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MiR-431 inhibits colorectal cancer cell invasion via repressing CUL4B

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Abstract. – OBJECTIVE: To detect the expression of microRNA-431 (miR-431) in epithelial-mesenchymal transition (EMT) in colorectal carcinoma and investigate its effects on the migration of colorectal cancer cells.

PATIENTS AND METHODS: MiR-431 expressions in colorectal cancer tissues and adjacent tissues were examined via Real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). The expression levels of cullin-4B (CUL4B) and EMT-related protein were evaluated by Western blotting assay. The invasive ability of the cells was examined via transwell method.

RESULTS: The expression level of miR-431 in colorectal cancer tissues was lower than that in adjacent tissues ($p<0.05$). After colorectal cancer cell line was transfected with miR-431 mimics, the expression level of e-cadherin was increased in the cells, and the expression levels of n-cadherin, vimentin, fibronectin (FN), and snail were reduced ($p<0.05$). The migration ability of colorectal cancer cells with miR-431 overexpression was significantly decreased. Moreover, the reverse effect was observed in cells transfected with miR-431 inhibitors.

CONCLUSIONS: The low expression of miR-431 in colorectal cancer patients is involved in the development of colorectal cancer by promoting the migration of colorectal cancer cells.

Key Words
miR-431, Colorectal cancer, EMT, Migration.

Introduction

Colorectal cancer (CRC) is one of the most common malignancies in the digestive tract worldwide. According to the WHO International Cancer Research Institute, the incidence and mortality of CRC have been increasing in recent years[1]. In China, due to the westernization of dietary structure, lifestyle changes, mental stress, and many other factors, CRC mortality ranks the fifth in malignant tumors[2,3]. Therefore, the pathogenesis, prevention, and treatment of CRC is of important clinical significance. Epithelial-mesenchymal transition (EMT) refers to the phenomenon in which epithelial cells transform to the interstitial cells in specific conditions. A study has found that EMT phenomenon is closely related to tumor invasion and metastasis, and plays an important role in the invasion and distant metastasis of various cancers[4].

In hepatocellular carcinoma, cullin 4B (CUL4B) is implicated in the cell proliferation and invasiveness by regulating Wnt/β-catenin signaling pathway *via* inhibiting Wnt antagonists[5]. CUL4B regulates CRC cell proliferation and apoptosis *via* inhibiting the Wnt/β-catenin signaling pathway[6]. Both CUL4B mRNA and protein levels in cancer samples are up-regulated, which may be markers for prognosis of patients with colon cancer[7].

MicroRNA-431 (miR-431) is able to inhibit the EMT of gastric cancer by targeting FoxM1[8]. Highly expressed miRNA-9 is involved in the EMT process of breast cancer[9]. Low-expression miR-431 is related to the characteristics of hepatocellular carcinoma, including lymph node metastasis and clinical stage[10]. However, so far, there are few studies on EMT-related miRNA-431 expression in the CRC samples and the potential role of CRC cells. We compared the expression of miR-431 in the CRC samples and observed its effect on the migration of CRC cells.

Patients and Methods

Patients

All tissue samples were obtained from surgical resection of rectal cancer in patients pathologically diagnosed with CRC in our hospital. Patients were aged 23 to 69 years old. Before surgery, they received no chemotherapy or radiotherapy. Cancer tissue specimens were controlled *in vitro* for 30 min, and adjacent tissues (approximately 4 cm from cancer tissue) were taken. There were

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no statistically significant differences in gender and age (p>0.05). This study was approved by the Ethics Committee of Xiantao First People's Hospital. Signed written informed consent was obtained from all participants before the study.

Experimental Methods

Specimens and RNA detection

Reverse transcription reaction and detection of CRC tissue and adjacent tissue obtained from surgical removal were immediately performed after removal of liquid nitrogen, and these tissues were stored in a refrigerator at -80°C. About 100 mg tissues were added into 1 mL TRIzol (Gibco, Rockville, MD, USA) and homogenized using a homogenizer. Then, the total RNA was extracted, the resulting RNA was dissolved in 20 μL of diethyl pyrocarbonate (DEPC) solution, and cDNA was extracted by reverse transcription using the reverse transcription kit (Thermo Fisher, Waltham, MA, USA). Polymerase chain reaction (PCR) conditions: denaturation at 95°C for 20 s, followed by 40 cycles at 60°C for 20 s and 70°C for 1 s. MiRNA-431 probe primer: 5'-CAGGCCGTCATGCAAA-3', U6 internal reference: 5'-CTCGCTTCGGCAGCACA-3' and 5'-AACGCTTCACGAATTTGCGT-3'. PCR was conducted using ABI's 7500 model real-time PCR instrument (Waltham, MA, USA).

Cell transfection

Human colorectal cancer cell lines HCT116 and SW480 (Shanghai Baili Biotechnology Co., Ltd., Shanghai, China) were sub-cultured with high glucose Dulbecco's modified Eagle medium (DMEM) (HyClone, South Logan, UT, USA) + 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA). When the confluence rate of cells was about 60-70%, cells were treated with miRNA-431 mimics and inhibitors, respectively, with liposomes 2000 (Invitrogen, Carlsbad, CA, USA). The interference efficiencies of the high-expression and low-expression miRNA-431 were detected at 48 h later. MiRNA-431 mimics and inhibitors were designed and synthesized by Guangzhou Rui Bo Biotechnology Co., Ltd. (Guangzhou, China).

Cell migration experiments

The cell suspension was prepared using cells in the logarithmic phase of growth. The cell density was adjusted to 5×10^5 cells/mL, and the cells were added to the upper layer of the transwell chamber (Corning Corporation, Corning, NY, USA) at 0.1 mL/well. 1 mL 10% serum culture medium (complete DMED) was added. After incubation for 24 h, the transwell chamber was removed and the supernatant was eliminated to allow the chamber to dry naturally at room temperature. After immobilization with ethanol, 0.1% crystal violet was used for staining for 30 min and the cells migrating to the lower chamber were observed under an inverted microscope (Zeiss, CFM-500, Oberkochen, Germany). Five visual fields were randomly selected to count the cells in the lower chamber. This experiment was repeated 3 times, and three wells were chosen each time.

Detection of protein levels via Western blotting

Cells were collected after transfection, and 1 × sodium dodecyl sulphate (SDS) cell lysate was added. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed for 120 min, followed by transfer to film at a constant voltage for 100 min, sealing at 37°C for 80 min and addition of rabbit anti-human epithelial cadherin (E-cadherin), N-cadherin, Vimentin, Fibronectin (FN), and Snail. After incubation at 4°C overnight, horseradish peroxidase (HRP)-labeled mouse anti-rabbit secondary antibody (Nanjing Shengxing Biology, Nanjing, China) (diluted at 1:2000) was incubated at 37°C for 30 min, and enhanced chemiluminescence (ECL) was detected. The rabbit anti-human β-actin (diluted at 1:3000) provided by Sigma-Aldrich (St. Louis, MO, USA) was selected as the reference protein. The content of the protein was scanned by Image-Pro-plus image analysis software (Version X, Media Cybernetics, Silver Springs, MD, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 software (Chicago, IL, USA) was used for statistical analysis. The observational data were measured by normality test and expressed as mean ± standard deviation ($\bar{x}\pm s$). p<0.05 suggested that the difference was statistically significant.

Results

Expression of miRNA-431 mRNA in the Patients With CRC and its Correction With Tumor Metastasis and Staging

The expression of miRNA-431 mRNA in CRC samples was significantly lower than that in para-cancerous tissues. The differentially expressed miR-431 was not associated with gender, age, etc., but it was significantly related to tumor metastasis and staging (Figure 1).

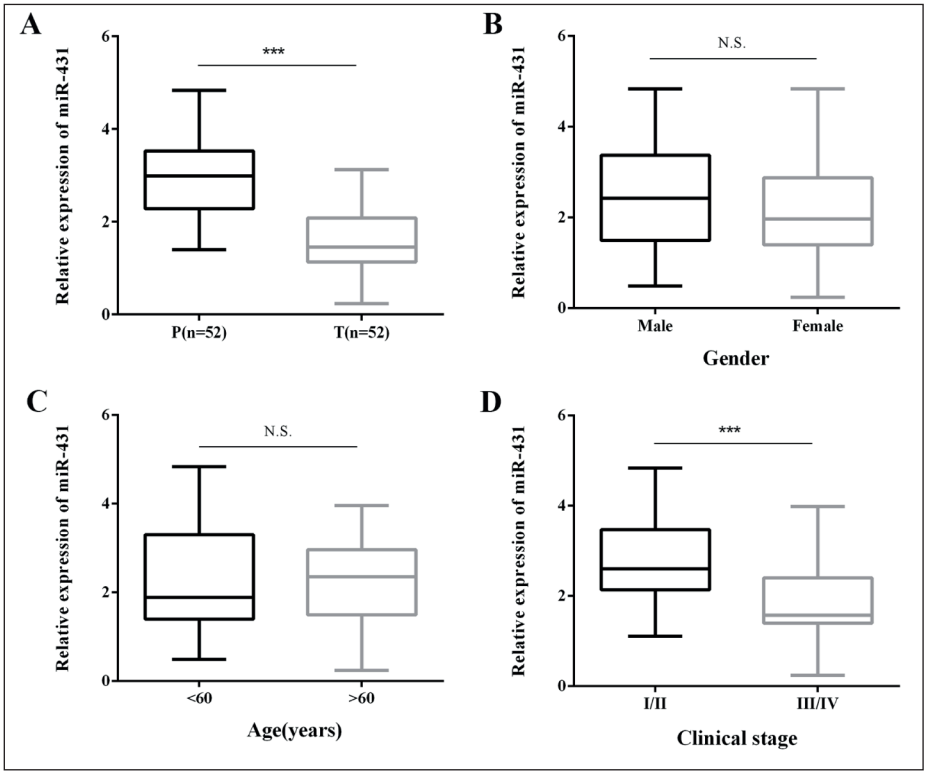

Figure 1. The expression of miRNA-431 mRNA in CRC patients (n=52) is related to tumor staging detected by RT-PCR assay. P: para-cancerous tissues; T: tumor tissues; N.S.: no significance. ***p<0.001.

Effect of Low-Expression miRNA-431 on the Migration Ability and EMT-Related Protein Level of CRC Cells

The results of comparison showed that miRNA-431 mRNA level was significantly decreased after CRC cells HCT116 were transfected with miRNA-431 inhibitor (p<0.05) (Figure 2A). The migration of cells was markedly promoted after the low expression of miRNA-431 in tumor cells (p<0.05) (Figure 2B). In addition, it was found that there were statistically significant differences in the lower expression level of E-cadherin and higher expression levels of N-cadherin, FN, Vimentin, and Snail in CRC cells with low expression of miRNA-431 compared with those in the transfection control group (p<0.05, Figure 2C).

Effect of Overexpression of miRNA-431 on Cell Migration Ability and EMT-Related Protein Levels

The results presented that the expression of miRNA-431 mRNA was increased after CRC cells SW480 were transfected with mimics of miRNA-431 (Figure 3A), and the difference was statistically significant (p<0.05). After the high expression of miRNA-431 in tumor cells, the cell migration ability was also remarkably attenuated (p<0.05, Figure 3B). In addition, the expression level of EMT-related E-cadherin in CRC cells overexpressing miRNA-431 was increased, and the expression levels of N-cadherin, Vimentin, FN, and Snail were reduced compared with those in the transfection control group (p<0.05, Figure 3C).

Negative Regulation of MiR-431 on CUL4B

The miR-431 binding site was found on the 3'-UTR of CUL4B using miRBase software predictions and bioinformatics analysis (Figure 4A). Fluorescence quantitative PCR demonstrated that CUL4B mRNA was significantly inhibited by miR-431 transfection. Western blotting assay revealed that the expression of CUL4B protein was negatively regulated by abnormal miR-431 expression. MiR-431 could negatively regulate the expression of CUL4B by binding to the 3'-UTR of CUL4B (Figure 4B and C).

Discussion

CRC is a malignant cancer of the digestive tract, with high incidence and bad lifestyle. The development of CRC is a multifactorial and complex process, and tumor metastasis is one of the major causes of CRC mortality[11]. EMT process

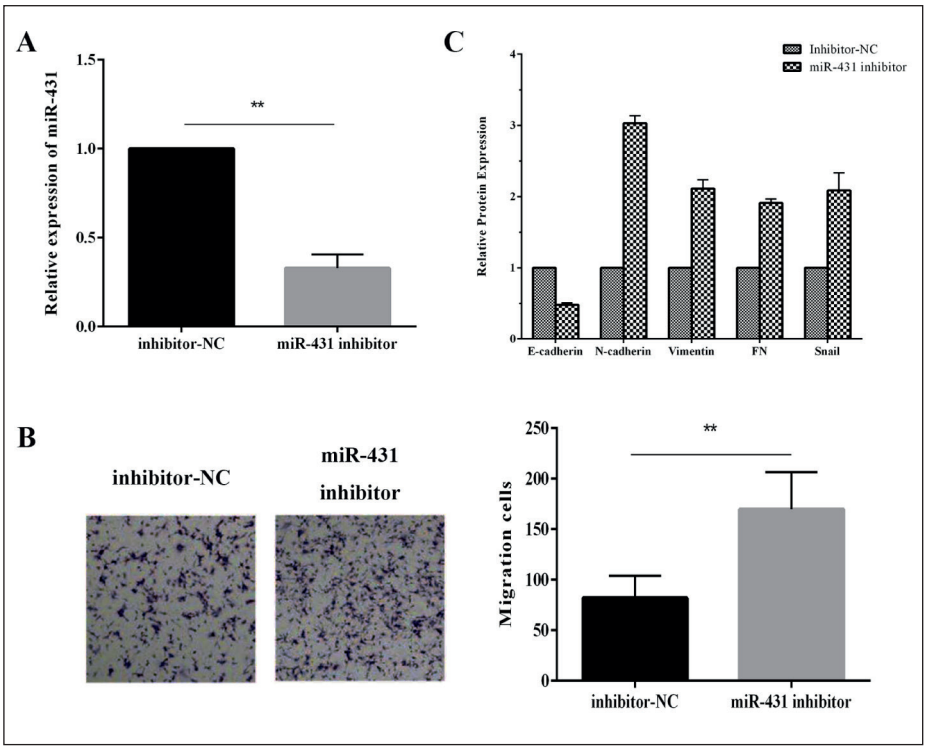

Figure 2. The effect of low-expression miRNA-431 on the migration ability and EMT-related protein level of CRC cells. **A**, The transfection effect is detected by RT-PCR assay. **B**, Cells invasiveness is explored by transwell invasion assay between inhibitor-NC (inhibitor negative control) and miR-431 inhibitor. **C**, EMT-related protein level of colorectal cancer cells is detected by Western blotting assay. $^{**}p<0.01$.

of CRC contains cytoskeleton rearrangement, cell adhesion structure enhanced by epithelial cells and cell polarity changes, leading to cell deformation, protruding filopodia and cell polarity loss, etc., so it plays an extremely pivotal role in the development of CRC[12]. Therefore, the search for new methods of specific early diagnosis of EMT-related molecular markers (such as gene markers) and gene targeting therapy has become a hot issue in the basic and clinical research of CRC.

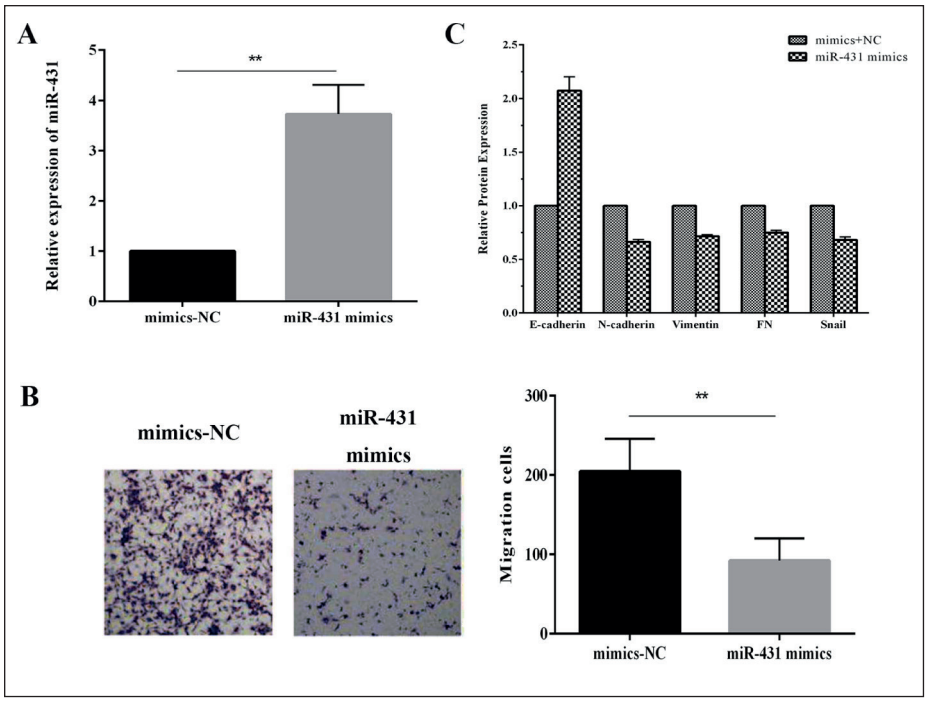

Figure 3. The effect of miRNA-431 up-regulation on the migration ability and EMT-related protein level of CRC cells. **A**, The transfection effect is detected by RT-PCR assay. **B**, Cells invasiveness is explored by transwell invasion assay between mimics-NC (mimics negative control) and miR-431 mimics. **C**, EMT-related protein level of CRC cells is detected by Western blotting assay. $^{**}p<0.01$.

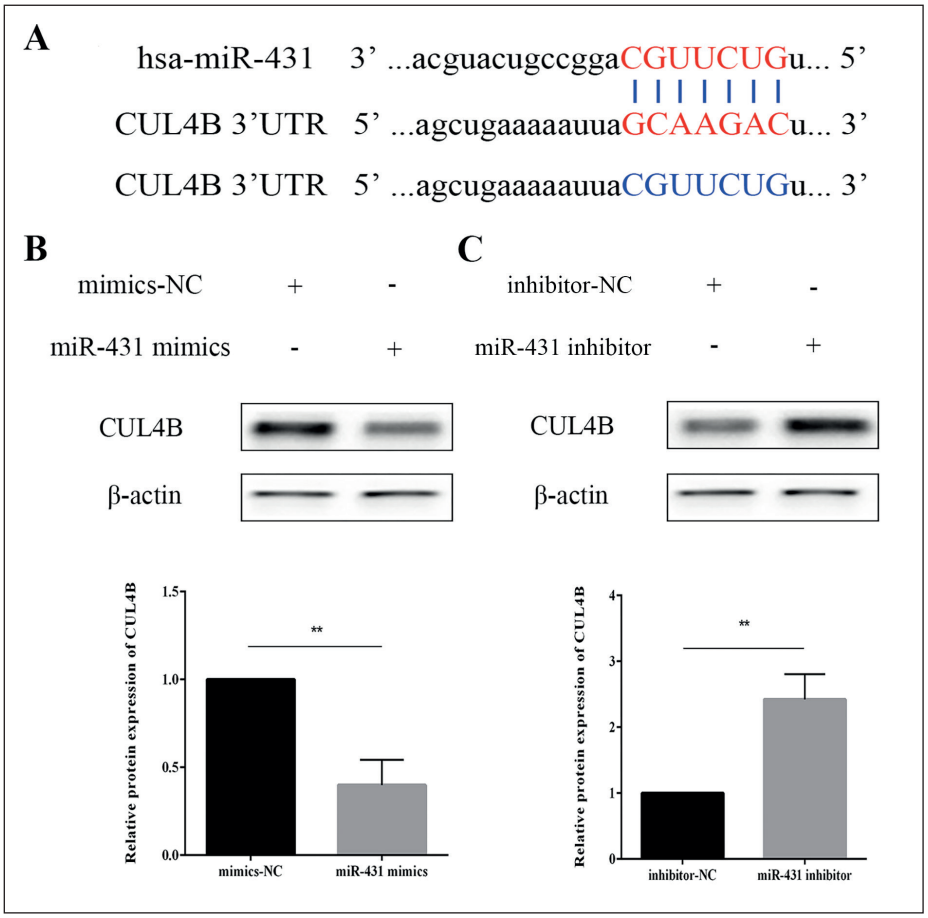

Figure 4. MiR-431 negatively regulates CUL4B. **A**, The miR-431 binding site is found on the 3'-UTR of CUL4B using miRBase software predictions and bioinformatics analysis. **B**, miR-431 overexpression inhibits CUL4B protein expression. **C**, miR-431 inhibition enhances CUL4B protein expression (**$p<0.01$).

MiRNA is a novel gene expression regulatory molecule causing degradation of specific target mRNA to inhibit protein synthesis and negatively regulate the expression of specific genes at post-transcriptional levels[13,14]. Scholars[15-17] have suggested that miRNAs can act as both oncogenes and tumor suppressor genes, and are widely involved in the pathogenesis of tumor cell invasion and metastasis. A large number of research[18,19] has manifested that miRNAs contribute to the development, progression, diagnosis, and prognosis of colorectal tumors. Geng et al[20] found that miRNA-103 is highly expressed in CRC and can promote its migration. However, the expression of miRNA-431 in CRC patients and its function have not been reported so far. The results presented that miRNA-431 expression in the CRC samples was lower than that in the adjacent samples, indicating that miRNA-431 was involved in the development, progression, and metastasis. In addition, the cell migration with low expression of miRNA-431 was significantly increased, and the migration level of cells with high expression of miRNA-431 was significantly decreased, indicating that miRNA-431 can inhibit CRC cell migration, and it may be involved in the process of CRC by influencing the migration of CRC cells. Studies have shown that the typical molecular biological performance of the EMT process of rectal cancer is the missing or weakening of E-cadherin protein and other epithelial markers. In CRC, the expression of EMT-related protein E-cadherin in cells with high expression of miRNA-431 was significantly increased, suggesting that miRNA-431 was involved in EMT process. Further analysis of miRBase software and bioinformatics showed that there was a potential miR-431 binding site on 3'-UTR of CUL4B. CUL4B mRNA was significantly inhibited by transfection of miR-431. Western blotting analysis showed that up-regulation of miR-431 inhibited the expression of CUL4B protein, and low expression of miR-431 enhanced the expression of CUL4B. MiR-431 could bind to 3'-UTR of CUL4B, and negatively regulate CUL4B expression.

Conclusions

MiRNA-431 may play a role in promoting colorectal tumor, participate in the processes of the tumor and CRC with EMT-related protein E-cadherin mainly through the migration of CRC cells. Detection of miRNA-431 in patients with CRC may have certain guiding significance in the clinical treatment and prognosis of CRC.

Conflict of Interest:

The authors declared no conflict of interest.

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