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MiR-127-3p inhibits proliferation of ovarian cancer in rats through down-regulating MAPK4

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Abstract. – OBJECTIVE: **To reveal the anti-tumor effect of micro ribonucleic acid (miR)-127-3p on epithelial ovarian cancer (EOC).**

PATIENTS AND METHODS: **The expression of miR-127-3p in 7 kinds of EOC cell lines and 10 cases of clinical samples of EOC patients was detected *via* quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). OVCAR-3 and Caov-3 cell lines were transfected with lentiviruses to overexpress endogenous miR-127-3p. Then, the anti-tumor effect of miR-127-3p on EOC cells was explored through the *in vitro* cell proliferation assay, bufalin sensitivity assay, wound healing assay, and invasion assay. In addition, whether the mitogen-activated protein kinase 4 (MAPK4) gene is a downstream target of miR-127-3p in EOC was verified *via* Dual-Luciferase reporter assay and qRT-PCR. The involvement of MAPK4 in regulating phenotypes of OVCAR-3 and Caov-3 cells was finally explored.**

RESULTS: **MiR-127-3p was downregulated in both EOC cell lines and EOC tissues (*p*<0.05). After lentivirus-mediated overexpression of miR-127-3p, in vitro proliferation and invasion of EOC cells were inhibited, and the sensitivity to bufalin was enhanced (*p*<0.05). MiR-127-3p directly regulated MAPK4 gene in EOC. Moreover, the upregulation of MAPK4 inhibited the anti-tumor effect of miR-127-3p on EOC, manifested as the remarkably enhanced cell proliferation and migration (*p*<0.05), and the weakened sensitivity to bufalin (*p*<0.01).**

CONCLUSIONS: **MiR-127-3p exerts an inhibitory effect on EOC cells *via* regulating MAPK4 level.**

Key Words:
MiR-127-3p, MAPK4, Epithelial ovarian cancer, Bufalin.

Introduction

Epithelial ovarian cancer (EOC) is one of the most malignant gynecological cancers in the world[1,2]. It is estimated that there are more than 20,000 new cases in the United States, and over 14,000 deaths of EOC[3]. Familial inheritance and genetic variations may play an important role in the occurrence of EOC[1]. Despite great efforts, the exact pathogenesis of EOC is still fundamentally unknown, and early diagnosis and novel therapies are lacked. Therefore, it is of great importance to explore the potential molecular mechanisms of occurrence, development, and metastasis of EOC, so as to find new therapeutic targets for EOC patients.

It has been observed that miRNAs play a crucial role in human cancers, and act as oncogenes or tumor-suppressor genes[4-7]. Differentially expressed miRNAs have been found in human EOC[8], indicating that the regulatory effects of miRNAs and related signaling pathways are complex in EOC.

MiR-127-3p, also known as miR-127, is often downregulated in human tumors and exerts an anti-tumor effect on breast cancer and gastric cancer[9,10]. By analyzing differentially expressed miRNAs in primary breast cancer tissues and normal adjacent tissues, miR-127-3p is found to be downregulated by more than twice in breast cancer tissues[11]. MiR-127-3p is also downregulated in clinical samples of EOC patients, besides breast cancer[12,13]. However, the functional mechanism of miR-127-3p has not been clarified yet in EOC. In the present study, the differential expression of miR-127-3p was evaluated in EOC cell lines and EOC samples. Then, it was hypothesized that miR-127-3p might possess an anti-tumor effect in EOC. OVCAR-3 and Caov-3 cell lines were transfected with lentiviruses to overexpress endogenous miR-127-3p. Moreover, the effects of miR-127-3p overexpression

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on proliferation, drug sensitivity (bufalin) and invasion of EOC were evaluated. It was also speculated that mitogen-activated protein kinase 4 (MAPK4) gene is a downstream target of miR-127-3p in EOC. Therefore, Dual-Luciferase reporter assay and quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) were adopted to verify our speculation. Next, the regulatory function of MAPK4 in EOC influenced by miR-127-3p was further studied.

Patients and Methods

Laboratory Materials

This study was approved by the Ethics Committee of Jinan City People's Hospital. Signed written informed consents were obtained from all participants before the study. According to international guidelines[14], normal ovarian epithelial tissues and paired ovarian cancer tissues were obtained from 10 EOC patients from April to October 2013. TaqMan reverse transcription kit from Biosystems (Foster City, CA, USA), RNeasy Mini kit from Qiagen (Hilden, Germany), TaqMan miRNA analysis kit from Biosystems (Foster City, CA, USA), mitomycin from Sigma-Aldrich (St. Louis, MO, USA), psiCHECK2 Luciferase vectors from Promega (Madison, WI, USA), Lipofectamine 2000 reagent from Thermo Fisher Scientific (Waltham, MA, USA), and miR-127-3p-mimic and control lentiviral transfection reagent from RiboBio Biotech (Guangzhou, China).

Objects of Study

EOC cell liens SKOV-3, OVCAR-3, Caov-3, ES-2, PA-1, MCAS, and OVCA432, and non-tumorigenic human ovarian cell line HS-832 were used in this study. The expression of miR-127-3p was upregulated in OVCAR-3 and Caov-3 cells, and the cells were divided into negative control (NC) group and mimic group. Besides, the peripheral blood samples were taken from 10 EOC patients and divided into Tumor group and Normal group. In Dual-Luciferase reporter assay, the cells were transfected with MAPK4-wild-type 3'-UTR (WT group) and MAPK4-mutant-type 3'-UTR (MUT group). Caov-3 cells were co-transfected with mimic + pcDNA/- as control group and mimic + pcDNA/MAPK4 as experimental group.

RNA Isolation and Purification, and Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

The total RNA was isolated from EOC cell lines or clinical samples of EOC patients using the RNeasy Mini kit. According to the manufacturer's instructions, 100 ng of RNA in each sample was synthesized into cDNA using the TaqMan reverse transcription kit, followed by qRT-PCR on the ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA, USA). The TaqMan miRNA analysis kit was used to detect miR-127-3p under the following reaction conditions: 95°C for 15 min, followed by 38 cycles at 95°C for 30 s and 62°C for 40 s, with U6 as an internal reference. Moreover, the SYBR Green PCR Master Mix kit (TaKaRa, Otsu, Shiga, Japan) was used to detect MAPK4 level under the following reaction conditions: 95°C for 15 min, followed by 38 cycles at 95°C for 30 s, 58°C for 40 s, and 70°C for 30 s, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as negative control. The relative gene expression was measured using the $2^{-\Delta\Delta Ct}$ method. The primer sequences were as follows: MiR-127-3p-F: 5'-TAGTTTGGAGTTAGGGGTAGGGTAT-3'; MiR-127-3p-R: 5'-AATAAATCAAAAAAAACACCTCCAC-3'. MAPK4-F: 5'-CCCAAGCTTATGGCTGAGAAGGGTGACTGCATC-3'; MAPK4-R: 5'-CCGCTCGAGTCACAGGGTACCAGCAAAGAGCATT-3'. U6-F: 5'-CTCGCTTCGGCAGCACA-3'; U6-R: 5'-AACGCTTCACGAATTTGCGT-3'. GAPDH-F: 5'-TGTTCGTCATGGGTGTGAAC-3'; GAPDH-R: 5'-ATGGCATGGACTGTGGTCAT-3'.

Cell Transfection with MiR-127-3p Mimic

To overexpress miR-127-3p in EOC cell lines, 5×10^5 OVCAR-3 or Caov-3 cells were transfected with L-miR-127-3p-mimic and infected with Polybrene (10 and 5 µg/mL) for 4 h, while those transfected with NC lentiviruses were considered as NC group. The cells were washed with phosphate-buffered saline (PBS) for 3 times and cultured for another 48 h. Finally, the cell precipitate was collected for subsequent experiments.

Cell Proliferation Assay

A total of 500 OVCAR-3 or Caov-3 cells were inoculated into a 96-well plate. Then, the growth of EOC cells was determined using CellTiter96®

Aqueous One cell proliferation assay according to the instructions. Briefly, 20 μL of proliferation solution was added into the cell culture for 1 h, and the absorbance was measured at 490 nm for 5 consecutive days. The changes in absorbance indicated the cell growth rate.

Bufalin Sensitivity Assay

OVCAR-3 or Caov-3 cells were incubated with bufalin at different concentrations (0, 0.1, 0.5, 1, 10, and 100 ng/mL) for 48 h. The sensitivity of EOC cells to bufalin was evaluated by relative cell viability determined *via* proliferation assay. Then, the absorbance was normalized to the control value (without bufalin treatment).

Cell Migration Assay

The cell migration ability was determined using wound healing assay as follows. OVCAR-3 and Caov-3 cells were inoculated into the 96-well plate (5000 cells/well). After fusion, the cells were incubated with mitomycin (10 μg/mL) for 2 h to terminate proliferation. Then, a defined area of wound was created using the 96-gauge needle, and the phase contrast images were captured at 0 h and 24 h. Finally, the changes in wound area were measured using Image J software at 0-24 h, so as to determine the cell migration ability.

Dual-Luciferase Reporter Assay

The human MAPK4-wild-type 3'-UTR and MAPK4-mutant-type 3'-UTR were cloned into the psiCHECK2 Luciferase vectors. The cells were inoculated into a 6-well plate and co-transfected with MAPK4-wild-type or MAPK4-mutant-type and NC or mimic lentiviruses. After co-transfection for 48 h, Dual-Luciferase reporter assay was performed according to the manufacturer's instructions.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA) was used for analyzing experimental data in this study. Experimental data were obtained from three replicates. Data were represented as mean ± SD (Standard Deviation). Differences between two groups were analyzed by the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test, followed by post-hoc test (Least Significant Difference). $p<0.05$ indicated the statistically significant difference.

Results

MiR-127-3p had a Significantly Low Expression in EOC Cells and Tissues

MiR-127-3p expression was detected *via* qRT-PCR in 7 kinds of EOC cells and non-tumorigenic HS-832 cells. The results revealed that miR-127-3p was significantly downregulated in 7 kinds of EOC cells compared with that in HS-832 cells ($p<0.05$). Besides, the expression of miR-127-3p was also determined *via* qRT-PCR in EOC tissues and adjacent normal ovarian epithelial tissues. It was found that miR-127-3p was downregulated in EOC tissues compared with that in normal tissues ($p<0.05$) (Figure 1).

Overexpression of miR-127-3p Inhibited EOC Growth and Enhanced Bufalin Sensitivity

OVCAR-3 and Caov-3 cells were transfected with miR-127-3p-mimic lentiviruses to overexpress endogenous miR-127-3p. Meanwhile, EOC cells transfected with NC lentiviruses were considered as NC group. After transfection, the expression of miR-127-3p was detected *via* qRT-

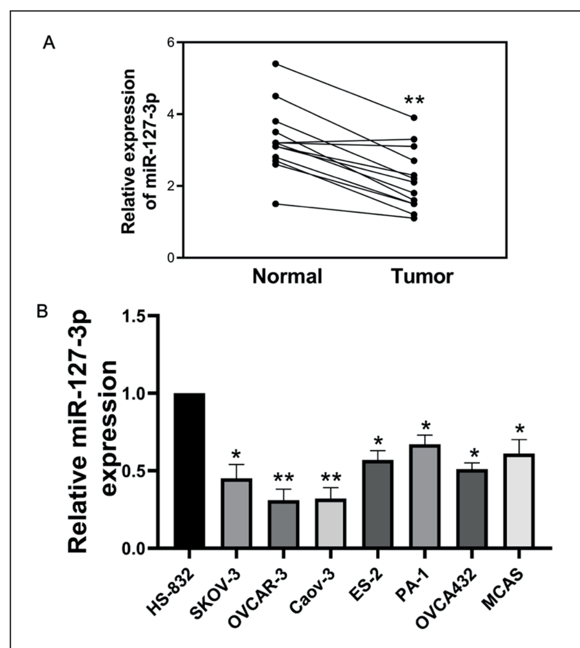

Figure 1. Expression of miR-127-3p in EOC cells and tissues detected *via* qRT-PCR. **A,** Expression of miR-127-3p in EOC tissues. The expression of miR-127-3p obviously declines in Tumor group compared with that in Normal group ($p<0.01$). **B,** Expression of miR-127-3p in EOC cells. The expression of miR-127-3p is obviously lower in EOC cells than that in HS-832 cells ($p<0.01$). ($^{*}p<0.05$, $^{**}p<0.01$).

PCR in EOC cells transfected with miR-127-3p-mimic or NC. The results showed that endogenous miR-127-3p was evidently upregulated in OVCAR-3 and Caov-3 cells transfected with miR-127-3p-mimic compared with that in cells transfected with NC ($p<0.01$). Then, the growth of EOC cells transfected with lentiviruses was compared. According to the results of proliferation assay, the growth of cancer cells was evidently inhibited by overexpression of miR-127-3p from 2 d after proliferation assay ($p<0.05$). Moreover, the effect of miR-127-3p on EOC sensitivity to bufalin was evaluated. It was confirmed that overexpression of miR-127-3p reduced viability of OVCAR-3 and Caov-3 cells, and evidently enhanced bufalin sensitivity ($p<0.05$) (Figure 2).

Overexpression of MiR-127-3p Suppressed EOC Cell Invasion

The effect of overexpressed miR-127-3p on EOC invasion was evaluated by wound healing assay in OVCAR-3 and Caov-3 cells transfected with lentiviruses. The results manifested that the cells transfected with miR-127-3p-mimic had a lower ability of wound healing than those tran-

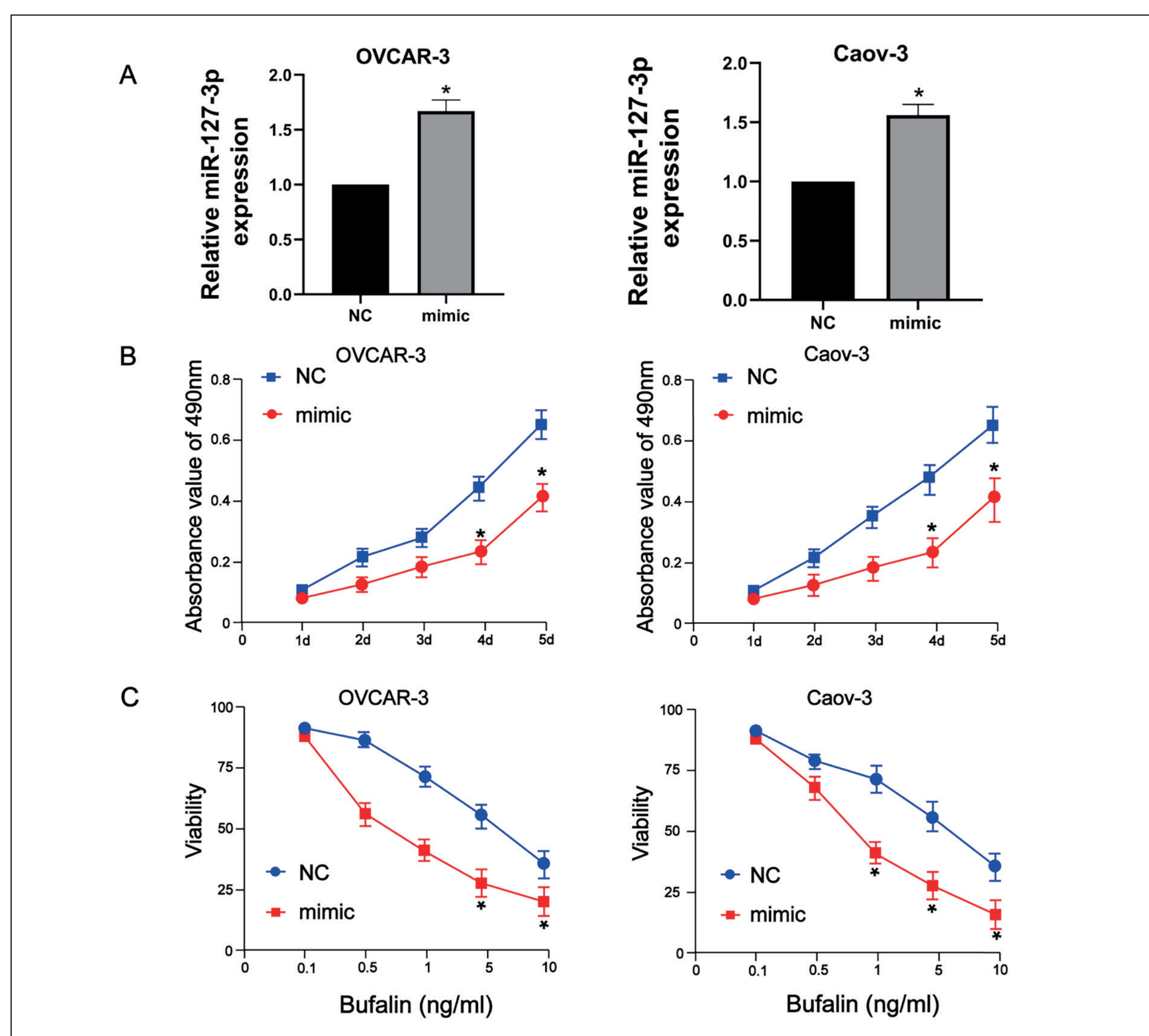

Figure 2. Effect of miR-127-3p on cell proliferation. **A,** OVCAR-3 and Caov-3 cells are transfected with miR-127-3p mimic lentiviruses (miR-127-3p-mimic) or control lentiviruses (NC), and it is found that the expression of miR-127-3p in mimic group is remarkably higher than that in NC group ($p<0.05$). **B,** Effect of miR-127-3p on cell proliferation. The cell proliferation rate in mimic group is remarkably lower than that in NC group ($p<0.05$). **C,** Effect of miR-127-3p on bufalin sensitivity. The cell viability in mimic group is lower than that in NC group ($p<0.05$). ($^*p<0.05$).

sfected with NC, indicating that overexpression of miR-127-3p markedly weaken the invasion ability of OVCAR-3 and Caov-3 cells ($p<0.05$) (Figure 3).

MAPK4 was Directly Related to MiR-127-3p Overexpression in EOC

The inhibitory effect of overexpressed miR-127-3p on EOC was proved as above, so the molecular target of miR-127-3p was explored. The target proteins of miR-127-3p were searched online in miRDB (www.mirdb.org) and TargetScan Human (www.targetscan.org), and MAPK4 was searched. The cells were co-transfected with MAPK4-wild-type 3'-UTR or MAPK4-mutant-type 3'-UTR, and NC or miR-127-3p-mimic lentiviruses, and the changes in the relative Luciferase activity were measured after co-transfection for 48 h. The results showed that the Luciferase activity in WT group was markedly lower than that in MUT group ($p<0.05$), confirming that MAPK4 is the target gene of miR-127-3p. In addition, the mRNA level of MAPK4 was determined in OVCAR-3 and Caov-3 cells overexpressing miR-127-3p. The results of qRT-PCR manifested that overexpression of miR-127-3p greatly inhibited the endogenous MAPK4 expression in EOC cells (Figure 4).

Upregulation of MAPK4 had the Opposite Effect to miR-127-3p Overexpression in EOC

MAPK4 was upregulated in Caov-3 cells. At 48 h after transfection, it was observed *via* fluorescence qRT-PCR that MAPK4 was markedly upregulated ($p<0.05$). Next, the changes in EOC cell proliferation and migration were analyzed after upregulation of MAPK4. It was proven that upregulation of MAPK4 restored the growth and migration ability of EOC cells overexpressing miR-127-3p ($p<0.05$). It can be seen that upregulation of MAPK4 has the op-

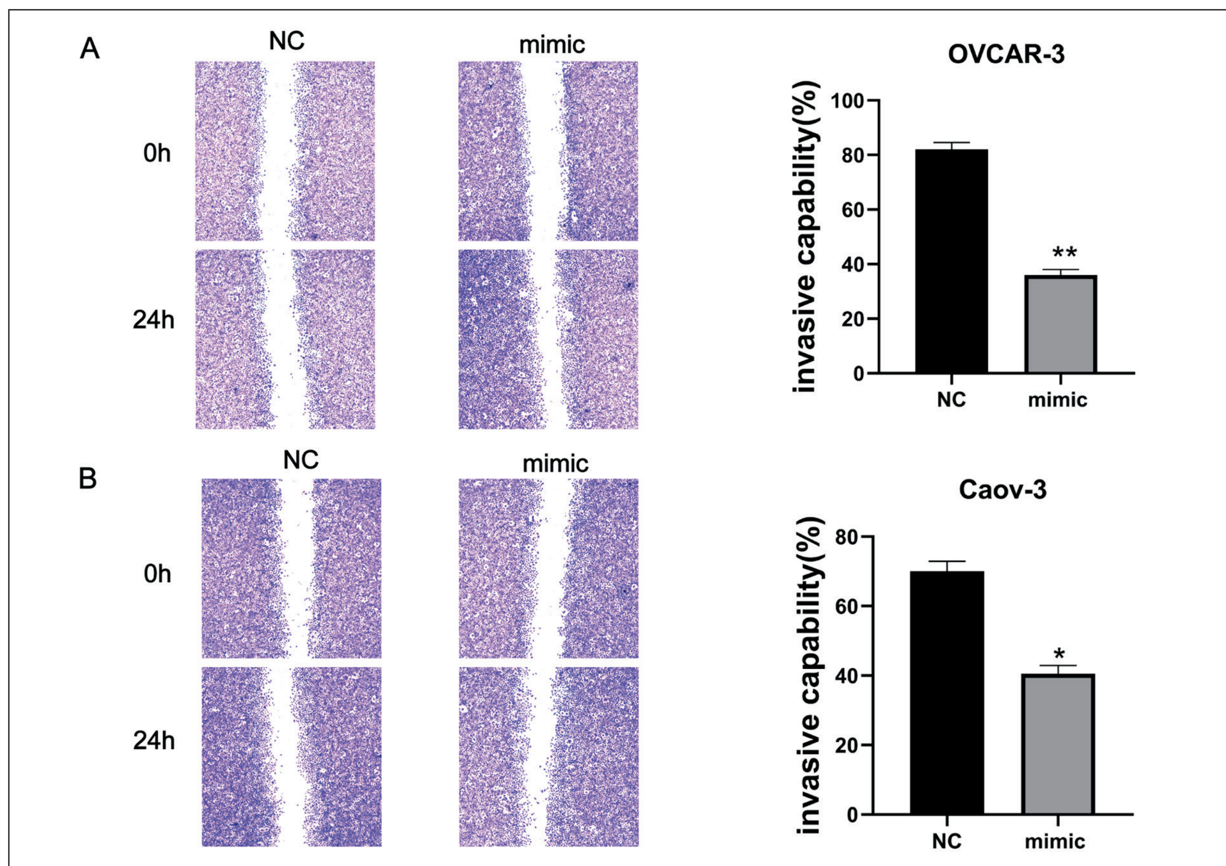

Figure 3. Effect of miR-127-3p on EOC cell migration. **A,** and **B,** Quantitative analysis of effect of overexpressed miR-127-3p on migration of OVCAR-3 and Caov-3 cells. The cells have a low ability of wound healing at 24 h in mimic group than those in NC group (magnification: 40×) ($p<0.05$). ($^*p<0.05$).

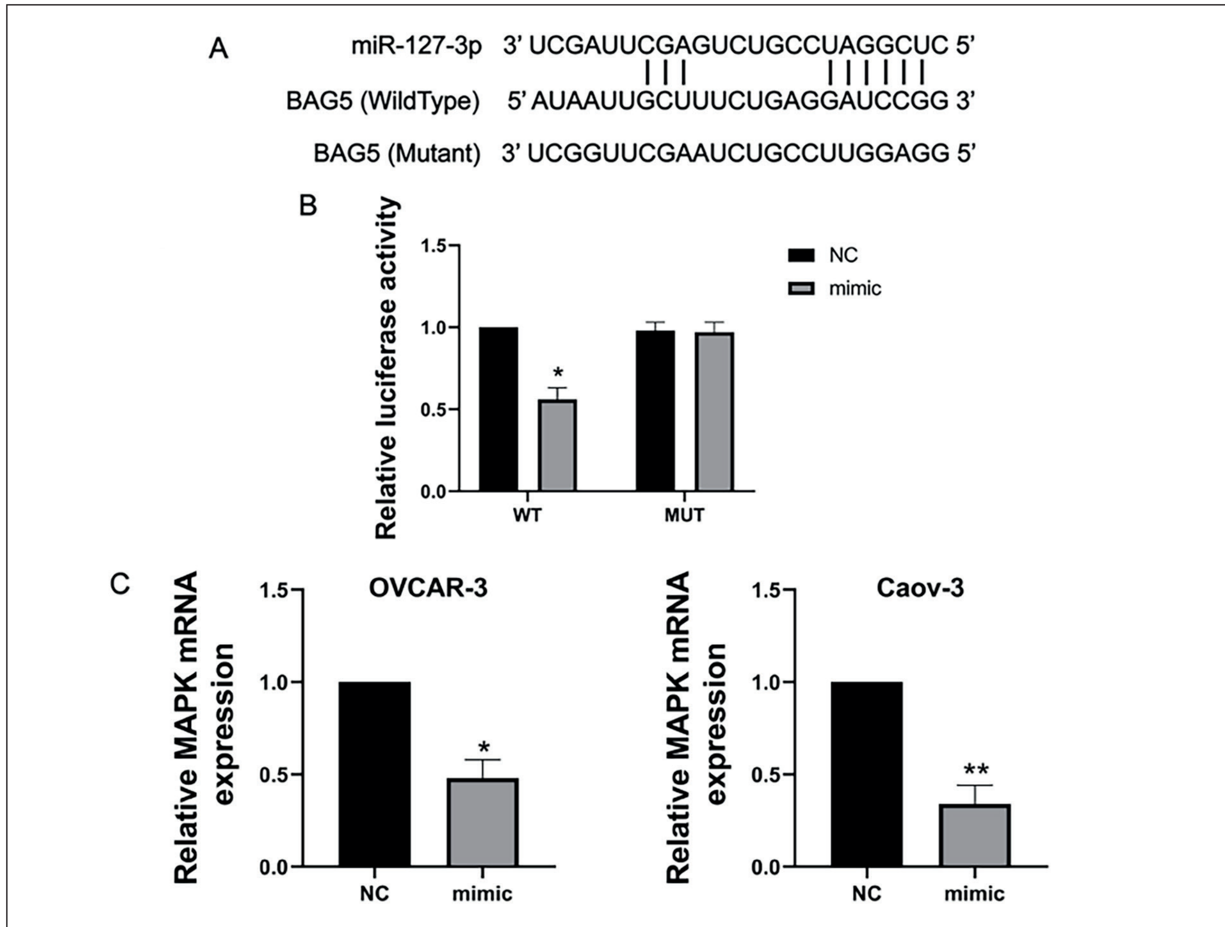

Figure 4. MiR-127-3p regulates MAPK4 expression in EOC cells. **A,** Binding sites of MAPK4-wild-type 3'-UTR and miR-127-3p. **B,** Changes in luciferase activity after co-transfection for 48 h. The luciferase activity in WT group is markedly lower than that in MUT group ($p<0.05$). **C,** Effect of miR-127-3p on MAPK4 mRNA expression in EOC cells determined using qRT-PCR. The expression of MAPK4 mRNA is significantly lower in mimic group than that in NC group ($p<0.01$). ($^{*}p<0.05$, $^{**}p<0.01$).

posite effect to miR-127-3p overexpression in EOC cells (Figure 5).

Discussion

MiRNAs have sparked a revolution in molecular biology, showing multiple functions in a variety of cellular processes[8,16]. Therefore, it is extremely important to understand the physiological and disease-related mechanisms of functions of these small single-stranded RNAs[15]. There are changes in the miRNA expression profile in cancer[9,10,17]. MiR-127-3p acts as a tumor-suppressor gene in human cancers and is downregulated in EOC[18,19]. However, the exact role of miR-127-3p in EOC remains unknown. In this study, the expression pattern of miR-127-3p was first evaluated *via* qRT-PCR. The results revealed that miR-127-3p was downregulated in *in vitro* EOC cell lines and *in vivo* clinical samples of EOC. OVCAR-3 and Caov-3 cell lines with stable overexpression of miR-127-3p were constructed. According to the results of subsequent functional experiments, overexpression of miR-127-3p inhibited proliferation, drug (bufalin) sensitivity, and invasion of EOC, indicating that miR-127-3p exerted an anti-tumor effect in EOC. To sum up, miR-127-3p plays a major anti-tumor role in various cancers[9,10,17]. In a study on breast cancer and bladder cancer, it was found that the main molecular target of miR-127-3p is the zinc finger repressor Bcl-6[9]. However, the results in this study revealed that the MAPK4 gene was a new candidate gene and might be a target gene for miR-127-3p in ovarian cancer.

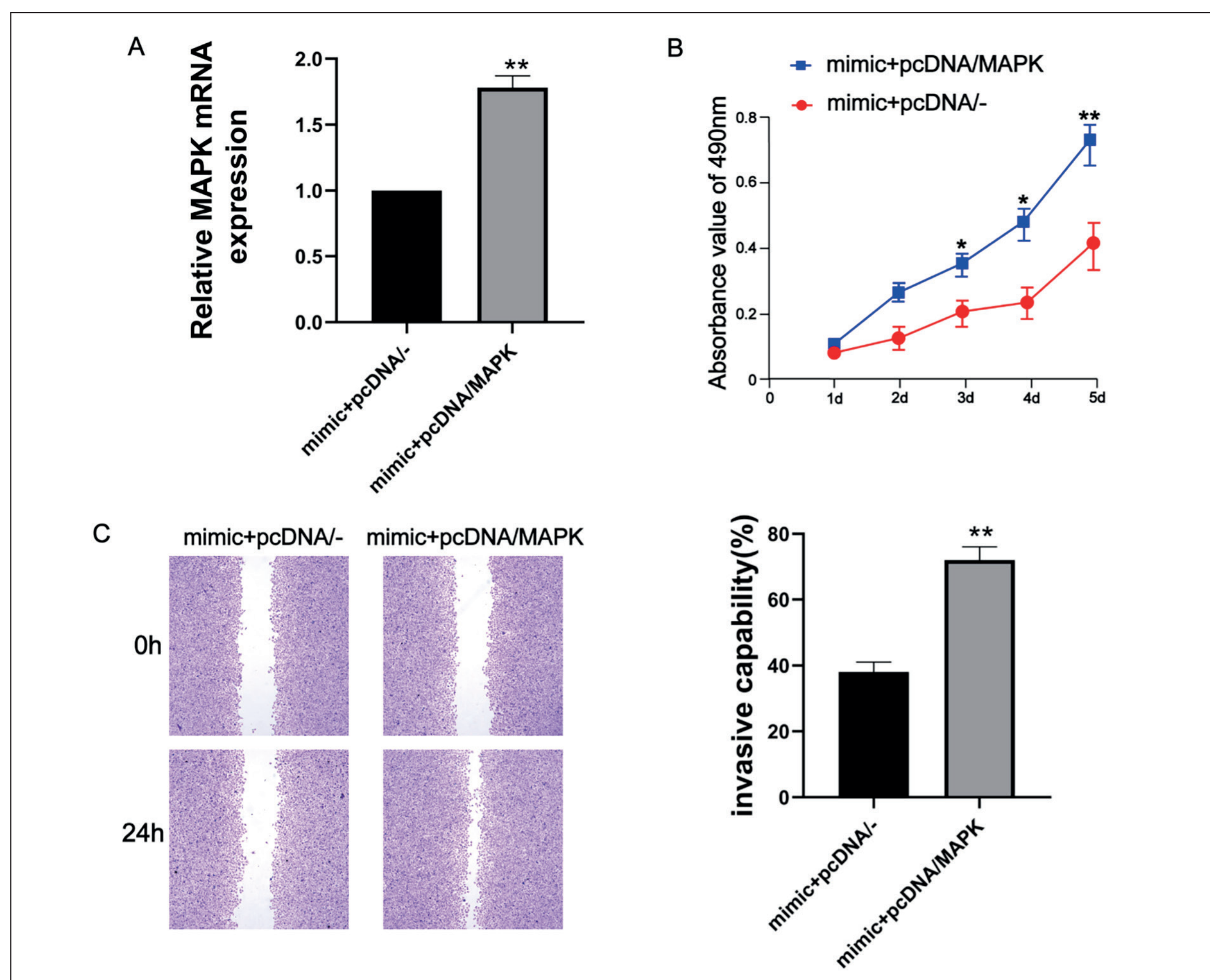

Figure 5. Effects of MAPK4 up-regulation and miR-127-3p overexpression on EOC proliferation. **A,** Changes in MAPK4 expression in Caov-3 cells transfected with pcDNA/MAPK4 plasmids. The mRNA expression of MAPK4 is remarkably higher in experimental group than that in control group ($p<0.01$). **B,** Effect of overexpressed MAPK4 on proliferation of Caov-3 cells. The cell proliferation rate is restored in experimental group compared with that in control group ($p<0.05$). **C,** Effect of overexpressed MAPK4 on migration of Caov-3 cells. The wound area is significantly smaller in experimental group than that in control group ($p<0.01$) (magnification: 40×) ($^{*}p<0.05$, $^{**}p<0.01$).

The miRNA targets were predicted using various computer-aided algorithms (TargetScan 4.0, RNA22 and miRanda), and all genes predicted were selected as candidate targets, among which MAPK4 with binding sites to miR-127 was considered as the potential target gene of miR-127. It was confirmed *via* Dual-Luciferase reporter assay that miR-127-3p indeed had binding sites to human MAPK4 gene. The results of qRT-PCR also demonstrated that overexpression of miR-127-3p in OVCAR-3 and Caov-3 cell lines downregulated MAPK4 gene. In addition, it was clearly shown *via* functional experiments that upregulation of MAPK4 inhibited the anti-tumor effect of overexpressed miR-127-3p in EOC. The MARK family is a key regulator of tubulin cytoskeleton stability, which mediates the growth factor signals, thereby regulating various cellular processes. Besides, the activation of MAPK4 signals is closely related to the invasion ability of gastric cancer (GC) cells, and the expression of p-MAPK4 is significantly higher in diffuse GC and GC with peritoneal metastasis and lymph node metastasis[20,21].

Conclusions

The novelty of this study was that the anti-tumor effect of miR-127-3p on EOC is discovered,

and MAPK4 is a new molecular target of miR-127-3p involved in the progression of EOC. The findings further help understand the epigenetic regulation in EOC and develop new targets for early diagnosis and treatment of EOC patients.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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