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MiR-574-3p exerts as a tumor suppressor in ovarian cancer through inhibiting MMP3 expression

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Abstract. – OBJECTIVE: The mortality rate of ovarian cancer (OC) has always been the highest among all female reproductive system malignant tumors. Currently, miRNAs have been verified to participate in the tumorigenesis and prognosis of OC. However, the expression and function of miR-574-3p in OC have not been fully elucidated.

PATIENTS AND METHODS: The expression level of miR-574-3p in OC tissues and cells was detected using quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). By transfection of miR-574-3p mimics or inhibitor, the expression of miR-574-3p in SW626 or A2780 cells was up-regulated or down-regulated, respectively. Cell counting kit-8 (CCK-8) and colony formation assays were used to measure the proliferation of transfected OC cells. Meanwhile, the transwell assay was applied to detect the migration and invasion abilities of OC cells. Furthermore, dual-luciferase reporter gene analysis and Western blot were utilized to explore the underlying downstream molecules for miR-574-3p in OC.

RESULTS: MiR-574-3p was lowly expressed in OC tissue samples when compared with para-tumor tissues. Meanwhile, the expression of miR-574-3p in OC-derived cells was significantly lower than normal control HOSE cells. The overexpression of miR-574-3p markedly reduced the proliferation, invasion, and migration of SW626 cells. However, the inhibition of miR-574-3p remarkably accelerated the growth and metastasis of A2780 cells. MMP3 was verified as a direct target for miR-574-3p in OC. In addition, miR-574-3p could reduce the protein expression of MMP3 by binding to its 3'-untranslated region (3'-UTR).

CONCLUSIONS: MiR-574-3p functioned as a tumor suppressor in OC, which might be served as a potential target for the diagnosis and therapy for OC.

Key Words:
MiRNA, MiR-574-3p, Suppressor, Ovarian cancer (OC), MMP3.

Introduction

Ovarian cancer (OC) is one of the most common gynecological malignancies in women. Its anatomical location is special and deep in the pelvic cavity. The pathogenesis of OC is still not fully understood, whose 5-year survival rate is only 40%-45%[1]. Due to no evident symptoms in the early stage, there is a lack of specific and sensitive diagnostic methods for OC in clinical practice. Meanwhile, more than 70% of patients have already been in the advanced stage when diagnosed[2]. Therefore, it is of great importance to explore the underlying mechanism of OC tumorigenesis and progression.

In human cancer, microRNAs (miRNAs) function as oncogenes or tumor suppressor genes to regulate the development of malignancies according to the roles of their target genes[3]. MiRNA binds to the 3'-untranslated region (3'-UTR) of target genes, induces mRNA degradation, and inhibits protein translation[4,5]. For instance, miRNA-101-5p inhibits cell growth and aggressiveness in non-small cell lung cancer by targeting CXCL6. In clear cell renal cell carcinoma, miR-122 promotes cell proliferation and invasion by suppressing Forkhead box O3[6,7]. Meanwhile, miR-1249 targets VEGFA and HMGA2 to induce tumor growth, angiogenesis, and metastasis. Furthermore, miR-26a and miR-144 reduce

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the proliferation and metastasis of esophageal squamous cell carcinoma (ESCC) by inhibiting cyclooxygenase-2[8,9].

MiR-574-3p has been verified as a tumor suppressor in several cancers. In prostate cancer, it can be up-regulated by Genistein, thereby inhibiting tumor development[10]. In bladder cancer, miR-574-3p inhibits the expression of mesoderm development candidate 1 (MESDC1) to reduce cell proliferation and metastasis[11]. In gastric cancer and ESCC, miR-574-3p functions as a predictor of postoperative outcome, showing evident biological significance[12,13]. In addition, the up-regulation of miR-574-3p suppresses the growth and induces the apoptosis of chronic myeloid leukemia cells *via* targeting IL6/JAK/STAT3 pathway[14]. However, the expression and function of miR-574-3p in OC still needs to be further elucidated.

Here, 64 paired OC tissues and adjacent para-tumor tissues were first collected. The relative expression of miR-574-3p in paired tissues was detected using quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The result showed that miR-574-3p was lowly expressed in OC tissues and cell lines. Meanwhile, we established that miR-574-3p-overexpressed SW626 cells and miR-574-3p-inhibited A2780 cells by the transfection of miR-574-3p mimics and inhibitor, relatively. Subsequent functional experiments were employed to detect the influence of miR-574-3p in OC *in vitro*. Furthermore, MMP3 was confirmed as a potential target for miR-574-3p in OC. Taken all together, we demonstrated miR-574-3p expression and function in OC for the first time. Our findings might propose a new sight for OC biological treatment.

Patients and Methods

Patients

64 paired OC tissues and adjacent para-tumor tissues were collected from OC patients who received treatment in The First Affiliated Hospital of Soochow University from August 2013 to October 2016. The average age of patients was 49.48 ± 6.24 years old. All tissue samples were examined and confirmed by two pathologists. This study was approved by the Ethics Committee of The First Affiliated Hospital of Soochow University. Each collected sample was immediately stored in -80°C liquid nitrogen after extraction.

Cells and Culture

OC-derived cell lines (A2780, OVCA433, SKOV3, CAOV3, SW626) and normal human ovarian surface epithelial cell line (HOSE) were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), and maintained in a 37°C, 5% CO_2 incubator.

Cell Transfection

SW626 and A270 cells were first randomly divided into 2 groups. Then, the cells were transfected with miR-574-3p mimics (Mimics), miR-574-3p negative control (NC), miR-574-3p inhibitor (Inhibitor), and miR-574-3p inhibitor negative control (Inhibitor NC), respectively. Cells in logarithmic growth phase were seeded into 96-well plates at a density of 5×10^5. Then, the cells were incubated in a 37°C, 5% CO_2 incubator to approximately 60% of cell fusion. Subsequently, the cell transfection was performed according to the instructions of Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). Transfection efficiency was verified by qRT-PCR.

RNA Isolation and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The total RNA in OC tissues and cells was extracted in strict accordance with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Subsequently, extracted RNA was reverse transcribed into complementary deoxyribose nucleic acid (cDNA), followed by amplification by PCR. The miR-574-3p primer was purchased from GenePharma (Shanghai, China). Amplification conditions were as follows: 93°C 30 s, 64°C 30 s, 73°C 40 s, for a total of 34 cycles, and 72°C 8 min. Experimental results were analyzed and compared using the fluorescence quantitative operating system (ABI 7500, Applied Biosystems, Foster City, CA, USA). The mRNA expression of miR-574-3p was quantified by the $2^{-\Delta\Delta Ct}$ method. Primer sequences used in this study were as follows: miR-574-3p, F: 5'-GAGGGACAGGCCTCCTTACTC-3', R: 5'-GCTTGTGTCCGAAGGAACGGCC-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'.

Cell Counting Kit-8 (CCK-8) Assay

Cells in the logarithmic growth phase were first collected and adjusted to the cell suspension. Then, the cells were added to 96-well plates at a density of 2×10^5 cells per well. After that, the cells were incubated at 37°C, 5% CO_2 incubator for 1 d, 2 d, 3 d, and 4 d, respectively. Next, the cells in each stage were taken and added with 10 μL of CCK-8 solution (Donjindo, Kumamoto, Japan), followed by incubation at 37°C for 2 h in the dark. Finally, absorbance at 470 nm was measured by a microplate reader.

Colony Formation Assay

The transfected cells in logarithmic growth phase were first blunted into individual cells. 200 cells/well were inoculated into 6-cm culture dishes containing complete culture medium. After 2 weeks of culture and the cell cluster visible in control group reached 50 cells, the cells were fixed with pure methanol and stained with crystal violet. Finally, the number of formed colonies with 50 cells or more was counted under a microscope.

Wound-Healing Assay

Cells in each group were collected and inoculated into 6-well plates at a density of 3×10^5 cell/L. After the cells were fused to 80%-90%, a 200 μL tip was scratched along the vertical direction of the dish. After that, the cells were washed 3 times with phosphate-buffered saline (PBS), followed by the addition of serum-free medium. The plate was cultured in an incubator for 24 h at 37°C containing 5% CO_2. Cell migration trajectory was observed, and the image data of cells were recorded and counted.

Transwell Assay

For invasion assay, 50 μL of diluted Matrigel (BD, Franklin Lakes, NJ, USA) was first added to the upper chamber of a transwell chamber (Millipore, Billerica, MA, USA) and incubated for 1 h to allow coagulation. 24 h after transfection, the cells were cultured with serum-free medium for 24 h. Then, they were trypsinized to prepare cell suspension at a density of 2×10^8/L. Subsequently, 100 μL of cell suspension was added to the upper chamber. Meanwhile, 500 μL of RPMI-1640 medium containing 10% FBS were added into the lower chamber, followed by incubation for 36 h in a 5% CO_2, 37°C incubator. A cotton swab was then used to erase non-invasive cells in the upper chamber. The cells were fixed with 5 mL of methanol for 20 min and stained with 0.5% crystal violet for 10 min. The number of cells on the chamber surface was observed and counted under a microscope. The experiment was repeated for 3 times.

For the migration assay, the transwell chamber was coated with nothing, and other steps were the same with invasion assay.

Luciferase Reporter Gene Assay

The 3'-UTR sequence of the MMP3 gene and its mutated 3'-UTR sequence were cloned into a p-GL3.0 plasmid. Subsequently, dual-luciferase reporter vector, wild type MMP3 3'-UTR (wild type), and mutant MMP3 3-'UTR (mutant) were constructed. Then, constructed vectors were co-transfected with miR-574-3p mimics into SW626 cells, followed by culture at 37°C, 5% CO_2 for 48 h. Finally, fluorescence value was detected by Dual Luciferase Assay (Promega, Madison, WI, USA). The experiment was repeated for 3 times.

Protein Extraction and Western Blot

After transfection for 24 h, SW626 and A2780 cells in each group were collected and lysed with radioimmunoprecipitation assay (RIPA) reagent (Beyotime, Shanghai, China). The total protein was then extracted, and the concentration of protein samples was determined. Subsequently, extracted protein samples were separated by 8% Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking in 5% skim milk for 1 h at room temperature, the membranes were immersed with primary antibodies of MMP3 or GAPDH (CST, Danvers, MA, USA) at 4°C overnight. On the next day, the membranes were incubated with horseradish peroxidase (HRP)-labeled secondary antibody at room temperature for 1 h. Finally, immune-reactive bands were visualized by enhanced chemiluminescence (ECL) kit (Invitrogen, Carlsbad, CA, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 15.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Experimental results were expressed as mean ± standard deviation (mean ± SD). The Bonferroni corrected *t*-test was used to compare the difference between the two groups. $p<0.05$ was considered statistically significant.

Results

MiR-574-3p Was Lowly Expressed in OC Tissues and Cells

We first detected the expression of miR-574-3p in 64 pairs of OC tissues and adjacent para-tumor tissues using qRT-PCR. As shown in Figure 1A, the expression of miR-574-3p in OC tissues was markedly lower than that of para-tumor tissues. Similarly, the expression level of miR-574-3p in OC-derived cell lines (A2780, OVCA433, SKOV3, CAOV3, SW626) was significantly lower than normal human ovarian surface epithelial (HOSE) cells (Figure 1B). To further study the influence of miR-574-3p in OC *in vitro*, we up-regulated and down-regulated miR-574-3p in SW626 cells and A2780 cells by transfecting miR-574-3p mimics and miR-574-3p inhibitor, respectively. MiR-574-3p was significantly over-expressed in SW626 cells transfected with miR-574-3p mimics, while reduced in A2780 cells transfected with miR-574-3p inhibitor (Figure 1C, 1D).

MiR-574-3p Influenced the Proliferation of OC Cells

To verify the effects of miR-574-3p on the proliferation of OC cells, colony formation assay and CCK8 assay were performed. The transfection of miR-574-3p mimics in SW626 cells significantly reduced the number of formed colonies when compared with NC group (Figure 2A). However, the number of formed colonies increased significantly in A2780 cells after miR-574-3p inhibition (Figure 2B). At the same time, the CCK8 assay

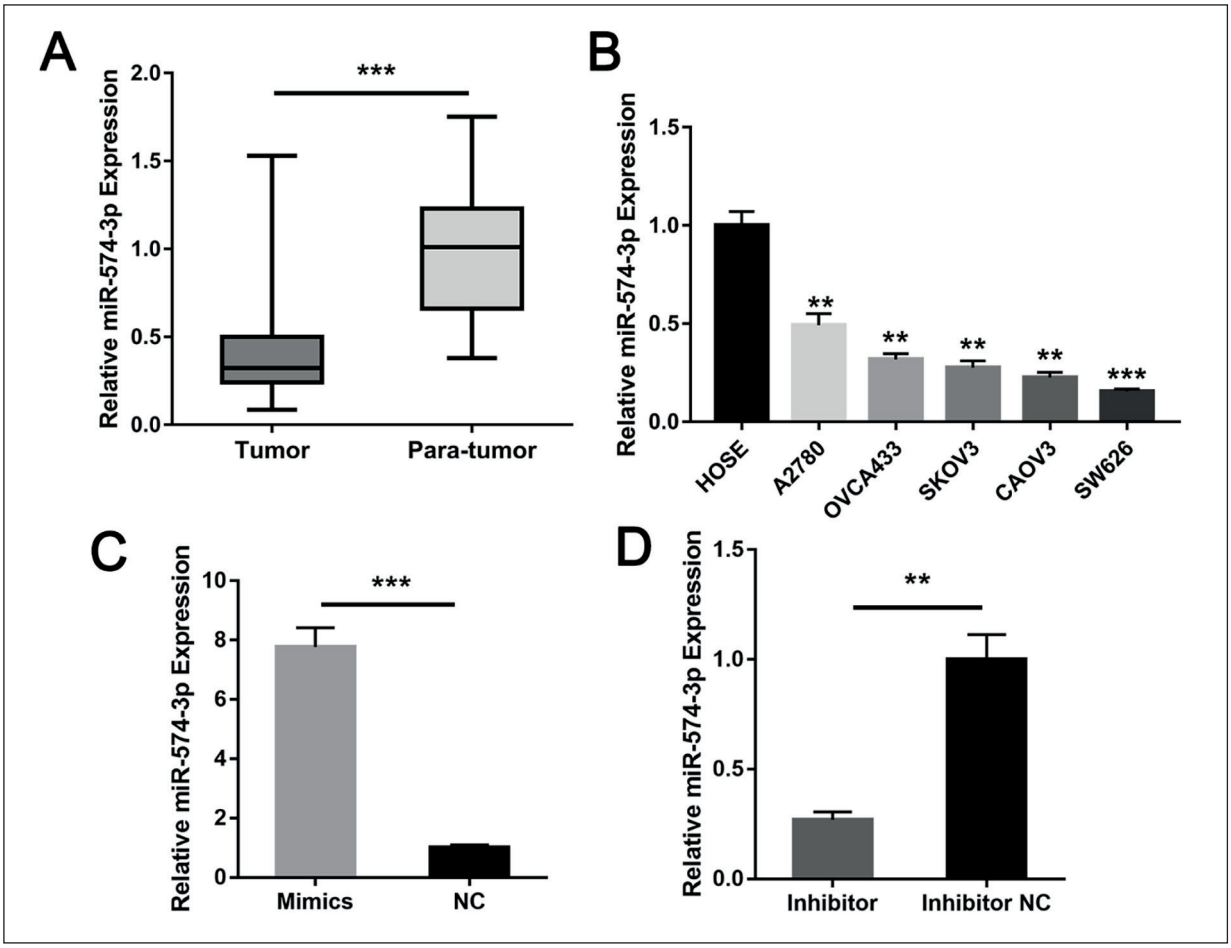

Figure 1. MiR-574-3p was lowly expressed in OC tissues and cell lines. ***A,*** The expression level of miR-574-3p in 64 paired OC tissues and adjacent para-tumor tissues. ***B,*** MiR-574-3p expression level in OC cell lines (SW626, OVCA433, SKOV3, CAOV3, A2780) and normal human ovarian surface epithelial line (HOSE). ***C,*** Expression of miR-574-3p in miR-574-3p mimics treated SW626 cells. ***D,*** Expression of miR-574-3p in miR-574-3p inhibitor-treated A2780 cells. *p<0.05, **p<0.01, ***p<0.001.

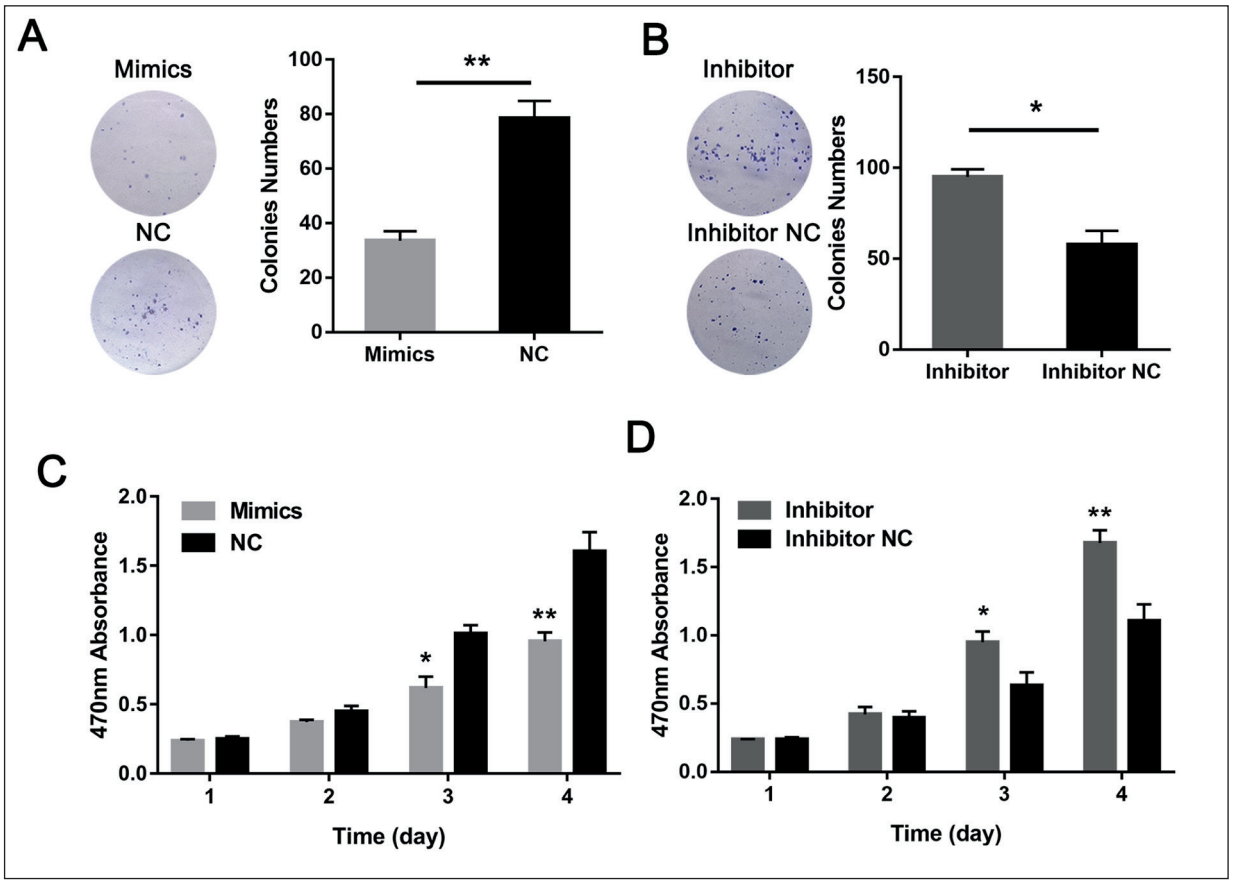

Figure 2. MiR-574-3p inhibited the proliferation of OC cells. ***A, B,*** Colony formation assay was performed to determine the growth of SW626 (***A***) or A2780 (***B***) cells transfected with mimics or inhibitor, respectively (magnification: 10×). ***C, D,*** CCK8 assay was performed to determine the proliferation of SW626 (***C***) or A2780 (***D***) cells treated with miR-574-3p mimics or inhibitors compared with negative control cells. *p<0.05, **p<0.01, ***p<0.001.

indicated that up-regulation of miR-574-3p remarkably decreased the growth of SW626 cells. However, the inhibition of miR-574-3p significantly increased the proliferation of A2780 cells when compared with control group. These results indicated that miR-574-3p could inhibit the proliferation of OC cells.

MiR-574-3p Affected the Invasion and Migration of OC Cells

Cell invasion and migration abilities were then detected by transwell assay. After miR-574-3p mimics transfection, the invasion of SW626 cells was markedly reduced when compared with negative control group (Figure 3A, 3B). However, A2780 cells showed significantly increased cell invasion capacity after transfection of miR-574-3p inhibitor (Figure 3A, 3C). Likewise, cell migration ability was markedly reduced by the miR-574-3p up-regulation while promoted by the miR-574-3p inhibition (Figure 3D, 3E, 3F). These data suggested that the invasion and migration of OC cells were inhibited by miR-574-3p.

MMP3 Was Identified as a Direct Target for MiR-574-3p in OC

Since we found that miR-574-3p inhibited OC cell proliferation and metastasis, we further explored the possible underlying mechanism. Multiple studies have reported that miRNAs exert their effects by binding to the 3-'UTR of target genes, thereby inhibiting protein expression. Subsequently, we searched several databases, including miRBase, TargetScan, PiTar, and miRWalk, according to the mechanism. Results showed that MMP3 was a potential target for miR-574-3p in OC. The binding site

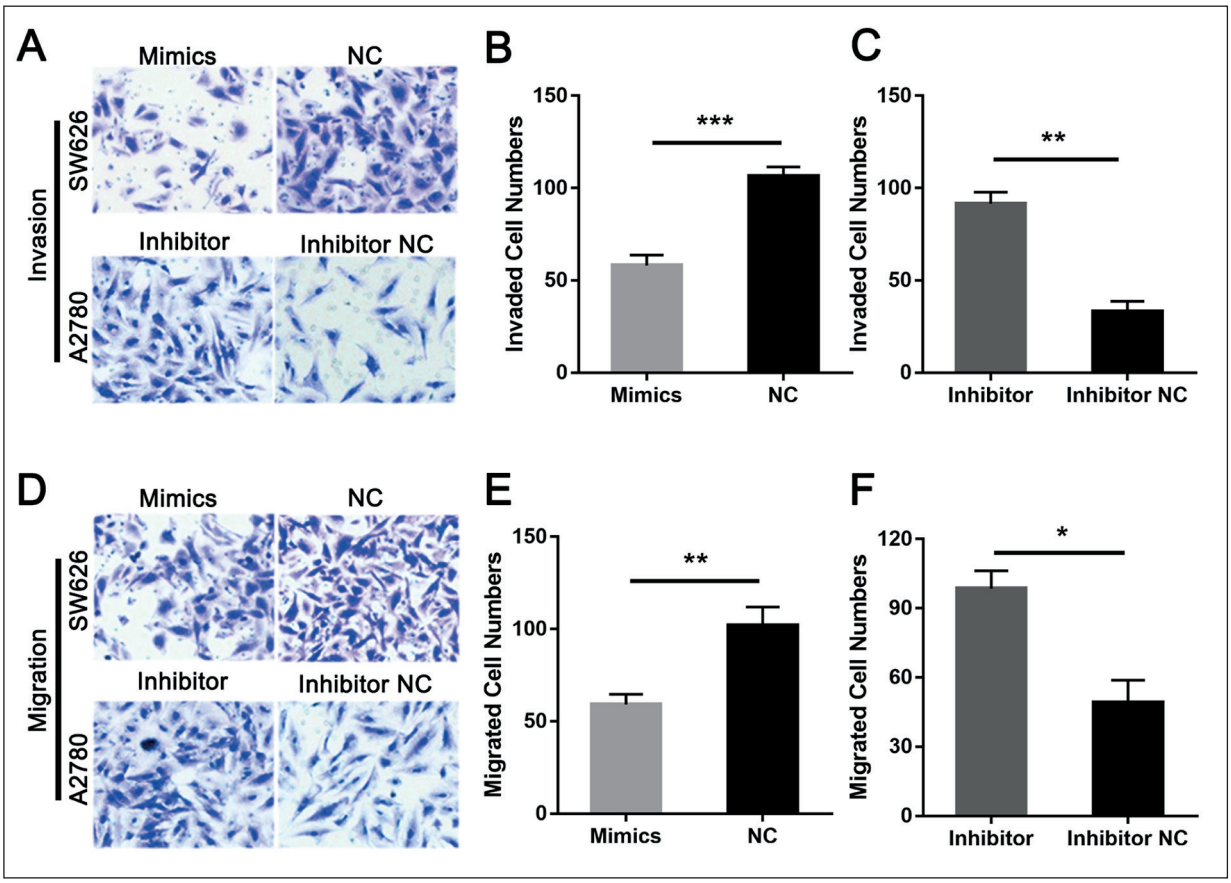

Figure 3. MiR-574-3p affected the invasion and migration of OC cells. ***A, B, C,*** Transwell invasion assay was used to detect the invasion ability of miR-574-3p mimics treated SW626 cells or miR-574-3p inhibitors treated A2780 cells (magnification: 40×). ***D, E, F,*** Transwell migration assay was used to detect the migration and invasion abilities of miR-574-3p mimics treated SW626 cells or miR-574-3p inhibitor-treated A2780 cells. Data were presented as mean ± SD of three independent experiments (magnification: 40×). *p<0.05, **p<0.01.

of MMP3 was shown in Figure 4A. To verify our assumption, we designed dual-luciferase assay using plasmid vector carrying wild-type or mutant 3'-UTR of MMP3. Luciferase activity decreased significantly in wild type group. However, no evident difference was observed in mutant group when compared with control group. These results indicated that miR-574-3p could directly bind to the 3'-UTR of MMP3 (Figure 4B). Next, we measured the protein level of MMP3 in transfected SW626 and A2780 cells. After the miR-574-3p overexpression, the protein level of MMP3 in SW626 cells decreased significantly. However, after the miR-574-3p inhibition, the protein level of MMP3 in A2780 cells was significantly up-regulated (Figure 4C, 4D, 4E). These experiments suggested that MMP3 was a direct target for miR-574-3p in OC.

The Proliferation and Metastasis of OC Cells Was Inhibited by MiR-574-3p Via Inhibiting MMP3 Expression

MMP3 was identified as a direct target for miR-574-3p. Furthermore, we restored the MMP3 expression to verify the findings. Using pcDNA for MMP3, we overexpressed MMP3 in miR-574-3p overexpressed SW626 cells. The protein level of MMP3 in SW626 cells inhibited by miR-574-3p mimics was markedly restored by MMP3 up-regulation (Figure 5A, 5B). Next, CCK8 assay showed that the proliferation ability of OC cells decreased by miR-574-3p overexpression was rescued by the MMP3 restoration (Figure 5C). Correspondingly, the cell invasion ability increased significantly after MMP3 overexpression co-treated with miR-574-3p up-regulation when compared with miR-574-3p mimics treatment individually (Figure

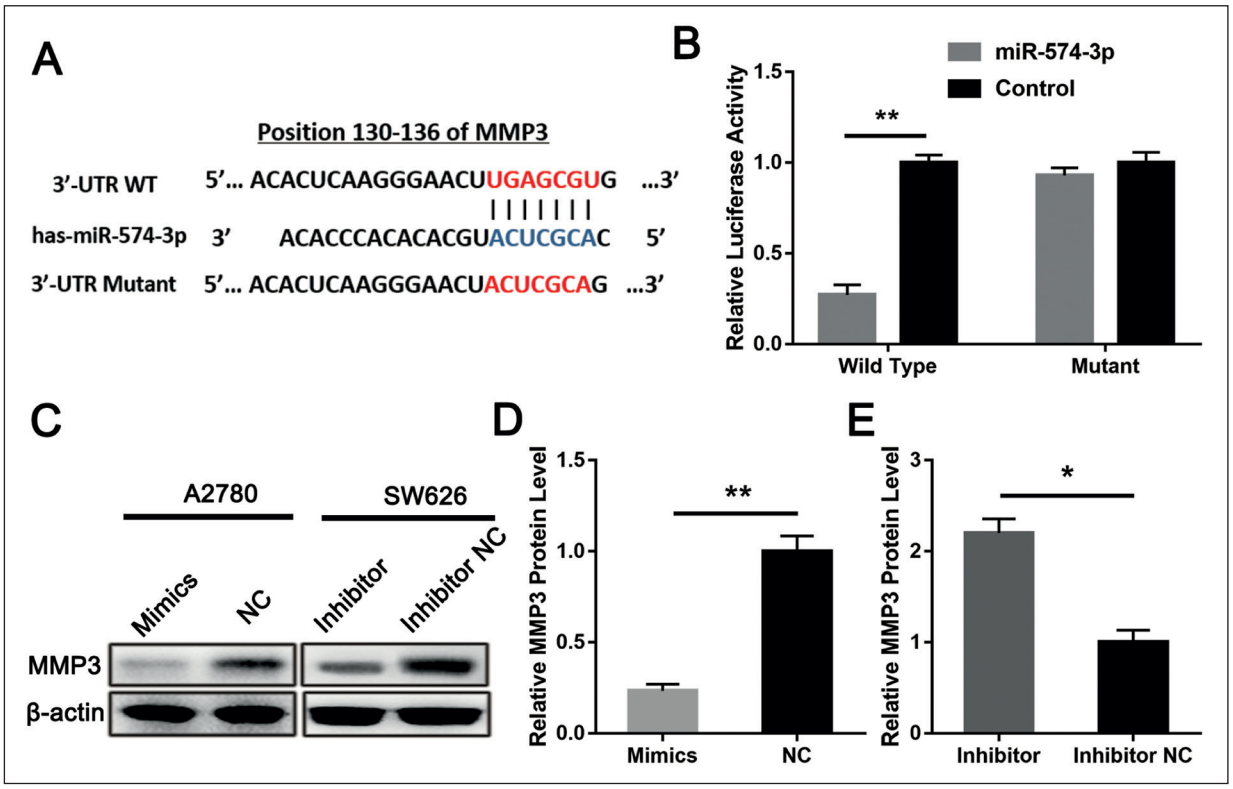

Figure 4. MMP3 was a direct target of miR-574-3p. ***A,*** Predicted binding sites of miR-574-3p in the 3'-UTR of MMP3. ***B,*** Dual-luciferase reporter assay was used to determine the binding site. ***C, D, E,*** Protein levels of MMP3 and GAPDH in miR-574-3p overexpression SW626 cells and miR-574-3p knockdown A2780 cells were measured by Western blot. The relative protein level of MMP3 compared to GAPHD. Data were presented as mean ± SD of three independent experiments. **p*<0.05, ***p*<0.01.

5D). These results indicated that miR-574-3p inhibited cell proliferation and metastasis *via* inhibiting MMP3 expression.

Discussion

OC is one of the most common malignant tumors in women, which is also a gynecological tumor with the highest mortality rate. Due to the concealment of clinical symptoms, most OC patients are diagnosed in the late stage with a poor prognosis[15,16]. According to different origins, OC can be divided into three types, including: epithelial tumor, sexual cord stromal tumor, and malignant germ cell tumor. Clinically, epithelial ovarian cancer is common. In recent years, clinical treatments, including surgery, chemotherapy, radiotherapy, and biotherapy, are evolving rapidly. However, the 5-year survival rate of OC remains 40%-50%[17,18].

The occurrence of OC is closely related to gene expression. Recently, miRNA is one of the research hotspots, which provides a new way for the development and progression of malignant tumors. MiRNA is a non-coding RNA with approximately 22 nt in length. It can regulate the gene expression by inhibiting the function of target genes or degrading target genes at post-transcriptional level[19]. Andreou et al[20] have shown that miRNAs are involved in a variety of complex biological processes, such as embryonic development, organ formation, tumorigenesis, and metastasis. Several miRNAs have been identified to affect OC development and progression. For example, miR-106a regulates the proliferation and differentiation of high-grade OC through repressing Rb tumor suppressor p130. MiR-30c down-regulates oncogene BCL9 and suppresses growth factor-induced proliferation. Meanwhile, miR-200c promotes the metastasis and epithelial-mesenchymal transition (EMT) of OC, while miR-137 and miR-34a inhibit EMT *via* targeting Snail. In addition, miR-145 and miR-135a exert tumor-suppressing functions through repressing TRIM2 and HOXA10 expression, respective-

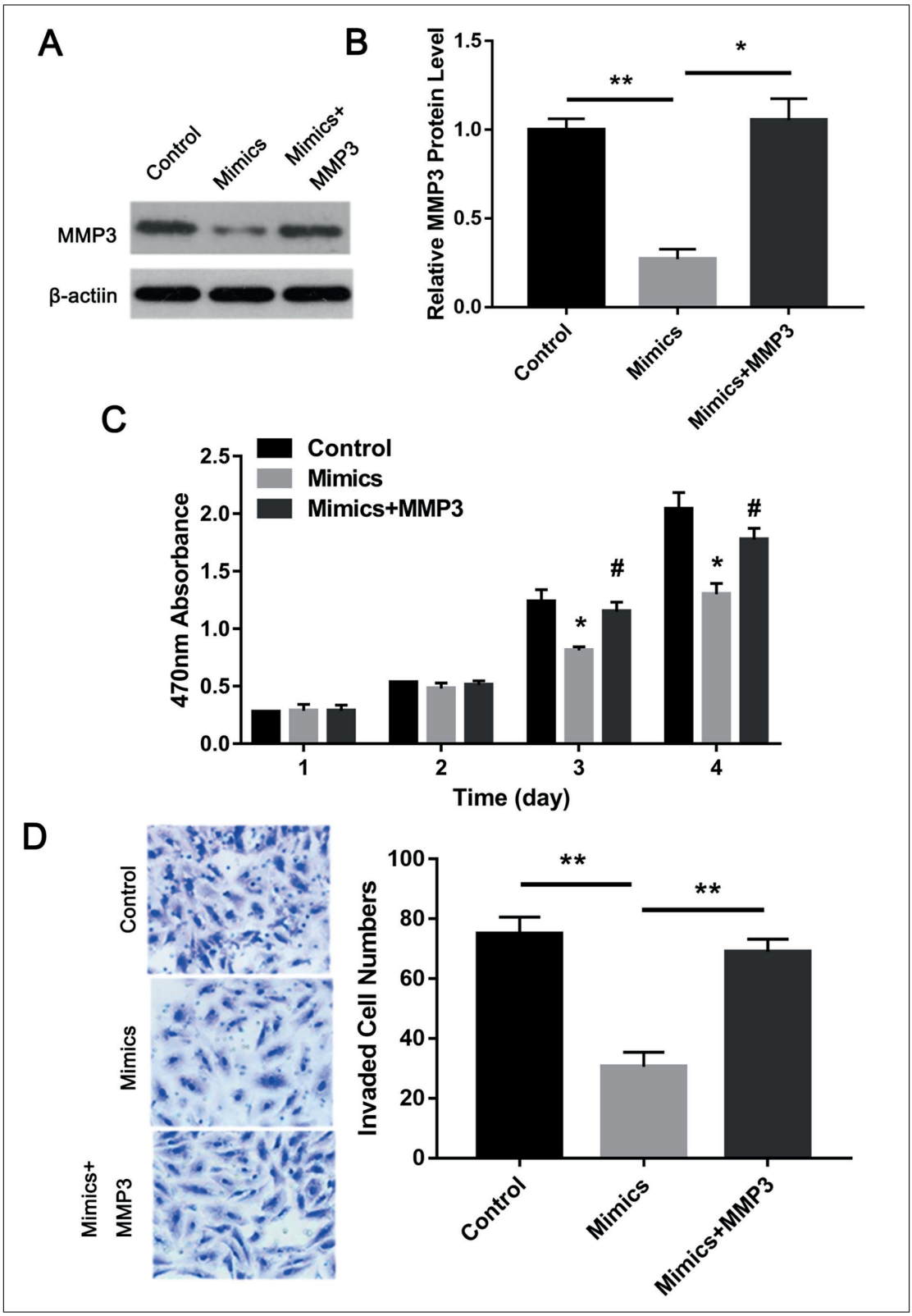

Figure 5. MMP3 rescued the effects of miR-574-3p mimics in SW626 cells. ***A, B,*** Western-blot analysis of MMP3. GAPDH was used as an internal control. ***C,*** Cell proliferation ability was detected by CCK8 assay in control, mimics, or mimics+MMP3 treated SW626 cells; ***D,*** Cell invasion ability was measured by transwell assay (magnification: 40×). Data were represented as mean ± SD of three replicates. *p<0.05, **p<0.01.

ly[21-25]. However, the exact role of miR-574-3p in OC has not been fully elucidated.

In our study, we demonstrated that miR-574-3p was lowly expressed in OC tissues compared with para-tumor tissues. Meanwhile, the miR-574-3p level was significantly down-regulated in OC cell lines. These results showed that miR-574-3p served as a tumor suppressor in OC. Next, we established miR-574-3p ectopic expressed cells using SW626 and A2780 cells, and conducted a series of functional experiments *in vitro*. Colony formation assay and CCK8 assay showed that miR-574-3p significantly inhibited the proliferation of OC cells. The transwell assay elucidated that miR-574-3p overexpression significantly reduced the invasion and migration of OC cells. These results revealed miR-574-3p could suppress OC tumorigenesis and metastasis *in vitro*.

Furthermore, we confirmed that MMP3 was a direct target for miR-574-3p in OC. As a member of the matrix metalloproteinases (MMPs) family, MMP3 is involved in a variety of biological processes in tumors, including cell growth, differentiation, apoptosis, migration, and invasion[26-28]. MMP3 expression was significantly elevated in OC tissues and cells. Meanwhile, it could significantly promote the progression of the growth and metastasis of OC. Previous studies[29-31] have indicated that it can change the state of the extracellular matrix, thereby promoting the transfer of OC cells. Studies[32] have also shown that MMP3 has the same potential as a tumor diagnostic indicator for CA125. Here, we found that miR-574-3p could directly reduce the expression of MMP3, eventually inhibiting the proliferation and metastasis of OC cells. Furthermore, restoration of MMP3 partially rescued the influence of miR-574-3p in OC, indicating the function of miR-574-3p/MMP axis. However, further *in vivo* studies are still needed to explore the role of miR-574-3p in OC.

Conclusions

We detected that miR-574-3p acted as a tumor suppressor in OC *via* inhibiting the expression of MMP3. Our findings might provide a new potential site for biological diagnosis, prognosis, and biotherapy of OC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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