MiR-202-5p suppressed cell proliferation, migration and invasion in ovarian cancer via regulating HOXB2

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Abstract. – OBJECTIVE: Ovarian cancer (OC) is still the third leading cause of death in reproductive system malignancies. In OC, the biological function of microRNA-202-5p (miR-202-5p) is unknown. Our current research mainly focuses on miR-202-5p in the OC progression.

PATIENTS AND METHODS: MiR-202-5p was determined to be down-regulated in OC by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Cell counting kit-8 (CCK-8) assay and colony formation assay were recruited to access the ability of miR-202-5p on cell proliferation. Cell migration and invasion were determined by transwell assay and Matrigel assay. Dual-Luciferase reporter assay was recruited, and it validated that HOXB2 was a downstream target of miR-202-5p. Epithelial-mesenchymal transition (EMT) hallmark genes and HOXB2 expression level were examined by Western blotting.

RESULTS: MiR-202-5p was down-expressed in OC. Receiver operating characteristic (ROC) curve indicated that miR-202-5p was positively related to HOXB2. MiR-202-5p over-expression led to a higher 5-year survival rate. Up-regulated miR-202-5p inhibited cell proliferation and metastasis in vitro. HOXB2 was a downstream target of miR-202-5p.

CONCLUSIONS: We verified that miR-202-5p suppressed cell proliferation, migration, and invasion in OC via regulating HOXB2. Our findings provide new insights into the underlying mechanism of OC progression and may be useful in finding biomarkers and therapeutic targets of OC.

Key Words: MiR-202-5p, Proliferation, Migration, Invasion, HOXB2.

Introduction

The incidence of ovarian cancer (OC) ranks third in reproductive system malignancies worldwide, while the mortality rate ranks first. High malignancy and rapid progression OC are still the main cause of death in OC patients. Diagnosis of ovarian cancer is in a difficult situation. About 70% of ovarian cancer patients have been diagnosed at advanced stage, and the emergence of microRNAs has brought insight into the diagnosis of ovarian cancer.

MicroRNAs (miRNAs) are a class of non-coding single-stranded RNAs which are approximately 19-22 nucleotides in length. MiRNA has been widely recognized to participate in cell development, differentiation, and metabolism; processes such as aging and defense play a positive role in human life activities. MiRNAs have the dual behavior of oncogene or tumor suppressor gene, and its abnormal expression may lead to a novel insight of diagnosis and treatment of ovarian cancer. Resnick et al. observed that circulating microRNAs can be taken as biomarkers for ovarian cancer. Zhou et al. reported that microRNAs in urine have specificity in the diagnosis of ovarian cancer.

We elucidated that miR-202-5p was down-expressed in OC. Besides, through the functional in vitro experiments, we found that miR-202-5p repressed cell proliferation and promoted cell metastasis in OC. We demonstrated that HOXB2 might serve as a downstream target of miR-202-5p by using bioinformatics analysis and luciferase reporter assay. The results of Western blotting and rescue assay indicated that miR-202-5p lessened cell proliferation and facilitated cell apoptosis by mediating HOXB2 and the epithelial-mesenchymal transition (EMT) in OC.

Patients and Methods

Clinical Samples

The Ethics Committee of Fuyang First Hospital empowered the present research. All volunteers involved in the current study signed the informed consents. All the clinical samples were
extracted from OC patients in Fuyang First Hospital during 2016-2017. A total of 55 paired OC and para-tumor tissues were engaged. All the clinical samples were collected in liquid nitrogen.

**Cell Culture**

Cell lines involved included 6 ovarian cell lines (OVCAR3, PEO1, A2780, 3AO, CAOV3, SKOV3). 1 human ovarian surface epithelial cells (HOSEpiCs) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco, Rockville, MD, USA) and supplemented by 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C with 5% CO₂.

**Cell Transfection**

Oligonucleotides were obtained for up-regulating miR-202-5p (GenePharma, Shanghai, China). The plasmid pcDNA-3.1(GenePharma, Shanghai, China) was used to up-regulate the expression of HOXB2, while the empty pcDNA-3.1 plasmid was taken as a control. The transfection efficiency was examined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR).

**QRT-PCR**

Total RNA of tissue specimens and cell lines were extracted through TRIzol reagent (Invitrogen, Carlsbad, CA, USA). All complementary deoxyribose nucleic acids (cDNAs) were synthesized via Reverse Transcription Kit (TaKaRa, Otsu, Shiga, Japan). MiR-202-5p expression level was assessed through SYBR Green real-time PCR, and glycerinaldehyde 3-phosphate dehydrogenase (GAPDH) was taken as a normalization. The primer sequences are listed as follows: miR-202-5p-F: 5’-TTC CATGCATATACCTTTG-3’; miR-202-5p-R: 5’-AGAGGCATAGGGCATGGAAAA-3’; U6-F: 5’-GCTTCGGCAGCACATATACTAAAAT-3’; U6-R: 5’-GCTTCGGCAGCACATATACTAAAAT-3’.

**Colony Formation**

Cells (1.0 × 10⁵) were planted into the culture plates (60 mm) and cultured for 2 weeks. Cells on the plates were then washed by phosphate-buffered saline (PBS; Gibco, Rockville, MD, USA) for twice and fixed in ice-cold 70% methanol for 15 min and crystal violet staining solution (Beyotime, Shanghai, China) was used to stain the cell colonies. All the colonies were subsequently photographed.

**Cell-Counting Kit-8 Assay (CCK-8)**

Cell Counting Kit-8 assay was used to examine the cell proliferation in OC. Transfected cells were planted into 96-wells plates (6 × 10³/well). Then, the CCK-8 solution (Beyotime, Shanghai, China) (10 μL/well) was used to stain cells for 2 h at 37°C. The optical density (OD) value (450 nm) was subsequently evaluated.

**Transwell Assay and Matrigel Assay**

We carried out the transwell assay to figure out the invasion ability of transfected OC cells. Transwell chambers and 24-wells plates were obtained from Corning (Corning, NY, USA). 1 ×10⁵ cells were suspended with serum-free medium (100 μL). In Matrigel assay, cells were transferred to the Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) coated upper chamber. After 36 h, the invasive cells were observed and counted.

**Western Blotting**

Total protein was isolated by radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific, Waltham, MA, USA) and phenylmethylsulfonyl fluoride (PMSF). Protein lysates separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were then transferred to polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). Then, the membrane was immunostained at 4°C by rabbit anti-HOXB2 (1:1000, CST, Danvers, MA, USA), anti-N-cadherin (1:1000, CST, Danvers, MA, USA), anti-Vimentin (1:1000, CST, Danvers, MA, USA), anti-β-catenin (1:1000, CST, Danvers, MA, USA), anti-MMP9 (1:1000, CST, Danvers, MA, USA) overnight. Rabbit anti-GAPDH (1:5000, CST, Danvers, MA, USA) was taken as a loading control. Protein relative expression level was evaluated by the Image J software (NIH, Bethesda, MD, USA).

**Statistics Analysis**

All investigations were performed three times independently at least. All data recorded were exhibited as mean ± standard deviation (SD). Pearson correlation analysis was performed for correlation analysis. Student’s unpaired t-test was used to undergo statistics analysis. In this study, p<0.05 was statistically significant.

**Results**

**MiR-202-3p Was Down-Expressed In OC**

We examined the miR-202-5p relative expression level in OC. As shown in Figure 1A, we found that miR-202-5p was down-expressed in tumor specimens. Next, miR-202-5p expression in
OC cells was detected by qRT-PCR (Figure 1B). As Figure 1C showed, miR-202-5p was positively correlated with the OC progression. Besides, the 5-year survival rate in miR-202-5p over-expression group was higher than miR-202-5p down-expression group (Figure 1D). Taken together, it turned out that miR-202-5p was over-expressed in OC.

**MiR-202-5p Lessened Cell Proliferation in OC Cell Lines**

After transfection with mimics, miR-202-5p expression was determined by qRT-PCR (Figure 2A). Through CCK-8 assay, the over-expression of miR-202-5p led to lower the OD value at 96 h compared with control group (Figure 2B). Similarly, the up-regulated miR-202-5p generated more colonies when compared with control group (Figure 2C). Hence, miR-202-5p promoted cell proliferation in OC.

**MiR-202-5p Over-Expression Lessened Cell Migration and Invasion In Vitro and Suppressed EMT**

Through the transwell assay, the ability of cell migration in miR-202-5p over-expression group was significantly repressed (Figure 3A, 3B). After that, the Matrigel assay was brought into the current study to examine the ability of miR-202-5p on cell invasion. As Figure 3A and B showed, miR-202-5p over-expression inhibited cell invasion in comparison with NC group. By using the Western blotting assay, we determined the protein expression level of hallmarks in EMT. It turned out that the over-expression of miR-202-5p suppressed EMT (Figure 4A). Herein, we considered that miR-202-5p suppressed cell migration and invasion in OC.

**Figure 1.** MiR-202-3p had a down-regulated expression level in OC tissues and cell lines. A, Expression level of miR-202-5p in OC tissues and normal tissues; B, Analysis of miR-202-5p expression level in OC cell lines. C, ROC curve showed miR-202-5p was positively correlated with OC progression. D, 5-year survival rate in two groups was exhibited. Data are presented as the mean ± SD of three independent experiments. *p<0.05, **p<0.01, ***p<0.001.
Figure 2. MiR-202-5p promoted cell proliferation in OC cell lines. A, Transfection efficiency was determined by qRT-PCR. B, Cell proliferation ability was determined by CCK-8 assay. C, Colony formation assay was recruited for detecting cell proliferation (magnification: 40×). Data are presented as the mean ± SD of three independent experiments. *p<0.05, **p<0.01.
HOXB2 was a Down-Stream Target of MiR-202-5p

We used three publicly available databases: TargetScan, miRWalk, and MiRanda to predict the downstream target. We considered that HOXB2 was a potential target of miR-202-5p. HOXB2 expression was down-regulated in miR-202-5p overexpression group (Figure 4B). After transfection of HOXB2 up-regulated plasmids, the HOXB2 expression level was significantly up-regulated (Figure 4C). Subsequently, we determined the expression level of HOXB2 in OC tissues, demonstrating that HOXB2 was up-regulated in OC tissues (Figure 4D). In the Pearson correlation analysis, the result indicated that miR-202-5p was negatively correlated with HOXB2 (Figure 4E). Therefore, we verified that HOXB2 was a directly down-stream target of miR-202-5p in OC.

MiR-202-5p Functioned as a Tumor Suppressor in OC Through Regulating HOXB2

We employed the rescue assay for validating whether miR-202-5p exerted the physiological function in OC by regulating HOXB2. We co-transfected with HOXB2 over-expression plasmid. As Figure 5A exhibited, the HOXB2 expression level in miR-202-5p+HOXB2 group was increased when compared with mimics+NC group. In the rescue assay, HOXB2 over-expression cancelled the promotion effect on cell proliferation caused by miR-202-5p over-expression (Figure 5B). Consistently, similar results emerged in the transwell assay (Figure 5C). Taken together, all the findings mentioned above suggested that miR-202-5p functioned as a tumor suppressor in OC by regulating HOXB2.
Discussion

Ovarian malignant tumors have diversity and heterogeneity, and the incidence rate ranks third in gynecological malignant tumors, but the mortality rate ranks first\(^1\). Although new chemotherapy drugs continue to emerge and targeted therapy has made new progress, the 5-year survival rate of ovarian cancer is still low. It has been determined that the poor prognosis of epithelial ovarian cancer is due to the invasion and migration of tumor cells\(^9\). Therefore, it is important to explore its pathogenesis and find effective therapeutic targets.

From the aspect of tumor cell invasion and metastasis, miRNA plays an important role as a tumor suppressor gene or oncogene\(^10,11\). Researches on miRNAs are essential for biomarkers and molecular therapeutic targets\(^12\). Abnormal expres-
Figure 5. MiR-202-5p functioned as a tumor suppressor in OC through regulating HOXB2. A, HOXB2 expression level in miR-202-5p+HOXB2 group was increased when compared with mimics+NC group. B, HOXB2 over-expression cancelled the effect of miR-202-5p up-regulation on cell proliferation. C, HOXB2 over-expression cancelled the effect of miR-202-5p up-regulation on cell migration (magnification: 40×). Data are presented as the mean ± SD of three independent experiments. *p<0.05.
sion of microRNA is related to the occurrence and development of tumors. In the work of non-small cell lung cancer, Kumar et al. detected that the expression of miRNAs in let-7 family was significantly decreased after K-RAS gene down-expressed, which led to the occurrence and development of lung cancer. Martello et al. showed that miR-107 over-expression was positively correlated with poor prognosis in breast cancer.

EMT plays a vital role in the metastasis of ovarian cancer. Therefore, the development of gene therapy on the inhibition of EMT is helpful for suppressing the metastasis of ovarian cancer. To clarify whether miR-202-5p repressed cell migration and invasion in vitro via regulating EMT process, hallmarks in EMT were examined by Western blotting assay. The results indicated that miR-202-5p inhibited the EMT process, thereby inhibiting cell migration and invasion in vitro.

Conclusions

We verified that miR-202-5p suppressed cell proliferation, migration, and invasion in OC via regulating HOXB2 and suppressing EMT. Our findings provide new insights into the underlying mechanism of OC progression and may be useful in finding biomarkers and therapeutic targets of OC.

Conflict of Interests

The Authors declared that they have no conflict of interests.

References