212-5p expression. A: Relative expression of miR-212-5p in tissues of CRC patients; B: Relative expression of miR-212-5p in CRC cells. Normal: the adjacent non-tumoural tissues of the CRC patients. Cancer: tissues of CRC patients; FHC: human normal colon epithelial cell line; HCT116: human colorectal cancer cell line. \* $P < 0.05$ , experiments were performed in triplicate.



**Figure 2.** Smad4 is a direct target of miR-212-5p. A: Interaction between miR-212-5p and 3'UTR of Smad4 was predicted using TargetScan; B: Luciferase activity of a reporter containing a wild-type Smad4 3'UTR or a mutant Smad4 3'UTR are presented. "Smad4-3'UTR-MUT" indicates the Smad4 3'UTR with a mutation in the miR-212-5p binding site. UTR, untranslated region. \* $P < 0.05$  vs. control. All data are presented as the mean  $\pm$  SD of three independent experiments.

For cell invasion ability detection, Transwell assay was performed. 24 h after transfection, cells were trypsinized and transferred to the upper chamber with matrigel-coated membrane matrix in serum-free medium. At the end of the tests, the non-invading cells on the upper membrane were struck off, and cells on the lower surface were fixed with 4% paraformaldehyde and stained with hematine. Cells were counted under microscope. Each experiment was independently performed three times.

### Western blot

We performed Western blot analysis to detect the expression of proteins in CRC cell lines. Transfected HCT116 cells were lysed using RIPA buffer (CST, USA). Proteins were resolved by 10% SDS-PAGE and then transferred to a PVDF membrane. After blotted overnight with a primary antibody against Smad4, N-cadherin, E-cadherin (CST, USA; dilution ratio, 1:1000) or  $\beta$ -actin (CST, USA; dilution ratio, 1:2000), the blots were then incubated with a secondary antibody (CST, USA; dilution ratio, 1:5000) at room temperature for 2 h. Protein bands were visualized using ECL Substrates (Millipore, USA) and imaged and then analyzed.

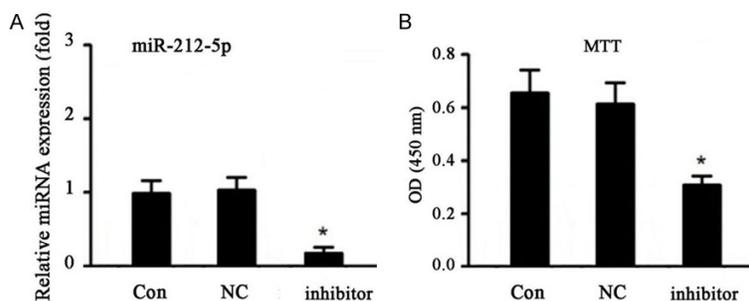
### Cell migration and invasion assay

For cell migration ability detection, cell scratch test was performed. 24 h after transfection, HCT116 cells were seeded in a 6-well plate ( $5 \times 10^5$  per well) and incubated in a normal cell culture condition with 5%  $\text{CO}_2$  at 37°C. When cells were in 80% confluence, wounds were formed by the 200- $\mu$ l pin. After washing with PBS, cells were cultured in media without serum. Pictures were captured using an inverted microscope (Olympus, Japan).

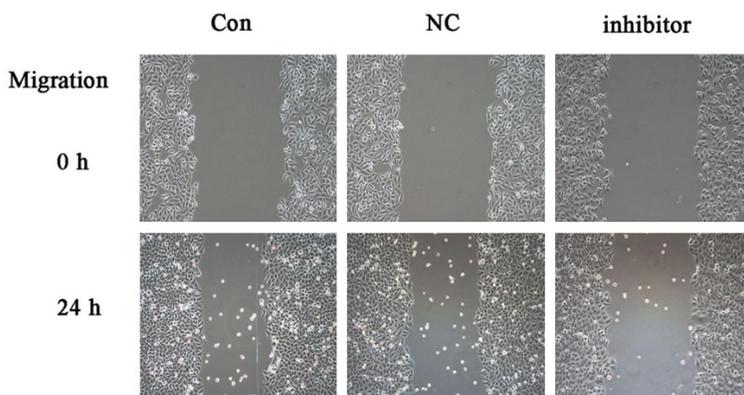
### QRT-PCR

Total RNAs were extracted from tissues and cells using Trizol reagent (Qiagen, USA) according to the manufacturer's protocol.  $\beta$ -actin or U6 was used as the internal control. The first-strand cDNA was generated by using the PrimeScript RT reagent Kit (TaKaRa, Japan) following the manufacturer's instructions. SYBR Premix Ex Taq (TaKaRa) was performed for real-time PCR analysis. The relative gene expression was calculated by using the Comparative

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**Figure 3.** MiR-212-5p down-regulation inhibits the proliferation in HCT116 cells. A: Relative expression of miR-212-5p in HCT116 cells transfected with miR-212-5p inhibitor or NC, respectively; B: Cell proliferation was analyzed by MTT assay in the HCT116 cells. Con, control; NC, negative control. \* $P < 0.05$  vs. Con. All results represent the means  $\pm$  SD of three independent experiments.



**Figure 4.** MiR-212-5p down-regulation inhibits the migration of HCT116 cells. The migration ability of the HCT116 cells was detected using cell scratch test at 24 h after the transfection of miR-212-5p inhibitor or NC, respectively. Con, control; NC, negative control. Experiments were performed in triplicate.

Ct method. Each experiment independently repeated for three times. The PCR primers for miR-212-5p and Smad4 were obtained from GenScript company (China) and shown in **Table 1**.

### Statistical analysis

Data are presented as the mean  $\pm$  SD. We performed SPSS 17.0 statistical software (SPSS, Chicago, IL, United States) for all statistical analyses. T-test was used to evaluate the difference between groups. A value of  $P < 0.05$  was considered statistically significant.

### Results

#### MiR-212-5p is up-regulated in human colorectal cancer

To investigate the role of miR-212-5p in the development of colorectal cancer, we first

detected miR-212-5p expression by qRT-PCR in the tumor tissues and adjacent non-tumoural tissues of the CRC patients. At the same time, the expression level of miR-212-5p in the human normal colon epithelial cell line FHC and the human colorectal cancer cell line HCT116 was measured. The findings suggested that compared with the control, miR-212-5p significantly increased in human colorectal cancer tissues and the HCT-116 cells, indicating that miR-212-5p was up-regulated in CRC (**Figure 1**).

#### MiR-212-5p directly targets Smad4

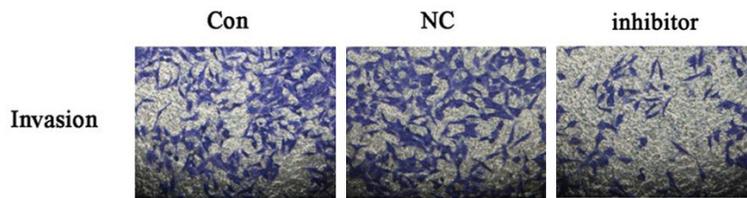
In order to explore the mechanism of miR-212-5p function in CRC, TargetScan and Miranda databases was used to predict the target gene of miRNA-212-5p, and Dual luciferase reporter Assay was performed to reveal our prediction. The miR-212-5p-Smad4-WT or miR-212-5p-Smad4-MUT reporter were co-transfected into 293T cells with miR-212-5p, and the results indicated that the luciferase

activity was significantly declined in miR-212-5p and miR-212-5p-Smad4-WT co-transfected 293T cells, but co-transfection of miR-212-5p and miR-212-5p-Smad4-MUT did not (**Figure 2**). The data revealed that miR-212-5p directly targets Smad4. In addition, the mRNA and protein expression of Smad4 were detected using qRT-PCR and western blotting. As shown in **Figure 6**, compared with the control, miR-212-5p low-expression markedly increased the expression of Smad4 at both mRNA and protein level. This once again proved Smad4 is a target gene of miR-212-5p.

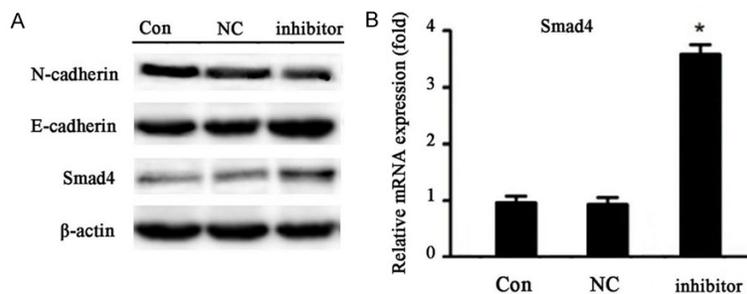
#### MiR-212-5p low-expression inhibits the proliferation of HCT116 cells

To explore the effect of miR-212-5p on CRC cell proliferation, miR-212-5p was down-regulated using miR-212-5p inhibitor in HCT116 cells. We performed QRT-PCR to determine the expres-

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**Figure 5.** MiR-212-5p down-regulation inhibits the invasion of HCT116 cells. The invasion ability of the HCT116 cells was detected using transwell assay at 24 h after the transfection of miR-212-5p inhibitor or NC, respectively. Con, control; NC, negative control. Experiments were performed in triplicate.



**Figure 6.** Expression level of Smad4, N-cadherin and E-cadherin in HCT116 cells. HCT116 cells were transfected with miR-212-5p inhibitor or NC, respectively. 24 h after the transfection, mRNA and protein expression of Smad4 were measured by QRT-PCR and western blotting. Protein expression level of N-cadherin and E-cadherin were measured by western blot analysis. A: Protein expression levels of Smad4, N-cadherin and E-cadherin in HCT116 cells; B: Relative mRNA expression levels of Smad4 in HCT116 cells. Con, control; NC, negative control. \* $P < 0.05$  vs. Con. All results represent the means  $\pm$  SD of three independent experiments.

sion level of miR-212-5p and the findings suggested that miR-212-5p was effectively down-regulated when HCT116 cells were transfected with miR-212-5p inhibitor (**Figure 3A**).

Subsequently, the proliferation ability of HCT116 cells was measured by CCK8 assay. Our results suggested that down-regulation of miR-212-5p significantly decreased the proliferation ability of HCT116 cells (**Figure 3B**). The data indicated that miR-212-5p down-regulation suppressed the proliferation of human colorectal cancer cells.

### *MiR-212-5p low-expression inhibits the migration and invasion of HCT116 cells*

The impact of miR-212-5p on migration and invasion of HCT116 cells were evaluated in vitro, and cell scratch test and the transwell assay were performed. The results of the cell scratch test suggested that compared with the control, miR-212-5p low-expression significant-

ly declined the migration ability of HCT116 cells (**Figure 4**). We also found that HCT116 cells transfected with miR-212-5p inhibitor invaded slower than those transfected with miR-NC. (**Figure 5**). The data indicated that miR-212-5p low-expression could suppress CRC cell migration and invasion.

### *MiR-212-5p low-expression affects the expression of N-cadherin and E-cadherin in HCT116 cells*

Protein expression of the mesenchymal marker (N-cadherin) and the epithelial marker (E-cadherin) were also determined by western blotting in our study. As shown in **Figure 6**, compared with the control, the protein expression level of N-cadherin was significantly decreased when miR212-5p was low-expressed in HCT116 cells, and the E-cadherin expression level remarkably increased.

## Discussion

In the present study, our results showed that miR-212-5p low-expression suppressed the proliferation, migration and invasion of colorectal cancer cells. MiR-212-5p directly targets Smad4 and miR-212-5p low-expression promotes its expression. MiR-212-5p low-expression thus indirectly inhibits the expression of N-cadherin and the E-cadherin expression level remarkably increased. First, we verified that miR-212-5p highly expressed in human colorectal cancer tissues and cells compared with the adjacent non-tumoural tissues and the human normal colon epithelial cell line FHC. We next revealed that miR-212-5p low-expression inhibited the proliferation, migration and invasion of colorectal cancer cells in vitro. Furthermore, we found a novel functional link between miR-212-5p and Smad4 in human colorectal cancer progress. These results indicated that miR-212-5p down-regulation plays essential roles in tumor suppression, thus, miR-212-5p may

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serve as a therapeutic target in the treatment of colorectal cancer.

A large amount of data indicate that miRNAs play essential roles in the diagnosis, therapy and prognosis of cancer, and they are associated with tumorigenesis and tumor metastasis [20-22]. Thus, miRNAs may be potential therapeutic targets for the treatment of cancer. A variety of studies have indicated that many miRNAs play important roles in the regulation of colorectal cancer cell proliferation, invasion and migration [19, 23-26]. However, to the best of our knowledge, the expression and the exact roles of miR-212-5p in human colorectal cancer have not been investigated. In our present study, the data strongly revealed that miR-212-5p expression was significantly up-regulated in both the tissues of CRC patients and human colorectal cancer HCT116 cells, and that miR-212-5p low-expression inhibited the proliferation, migration and invasion of HCT116 cells.

As a kind of tumor suppression genes, the genetic mutation, missing or negative expression of Smad4 can result in the deactivation, and play an important role in the development of tumor [27]. Studies have found that Smad4 play critical roles in inhibiting the metastasis of colorectal cancer cell [28]. In the process of Epithelial-mesenchymal transition (EMT), epithelial cells lose their polarity and cell-cell adhesion, and mesenchymal cells acquire migratory and invasive properties [29, 30]. Thus, EMT is essential for the initiation of cancer metastasis. In the present study, we found that miR-212-5p low-expression decreased the protein expression of mesenchymal marker N-cadherin but the protein expression of epithelial marker E-cadherin was increased, indicating that the inhibition of migration and invasion caused by miR-212-5p down-regulation was associated with its inhibitory effect on EMT. Multiple mechanisms are involved in regulating the progress of EMT and metastasis, including TGF- $\beta$ 1/SMAD pathways. Our results suggested that Smad4 is a target gene of miR-212-5p, and it can be proved by miR-212-5p down-regulation.

In conclusion, we proved for the first time that miR-212-5p was up-regulated in CRC, and its down-regulation can inhibit the proliferation, migration and invasion of colorectal cancer cells via directly targeting Smad4. MiR-212-5p

may play as a therapeutic target for CRC treatment.

### Acknowledgements

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### Disclosure of conflict of interest

None.

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