

MiRNA-155 promotes the invasion of colorectal cancer SW-480 cells through regulating the Wnt/ β -catenin

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Abstract. – OBJECTIVE: To investigate the role of microRNA-155 (miR-155) in the potential invasion of colon cancer cell and the underlying mechanism.

PATIENTS AND METHODS: The expression level of miR-155 in colon cancer and adjacent normal tissues was detected by Real-time quantitative polymerase chain reaction (RT-PCR). miR-155 mimics (miR-155), or siRNA against β -catenin (β -catenin siRNA), was transfected into human colon cancer cell line SW-480 using Lipofectamine 2000, respectively. RT-PCR was used to measure the expression levels of miR-155 and β -catenin mRNA, and β -catenin protein expression level was detected by Western blot. The *in-vitro* cell invasion abilities were determined by transwell invasion assays after up-regulating miR-155 or knocking down of β -catenin.

RESULTS: MiR-155 directly regulates β -catenin at the transcriptional level, and promotes the invasion potential of colon cancer cell, at least partly through the upregulation of β -catenin.

CONCLUSIONS: The findings of this study suggest that miR-155 and β -catenin may have a unique potential as a novel biomarker candidate for diagnosis and treatment of tumor metastasis.

Key Words:

Micro RNA-155, Wnt/ β -catenin, Colon cancer, Invasion.

colon. In China, the incidence rate of colorectal cancer ranks 3rd in malignant tumors, which is only inferior to the incidence rates of lung cancer and liver cancer, threatening the health of human beings. Multiple genes and procedures are involved in the complex regulation of occurrence and progression of colorectal cancer. According to the previous studies, β -catenin, a key signaling factor mediating the intercellular adhesion and genetic transcription regulation, is involved in multiple malignant biological behaviors including the growth, invasion and distant metastasis of malignant tumors like gastric cancer, breast cancer, prostate cancer and colorectal cancer. Latest research indicated that the expressions of miR-155 are elevated in the carcinoma tissues and cells in colorectal cancer, suggesting that miR-155 might be involved in the occurrence and progression of disease. In this study, we aimed to observe the effect of miR-155 on the invasion capability of colorectal cell SW-480, and the regulatory role of Wnt/ β -catenin signal pathway in this process, thereby providing experimental evidence for further elucidating the pathogenesis and development mechanism of distant invasion and metastasis of colorectal cancer.

Introduction

Colorectal cancer is the common malignant tumor in digestive system, which frequently occurs in conjunction between rectum and sigmoid

Patients and Methods

Patients

Thirty-four human colorectal cancer and corresponding para-carcinoma samples were col-

lected from the resected tissues of colorectal cancer patients who received surgical resection in Gastrointestinal Surgery of our hospital. The clinical samples enrolled in this study were approved by the Ethical Committee of our hospital. Fresh samples that were initially preserved in the liquid nitrogen were used for extraction of tissue RNA, and human colorectal cancer cells SW-480 were cultured with Iscove's Medium Dulbecco Medium (IMDM) supplemented with 10% fetal bovine serum (FBS) in an incubator (37°C and 5% CO₂).

Materials

Colorectal cancer cell strain SW-480 (China Center for Type Culture Collection, Wuhan University, Wuhan, Hubei, China); Iscove's Modified Eagle Medium (IMEM) culture medium (Gibco, Rockville, MD, USA); miR-155 mimic sequence (5'-GGCCAGUUUCCCAAGAAUCCCU-3', 3'-GUCCAGUUUUCGAGGAAUCCCU-5'), and the sequence of negative control (5'-CAGUACUUUUGUGUAGUACAA-3', 3'-CAGUACCUGUACUUAUCGAACA-5'); miR-155 qPCR Quantitation Kit and U6 snRNA qPCR Normalization Kit (Guangdong RiboBio Co., Ltd, Guangzhou, Guangdong, China); β -catenin-specific siRNA (synthesized by Shanghai GeneChem Co., Ltd., Shanghai, China); TRIzol kit (Invitrogen, Carlsbad, CA, USA); PCR primer and standard substance of nucleic acid (TaKaRa, Dalian, Liaoning, China); rabbit anti-human β -catenin polyclonal antibody (Abcam, Cambridge, MA, USA); rabbit anti-human GAPDH polyclonal antibody Santa Cruz Biotechnology (Santa Cruz, CA, USA); Protein marker of moderate molecular weight (Thermo Fisher Scientific, Waltham, MA, USA); enhanced chemiluminescence (ECL) kit (Millipore, Billerica, MA, USA); radio-immunoprecipitation (RIPA) kit and bicinchoninic acid (BCA) kit (Thermo Fisher Scientific, Waltham, MA, USA); 24-well transwell chamber (8 μ m; Corning Costar, Corning, NY, USA); Matrigel (Hyclone, Logan, UT, USA); thermostatic cell incubator (Sanyo, Tokyo, Japan); polymerase chain reaction (PCR) apparatus and electrophoresis apparatus for Western blotting assay (Bio-Rad, Hercules, CA, USA).

Cell Transfection

In accordance with the Lipofectamine[®] LTX instruction, gastric cells SGC-7901 were transfected by miR-10b mimics and the empty vector for negative control, and miR-10b inhibitor and

the empty vector for negative control with Lipofectamine 2000. After 24 h of transfection, cells were regularly cultivated and preserved at -80°C for use in sequential experiments.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Primers of β -catenin and glyceraldehyde-phosphate dehydrogenase (GAPDH) were synthesized by Beijing Tianyi Huiyuan Biotech Co., Ltd., (Beijing, China) and the primer sequences are shown as follows: β -catenin: forward 5'-CTCAAGCCTTCCAACCTC-3', reverse 5'-TTCCACGGCACCTTATTT-3'; GAPDH: forward 5'-TGAACGGGAAGCTCACTGG-3', reverse 5'-TCCACCACCCTGTTGCTGTA-3'. The commercialized miR-155 kit was purchased from Guangdong RiboBio Biotech Co., Ltd (miRQ0000075-1-3) (Guangzhou, Guangdong, China), and the target sequence was 5'-GUGCAUUGUAGUUGCAUUGCA-3'. miR-155 and U6 primers were used for Real-time PCR on the fluorescent quantitative PCR apparatus (Lightcycler480) with U6 as internal reference. PCR reaction conditions were set as follows: initial denaturation at 95°C for 3 min; denaturation at 92°C for 12 s, annealing at 60°C for 1 min and extension at 72°C for 30 s, for a total of 40 cycles; extension at 72°C for 5 min. The amplified product was preserved at 4°C. Statistical analysis was performed for the Real-time qPCR results.

Western Blotting Assay

Cells adhering to the wall that had been treated were washed with pre-warmed phosphate buffered saline (PBS) and transferred into the Eppendorf (EP; Hamburg, Germany) tubes for centrifugation at 12000 rpm for 5 min. The supernatant was discarded, and the collected cells were lysed using radioimmunoprecipitation assay (RIPA). Then, the supernatant was preserved at -20°C. Samples were loaded for electrophoresis at 80 V to coagulate the proteins which were then isolated and transferred to the membrane at 100 V. The membrane was blocked with 5% skimmed milk for 1 h. Rabbit anti-human β -catenin polyclonal antibody (1:500) and rabbit anti-human β -catenin polyclonal antibody (1:1000) were added to the membrane for incubation at 4°C overnight. The membrane was washed with Tris-buffered saline-Tween 20 (TBST) 3 times (5 min/time) on a decoloring shaker, and incubated using the horseradish peroxidase (HRP) labeled goat anti-rabbit immu-

noglobulin G at room temperature for 1 h. After the membrane was washed 3 times (5 min/time) using Tris-buffered saline-Tween 20 (TBST), ECL reagent was added onto the membrane for 1 min of reaction followed by exposure under X-ray, fixation and color development.

Transwell Cell Invasion Experiment

Before experiment, the Matrigel was melted at 4°C and diluted with serum-free culture medium (1:3). On the surface of polycarbonate membrane, 40 μ L diluted matrigel was spread and placed in the incubator for 4 h of coagulation for later use. After SW-480 cells in logarithmic phase were starved in the serum-free Iscove's Medium Dulbecco Medium (IMDM) for 24 h, cells were digested using 0.25% ethylene diamine tetraacetic acid (EDTA) trypsin for preparation of single-cell suspension using the serum-free Iscove's Medium Dulbecco Medium (IMDM) culture medium, in which cell density was adjusted to 4×10^5 /mL. In the upper transwell chamber, 200 μ L serum-free cell suspension was added, and the wells were grouped according to the experiment requirement with 3 replicative wells in each group. In the lower transwell chamber, 600 μ L Dulbecco's modified eagle Medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS) were added into each well for 24 h of culture in an incubator. The chambers were taken out and washed with phosphate buffered saline (PBS) to remove the medium. In the upper chamber, cells that failed to pass through the membrane were scrubbed using a wet cotton swab followed by 20 min of fixation in methanol drying at room temperature. Furthermore, cells were stained using crystal violet for 20 min, and placed under the inverted microscope to observe the quantity of cells that passed through the membrane. Cell count was performed in 5 high magnification vision (400 \times), and the average cell count was used as the result.

Statistical Analysis

All data were presented as mean \pm standard deviation, and one-way analysis of variance (ANOVA) was performed using the SPSS 17.0 (SPSS Inc., Chicago, IL, USA). $p < 0.05$ suggested that the difference had statistical significance. Tukey's HSD (honestly significant difference) test is used in conjunction with an ANOVA to find means that are significantly different from each other.

Results

Expression of miR-155 in Colorectal Cancer Tissues

RT-PCR was carried out to detect the expressions of miR-155 in the colorectal cancer tissues and normal para-carcinoma tissues, and the results showed that the expression of miR-155 in the colorectal cancer tissues was significantly elevated in comparison with the normal para-carcinoma tissues ($p < 0.01$; Figure 1).

miR-155 Expression is Upregulated in SW-480 Cells that Were Transfected with miR-155 Mimics

After SW680 cells were transfected with miR-155 mimics for 24 h, the result of fluorescent detection showed that in cells that were transfected with miR-155 mimics, green fluorescent protein (GFP) could be identified, suggesting that cells were successfully transfected (Figure 2A). Then, the total mRNA was extracted from the cells for RT-PCR. The results showed that compared with the empty group and the negative control group, the miR-155 expression in the SW680 cells that were transfected with miR-155 mimic was significantly elevated (Figure 2B).

Upregulation of miR-155 Expression Affects the mRNA and Protein Expression of β -catenin

After cells were transfected with miR-155 mimic for 48 h, the total protein was extracted for detecting the effect of overexpression of

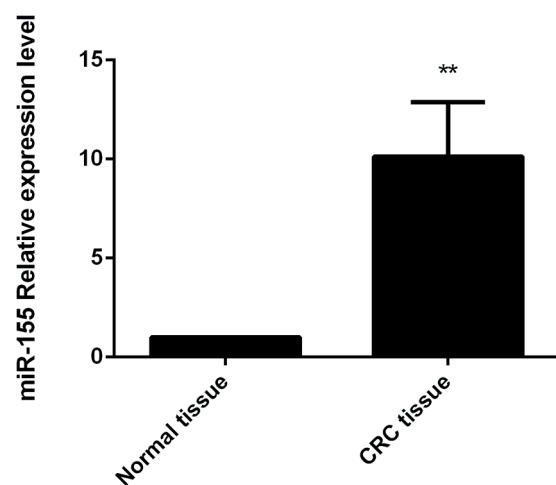


Figure 1. The expression level of miR-155 in colon cancer tissue and normal adjacent colon tissue.

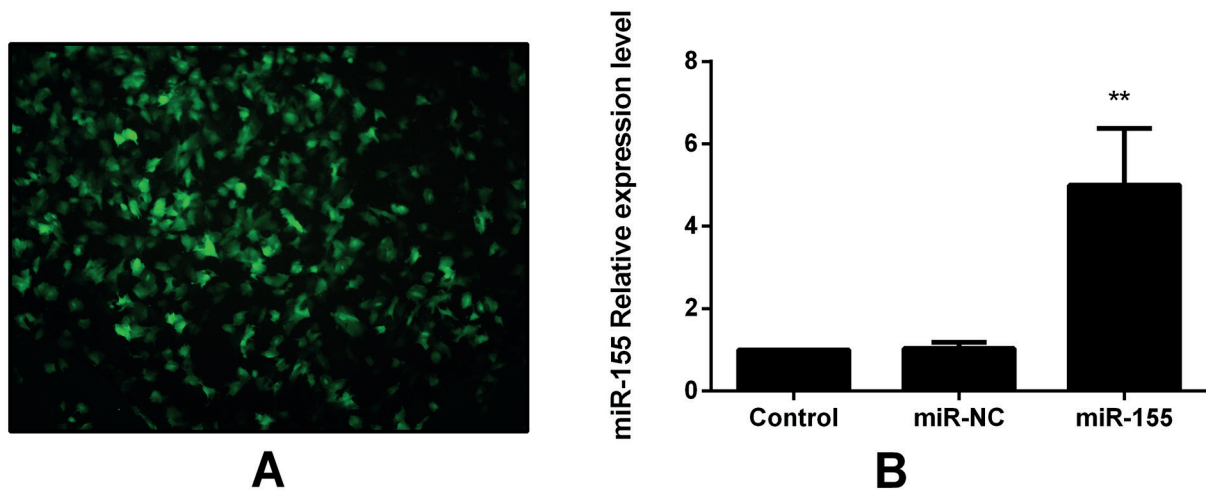


Figure 2. The expression level of miR-155 after transfected with miR-155 mimics.

miR-155 on mRNA and protein expressions of β -catenin, and the results showed that compared with the empty group and the negative control group, in the SW680 cells that were transfected

with miR-155 mimic, the mRNA and protein expressions of β -catenin were increased, and the difference had statistical significance ($p < 0.05$; Figure 3).

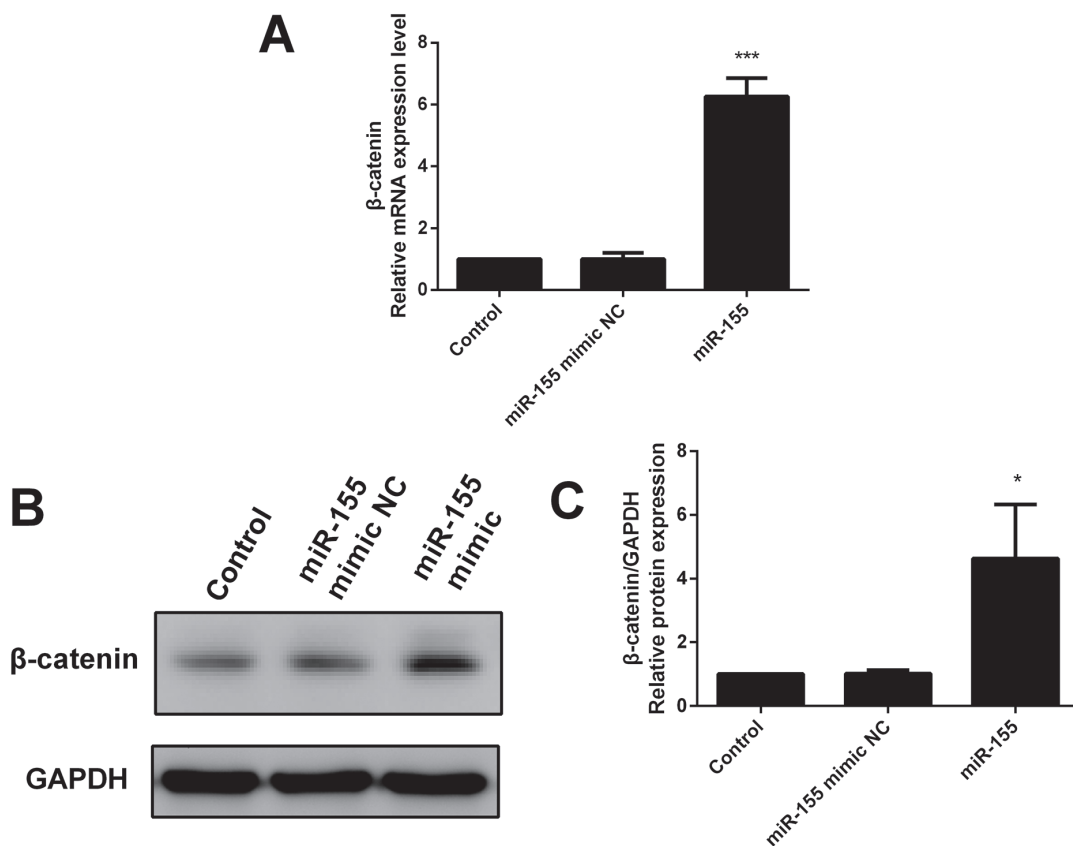


Figure 3. Effects of transfecting miR-155 mimic on expression level of β -catenin mRNA and protein in SW680 cells.

Transfection of miR-155 Mimic Affects the Invasion Capability of SW680 Cells

Transwell experiment of cell invasion was carried out for detecting the effect of miR-155 on invasion capability of SW680 cells, and the results showed that after 48 h of culture, the quantity of cells that passed through the microporous membrane of transwell in the miR-155 mimic group was significantly higher than those in the empty group and the negative control group, and the difference had statistical significance ($p < 0.05$; Figure 4).

β -catenin siRNA Affects the Cell Invasion Capability Mediated by miR-155

SW680 cells that were transfected with miR-155 mimic were also simultaneously transfected with β -catenin siRNA, and the results showed that β -catenin siRNA could significantly reverse the enhancing effect of miR-155 mimic on invasion capability of SW680 cells, and significantly weaken the invasion capability of colorectal cancer cells. The difference had statistical significance ($p < 0.05$; Figure 5).

Discussion

Colorectal cancer, a common type of malignant tumor in gastrointestinal tract in China, ranks 3rd in the incidence rate of all tumors in gastrointestinal tract, and is usually characterized by the high malignancy, infiltrative growth, susceptibility to distant invasion and metastasis, and recurrence¹⁻³. The occurrence and pathogenesis of colorectal cancer are co-regulated by multiple genes and factors. With the development in medical science, clinical physicians have gained new understanding on the formation, development, prognosis, and treatment of colorectal cancer, but as for the cure and prophylaxis of recurrence of colorectal cancer, precise understanding of the pathogen and pathogenesis of colorectal cancer are key problems, in which the distant invasion and metastasis of colorectal cancer cells play an important role. Thus, it is of great significance for improving the clinical efficacy and preventing the recurrence of colorectal cancer to perform in-depth research on the molecular mechanism of invasion and metastasis of colorectal cancer cells,

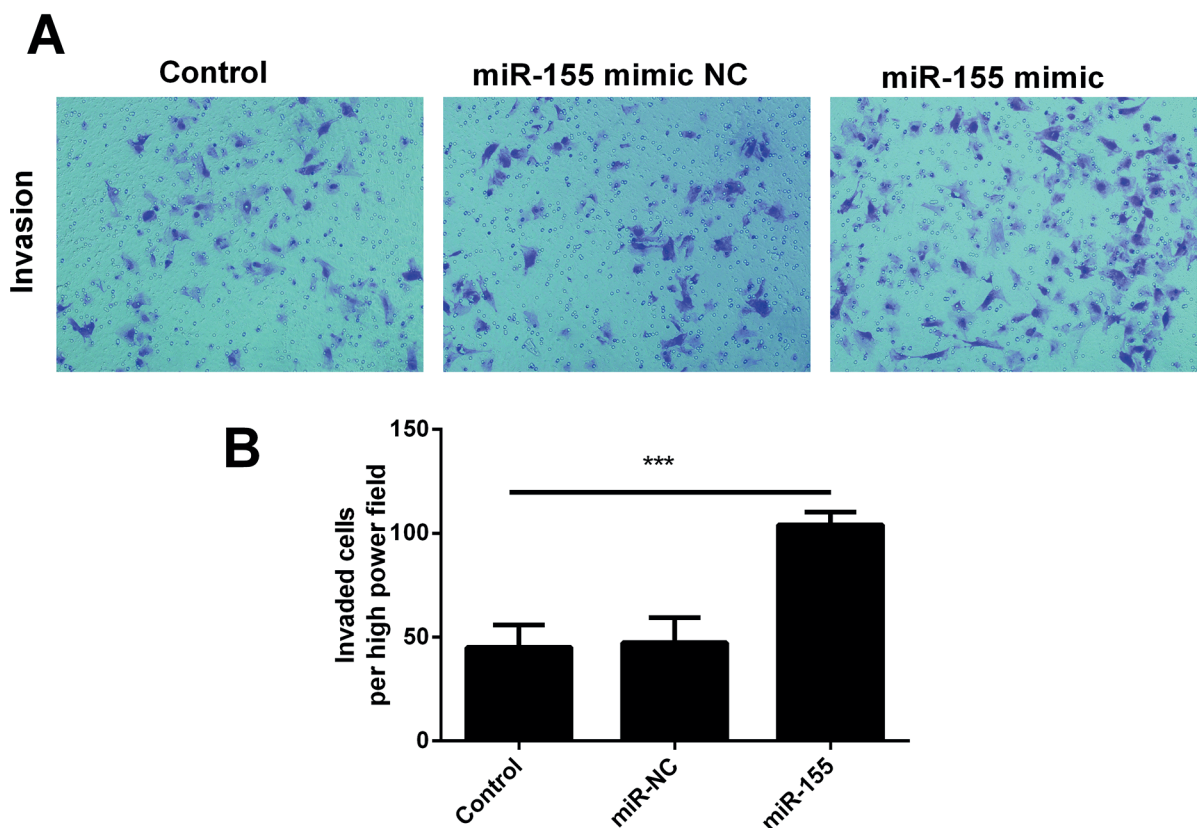


Figure 4. Effects of transfecting miR-155 mimic on invasion abilities in SW680 cells (crystal violet staining $\times 200$).

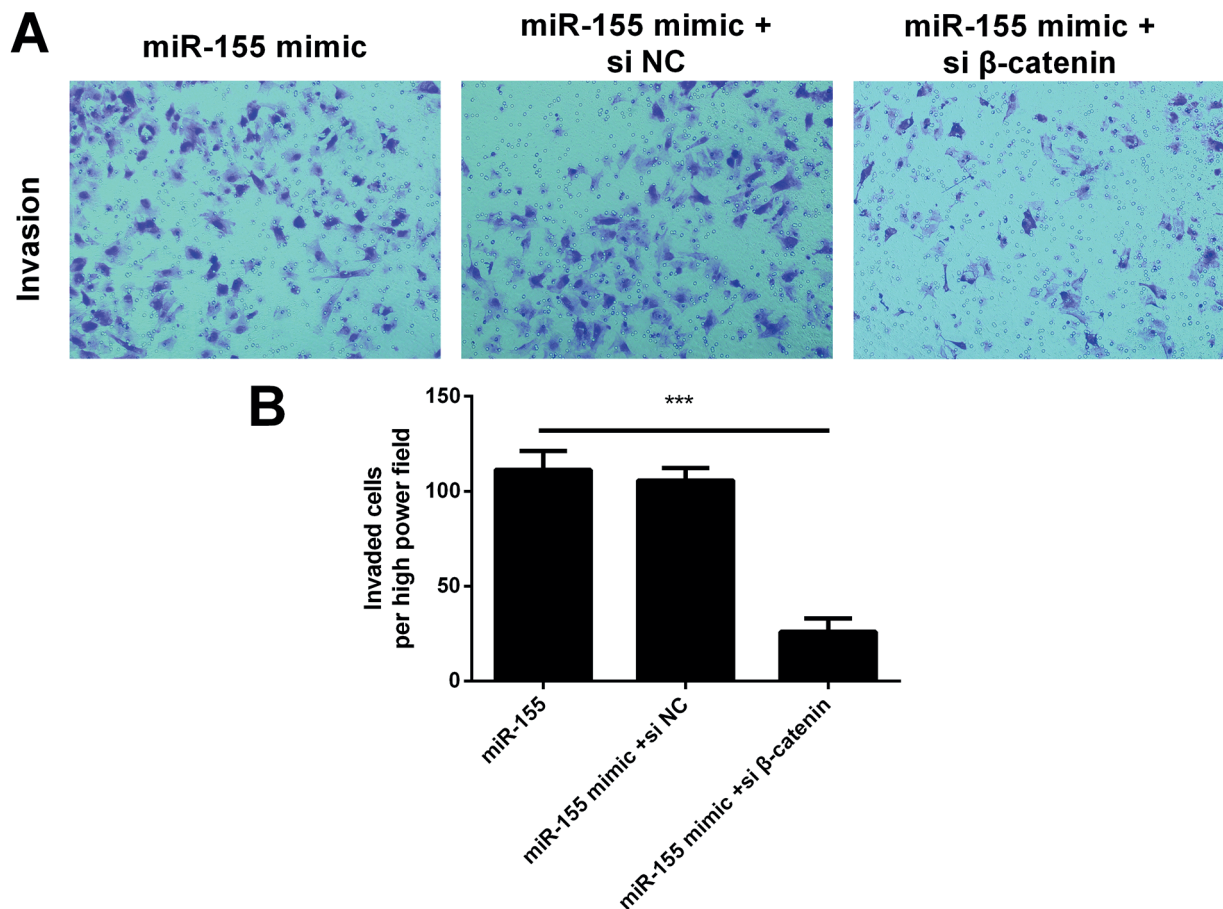


Figure 5. Effect of β -catenin siRNA on invasion abilities in SW680 cells after being transfected with miR-155 (crystal violet staining $\times 200$).

through which we wish to identify more effective therapeutic target with high specificity, and search for new intervention strategy. This is also the key point in treating colorectal cancer and improving clinical prognosis. MicroRNA (miRNA) is a kind of small endogenous RNA in length of 20-24 nucleic acids that is generated from the single-chain hairpin-shaped RNA precursor in length of 70 to 90 bp that is processed by Dicer⁴⁻⁶. Many studies have indicated that the abnormal expression of miRNA might be involved in a variety of diseases including tumors⁷⁻⁹. Dysregulation in expression of miRNA has been identified in tumors, and different miRNAs are involved in regulation of cell proliferation^{10,11}, apoptosis^{12,13}, invasion and metastasis^{14,15} through acting as the oncogene or anti-oncogene, thereby affecting the occurrence and progression of tumors. Previous studies have shown that miR-155 is extensively expressed in multiple tissues^{16,17}, and highly expressed in the

tumor tissues, suggesting that it can be served as a tumor promotor. In addition, several latest studies also proved its key role in facilitating the occurrence and progress of tumors. miRNA also greatly contributes to the metastasis and invasion of tumors^{18,19}. In breast cancer, p53 mutant can accelerate the invasion and metastasis of tumor cells through upregulating the expression of miR-155²⁰. Overexpression of miR-155 can promote the proliferation and invasion of squamous cells in human salivary gland through specific regulation of SOCS1 and STAT3 expressions²¹. In liver cancer after liver transplantation, upregulation in expression of miR-155 can facilitate the invasion and metastasis of liver cancer cells, which is negatively correlated with the prognosis²². Wnt/ β -catenin signal pathway participates in a variety of cellular information regulation, and plays key roles in cell proliferation, tissue repair, embryonic development, occurrence and

progression of various benign and malignant tumors²³⁻²⁶. Research shows that β -catenin is closely correlated to the occurrence and progression of diverse tumors, including retinoblastoma²⁷, liver cancer²⁸, gastric cancer²⁹ and colorectal cancer³⁰. These studies have indicated the high expression of β -catenin in these tumor tissues. A paper has implicated that β -catenin, as the target gene in downstream regulation of various miRNAs, can further regulate the downstream target genes to intervene in a series of biological events, such as cell proliferation, differentiation, apoptosis and invasion, thereby affecting the occurrence and progression of many kinds of tumors³¹⁻³⁴. In papillary thyroid cancer, the upregulation in expression of miR-155 can promote the progression of disease through activating β -catenin³⁵. Additionally, hepatitis virus can also regulate the Wnt/ β -catenin signal pathway through upregulating the expression of miR-155 to induce the liver cancer³⁶. However, there remains no study reporting whether miR-155 is involved in the pathogenesis of colorectal cancer, and its role as well as the relevant molecular mechanism in invasion of colorectal cancer cells. In this work, we firstly collected the samples from the colorectal cancer tissues and para-carcinoma tissues, and verified whether the miR-155 was abnormally activated in colorectal cancer at the level of tissue. The results showed that compared with the para-carcinoma tissues, the expression of miR-155 in colorectal cancer tissues was significantly elevated. Next, we performed a series of *in-vitro* experiments using the colorectal cancer cell line to examine the effect of miR-155 on the invasion capability of SW-480 cells, and to discover the potential molecular mechanism. After the cells were respectively transfected by miR-155 mimic and relevant NC plasmid, we found that the expression of miR-155 in the miR-155 mimic group was remarkably elevated. Thereafter, we further detected the effect of miR-155 mimic on β -catenin expression, and the results showed that compared with the cells that were transfected by empty plasmid and negative control plasmid, the mRNA and protein expressions of β -catenin in the cells that were transfected with miR-155 mimic were significantly elevated. The *in-vitro* transwell experiment of cell invasion showed that the SW-480 cells that were transfected with miR-155 mimic gained stronger invasion capability. Furthermore, we investigated the molecular mechanism that miR-155 mediated cell invasion; the result showed that, after the transfection

of β -catenin siRNA, the invasion capability of SW-480 cell mediated by miR-155 mimic could be remarkably attenuated, suggesting that the activation of β -catenin plays an important role in cell invasion mediated by miR-155. Based on the results mentioned above, we postulated that miR-155 regulates the activation of downstream target gene transcription through activating the Wnt/ β -catenin signal pathway, thereby facilitating the distant invasion and metastasis of SW-480 cells in colorectal cancer, finally promoting the occurrence and progression of colorectal cancer.

Conclusions

The results of this study preliminarily confirmed that upregulating the expression of miR-155 in colorectal cancer cell SW-480 can promote the distant invasion and metastasis of tumor cells through activating the Wnt/ β -catenin signal pathway, which is conducive to deepening the understanding of pathogenesis of distant invasion and infiltration of colorectal cancer. Thus, to inhibit the invasion and metastasis of tumor cells, we should perform more in-depth studies to discover the role and regulation mechanism of miR-155 and Wnt/ β -catenin signal pathway in the occurrence and progression of colorectal cancer. Also, more investigations are needed to develop the technique to specifically block the expression of miR-155 and the activity of Wnt/ β -catenin, which will provide new ideas and evidence for individual treatment for colorectal cancer, with a wide clinical significance and promising application prospect.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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