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MicroRNA-149 suppresses the malignant phenotypes of ovarian cancer *via* downregulation of MSI2 and inhibition of PI3K/AKT pathway

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Abstract. – OBJECTIVE: Ovarian cancer (OC) is one of the most lethal gynecologic malignant tumors. Emerging evidence has indicated that the dysregulation of microRNAs (miRNAs/miRs) participates in the OC progression. It has been revealed that miR-149 acts either as an oncogene or a tumour suppressor in various human tumors. The current study focused on the biological roles and potential mechanism of miR-149 in OC.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was performed to examine the level of miR-149 expression in 72 pairs of OC tissues and para-cancerous specimens. We further measured the miR-149 levels in OC cells. As we indicated that miR-149 inhibited OC cell viability, we further explored the roles of miR-149 in OC cell invasion and migration by performing the transwell assays. As we suggested that MSI2 was one target for miR-149 in OC cell lines, the expressions and clinical significance of MSI2 in OC were further investigated.

RESULTS: We first detected miR-149 expressions in the OC tissues using quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and the data showed that miR-149 was dramatically downregulated in the OC tissue samples in comparison to matched normal tissue samples. Additionally, the downregulation of miR-149 in OC was found to be related to the poor prognosis and malignant clinicopathologic characteristics of patients with OC. MiR-149 overexpression significantly suppressed the OC cell proliferation, invasion, and migration as determined by functional assays, including MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assays and transwell assays. Furthermore, the dual-luciferase reporter assay demonstrated that MSI2 was an efficient target of miR-149 in OC cells. Finally, some findings also revealed that miR-149 exerted its biological function in OC cells *via* direct regulation of phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT).

CONCLUSIONS: Collectively, miR-149 exerted anti-OC roles at least partially by regulating MSI2 *via* PI3K/AKT. The findings of this study suggested that miR-149 might be a promising target in the diagnosis and prognosis for OC patients.

Key Words:
Ovarian cancer, MiR-149, MSI2, PI3K/AKT.

Introduction

Ovarian cancer (OC) is the most life-threatening malignancy in the reproductive tract in women[1]. OC accounts for approximately 90% of malignant gynecologic tumors, with the highest mortality rate. Due to the lack of early detection strategies, the mortality of the patients with OC is very high[2]; moreover, most OC diagnoses are in the advanced metastatic stage because of the lack of early symptoms, and the 5-year survival rate remains unsatisfactory[3]. OC is a group of heterogeneous cancers with extensive morphological and clinical manifestations[4]. Despite significant advances in the diagnostic and therapeutic strategies, the clinical outcomes of OC patients remain poor. Therefore, developing reliable and novel biomarkers are of great clinical importance to improve the efficacy of the strategy treatments of OC patients.

MicroRNAs (miRNAs/miRs) are small non-coding RNAs of approximately 19-25 nucleotides. It has been demonstrated that miRNA is involved in the regulation of the expressions of the target genes *via* inducing cleavage or inhibiting the translation of miRNAs[5]. Even though their precise role is limited, increasing studies have indicated that miRNAs play an important

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role in various biological processes, including cell proliferation, invasion, growth, and survival[6-8]. Aaccumulating studies have demonstrated the dysregulation of multiple miRs in various human tumors, which are associated with tumorigenesis[9]. Particularly, miRNAs may play anti-tumor or carcinogenic roles in tumorigenesis depending on the functions of the target genes[10,11]. Here, we investigated the expressions and biological functions of miR-149 in the OC progression.

Musashi 2 (MSI2) is one member of the RNA-binding protein Musashi (MSI). MSI could maintain the state of stemcells and suppress the translations of certain mRNAs, also playing regulatory roles in tumorigenesis and cell differentiation[12]. Moreover, it was also found that MSI2 was expressed in cancers and participated in epithelialmesenchymal transition (EMT) and cell differentiation[13]. In particular, MSI2 is mainly expressed in the hematopoietic stem cells (HSCs), regulating HSCs depletion and engraftment. Additionally, the overexpression of MSI2 in myeloid leukemia regulated cell apoptosis and proliferation, indicating a poor prognosis[14]. It has also been demonstrated that MSI2 is implicated in certain solid tumors. MSI2 expression were increased in esophageal squamous cell carcinoma[15] and gastric carcinoma[16], and the upregulation of MSI2 could promote cell invasion and growth[17].

Patients and Methods

Clinical Tissue Samples

The tissue samples were surgically resected from OC patients with informed consent at the Affiliated Hospital of Chengde Medical University between July 2016 and June 2018. No patients received any treatment before the tissue collection. The tissues were frozen in liquid nitrogen immediately, followed by being preserved at -80°C. This study was approved by the Ethics Committee of the Affiliated Hospital of Chengde Medical University. The signed written informed consents were obtained from all participants before the study.

Cell Lines and Culture

The OC cell lines (SKOV3, OVCAR3, HO8910, and A2780) and the normal immortalized human ovarian surface epithelial cells IOSE29 were purchased from the American Type Culture Collection (Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, USA) including 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) at 37°C and 5% CO_2.

Transfections

MiR-149 mimic, the inhibitors, as well as the negative control (NC), were synthesized by GenePharma (Shanghai, China). The OC cell lines were cultured overnight and transfected with the corresponding miRs by Lipofectamine®2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). 48 hours after transfection, the cells were harvested for subsequent analysis.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The total RNA was isolated from the tissue samples and the cultured cell lines by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then, the M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) was used to synthesize complementary deoxyribose nucleic acid (cDNA). qRTPCR was conducted on ABI 7500 thermocycler (Applied Biosystems, Foster City, CA, USA) with the SYBR Premix Ex Taq™ kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). The expressions were quantified by the $2^{-\Delta\Delta Ct}$ method. The sequences of the primers were described in Table I. U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were endogenous controls.

Western Blotting

The treated cells were lysed in radioimmunoprecipitation assay lysis (RIPA) buffer for total protein extractions. A bicinchoninic acid protein (BCA) kit (Thermofisher Scientific, Waltham, MA, USA) was utilized to quantify the protein concentration. After separation with 10% SDS-PAGE, the protein was transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, Chicago, IL, USA). The membrane was blocked with 5%-skimmed milk in TBST. Thereafter, the specific primary antibody was incubated at 4°C overnight: AKT (1:1000, Abcam, Cambridge, MA, USA), p-AKT (1:1000, Abcam, Cambridge, MA, USA), PI3K (1:1000, Abcam, Cambridge, MA, USA), p-PI3K (1:2000, Abcam, Cambridge, MA, USA), E-cadherin (1:2000, Abcam, Cambridge, MA, USA), N-cadherin (1:1000, Abcam, Cambridge, MA, USA), Vimentin (1:1000, Abcam, Cambridge,

Table I. Primer sequences for qRT-PCR.

Primer	Sequence
miR-149 forward	5'-TCTGGCTCCGTGTCTTCACTCCC-3'
miR-149 reverse	5'-AGTGGTTGTTCTGCTCTCTGTGTC-3'
U6 forward	5'-CTCGCTTCGGCAGCACA-3'
U6 reverse	5'-AACGCTTCACGAATTTGCGT-3'
MSI2 forward	5'-AGGGGAAGAAAAAAA GAAAATAAGAG-3'
MSI2 reverse	5'-AACTCTCCTCACACATACAATATCAA-3'
GAPDH forward	5'-ATGGGGAAGGTGAAGGTCG-3'
GAPDH reverse	5'-GGGTCATTGATGGCAACAATATC-3'

U6: small nuclear RNA, snRNA; MSI2: Musashi 2; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

MA, USA) and GAPDH (1:1000, Abcam, Cambridge, MA, USA). Thereafter, the membranes were subjected to incubation with secondary antibody (1:4,000, ab7090; Abcam, Cambridge, MA, USA) for 2 h at room temperature. GAPDH was an internal control. The enhanced Chemifluorescence Western blotting kit (Pierce; Rockford, IL, USA) was utilized to detect the protein bands.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide) Assay

MTT assays (Sigma-Aldrich, St. Louis, MO, USA) were used to determine the proliferation of the OC cells. Briefly, the transfected OC cells were seeded into a 96-well plate and cultured for 24 h, 48 h, 72 h, respectively. After that, MTT reagent (5 mg/mL) was added into the wells and incubated at 37°C for 4 h. Then, 150 μL dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) was added into the plates and the optical density $(OD)_{490}$ was examined with a microplate (Bio-Rad Laboratories, Hercules, CA, USA).

Transwell Assay

The OC cells were plated into the top chamber of Matrigel-coated or non-coated Transwell chambers (8 μm pore size; Coning, Coning, NY, USA) in FBS-free DMEM medium for the evaluation of cell invasion or migration, respectively. Then, the medium containing 10% FBS was added into the bottom chambers. After incubation for 24h, the non-invaded or non-migrated cells were scraped off with cotton swabs. In the meantime, the cells (invading/migrating) on the bottom sides were fixed and stained with methanol and crystal violet. The results were photographed and counted under a microscope (Olympus, Tokyo, Japan) in five randomly selected fields.

Dual-Luciferase Reporter Assay

The OC cells were seeded into a 24 well plate and incubated for 24 h at 37°C. Then, the wide-type (WT) or mutant (MUT) MSI2 3′-untranslated region (3′-UTR) luciferase reporter vector, combined with miR-149 mimics, was co-transfected into the cells. 48 hours post-transfection, the luciferase activity was detected.

Statistical Analysis

All experiments were repeated at least thrice. The statistical analysis was performed with the Statistical Product and Service Solutions (SPSS) software version 17.0 (SPSS Inc., Chicago, IL, USA). The comparison between multiple groups was done using the One-way ANOVA test followed by the post-hoc test (Least Significant Difference). The differences between the two groups were analyzed *via* Student's *t*-test. The Kaplan-Meier analysis followed by the log-rank test was used to analyze the overall survival (OS) of OC patients. $p<0.05$ indicated a statistically significant difference.

Results

MiR-149 Expressions Were Inhibited in OC Tissues

The miR-149 expressions in OC tissues were detected. The results demonstrated that miR-149 expressions were significantly decreased in the OC tissues in comparison to the normal tissue samples (Figure 1A). Then, the prognosis-predictive role of miR-149 in the OS of OC patients was investigated during a follow-up period of 60 months. All the patients were separated into high- and low-miR-149 groups on the basis of the median miR-149 expression. As shown in Figure

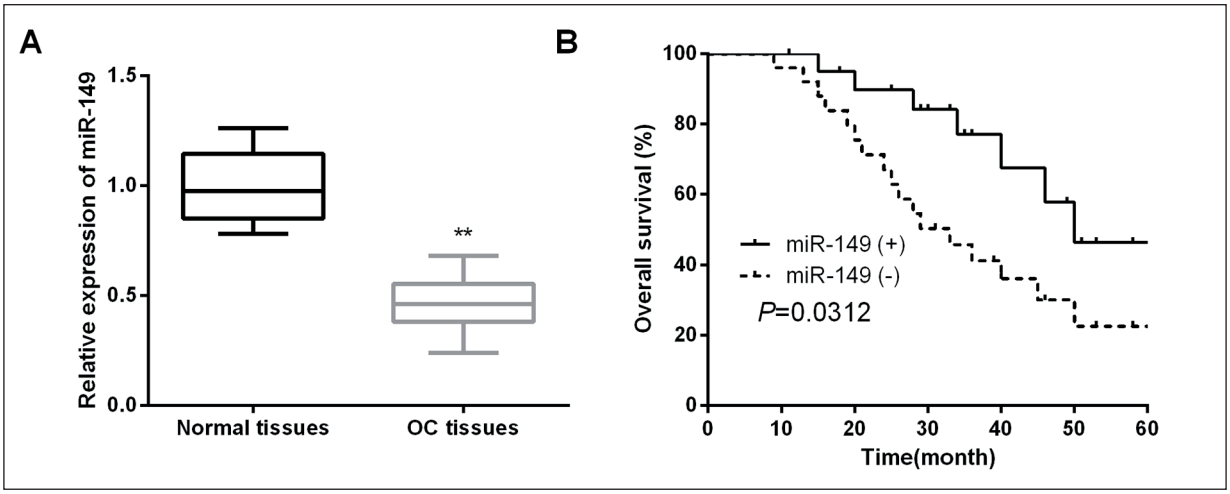

Figure 1. Downregulation of miR-149 in OC tissues indicated poor prognosis of OC patients. **A,** qRT-PCR analysis of miR-149 expressions in the OC tissues was performed. **B,** The Kaplan-Meier survival analysis was performed to determine the association between miR-149 expressions and the overall survival rate of OC patients. **$p<0.01$.

1B, the Kaplan-Meier survival analysis showed that OC patients with low miR-149 expressions exhibited shorter OS than those with high miR-149 expressions. We also assessed the clinical significance of miR-149 in OC patients. As shown in Table II, low miR-149 level in the OC tissues was associated with the adverse clinicopathologic features, which were indicators of poor prognosis.

Table II. Correlation of miR-149 expression with the clinicopathological characteristics of the ovarian cancer patients.

Clinicopathological features	Cases (n = 46)	miR-149[b] expression		p-value
		High (n = 18)	Low (n = 28)	
Age (years)				0.5769
> 60	24	9	15	
≤ 60	22	9	13	
Family history of cancer				0.4146
Yes	23	7	16	
No	23	11	12	
Tumor size (cm)				0.3725
≥ 5.0	23	8	15	
< 5.0	23	10	13	
TNM stage				0.0013*
I-II	21	15	6	
III	25	3	22	
Lymph-node metastasis				0.0018*
Yes	22	3	19	
No	24	15	9	
Pausimenia				
Yes	24	10	14	0.6456
No	22	8	14	
FIGO stage				0.0019*
I-II	20	14	6	
III-IV	26	4	22	
Distant metastasis				0.0022*
Yes	25	4	21	
No	21	14	7	

TNM: tumor-node-metastasis; FIGO: International Federation of Gynecology and Obstetrics; [b]The median expression level of miR-149 was used as the cutoff; *Statistically significant.

MiR-149 Upregulation Suppressed OC Cell Proliferation

We further measured the miR-149 levels in the OC cells. According to qRT-PCR analysis, the miR-149 expression levels in OC cells were substantially downregulated, compared to the normal IOSE29 (Figure 2A). OVCAR3 cells were selected for further assays. In brief, to investigate the functions of miR-149 in OC, we first established miR-149 stably-expressed or stably-inhibited OVCAR3 cells by the transfection of miR-149 mimics or inhibitor. The successful increase or decrease of miR-149 expressions in OVCAR3 cells was verified by qRT-PCR analysis (Figures 2B and 2C). After that, the MTT assays were performed to determine the roles of miR-149 in OC cell viability ability. As expected, the increase of miR-149 dramatically repressed OVCAR3 cell proliferation (Figure 2D). In the meantime, we found that the proliferation ability of OVCAR3 cells was prominently facilitated by a decrease of miR-149 (Figure 2E).

MiR-149 Overexpression Inhibited OC Cell Invasion and Migration

As we found that miR-149 inhibited the OC cell viability, we further explored the roles of miR-149 in OC cell invasion and migration by performing the transwell assays. As expected, our findings showed that miR-149 restoration significantly suppressed OVCAR3 cell invasion and migration (Figures 3A and 3B). Consistently, miR-149 inhibition remarkably promoted the invasion and migration capacities of the OVCAR3 cells (Figures 3C and 3D).

MSI2 Was Directly Targeted by MiR-149 in OC Cells

TargetScan was used to predict the potential targets of miR-149, and we found that MSI2 was a functional target of miR-149 (Figure 4A). In order to further indicate the association between MSI2 and miR-149, we conducted the luciferase reporter assays by co-transfecting miR-149 mimics and MSI2-3'UTR-WT or -MUT into the OC cells. MiR-149 upregulation was found to dramatically inhibit the luciferase activity of the OC cells transfected with MSI2-3'UTR-WT, while the luciferase activities of the OC cells transfected with MSI2-3'UTR-MUT were not significantly influenced by miR-149 mimics (Figure 4B). Similarly, the qRT-PCR analysis was utilized

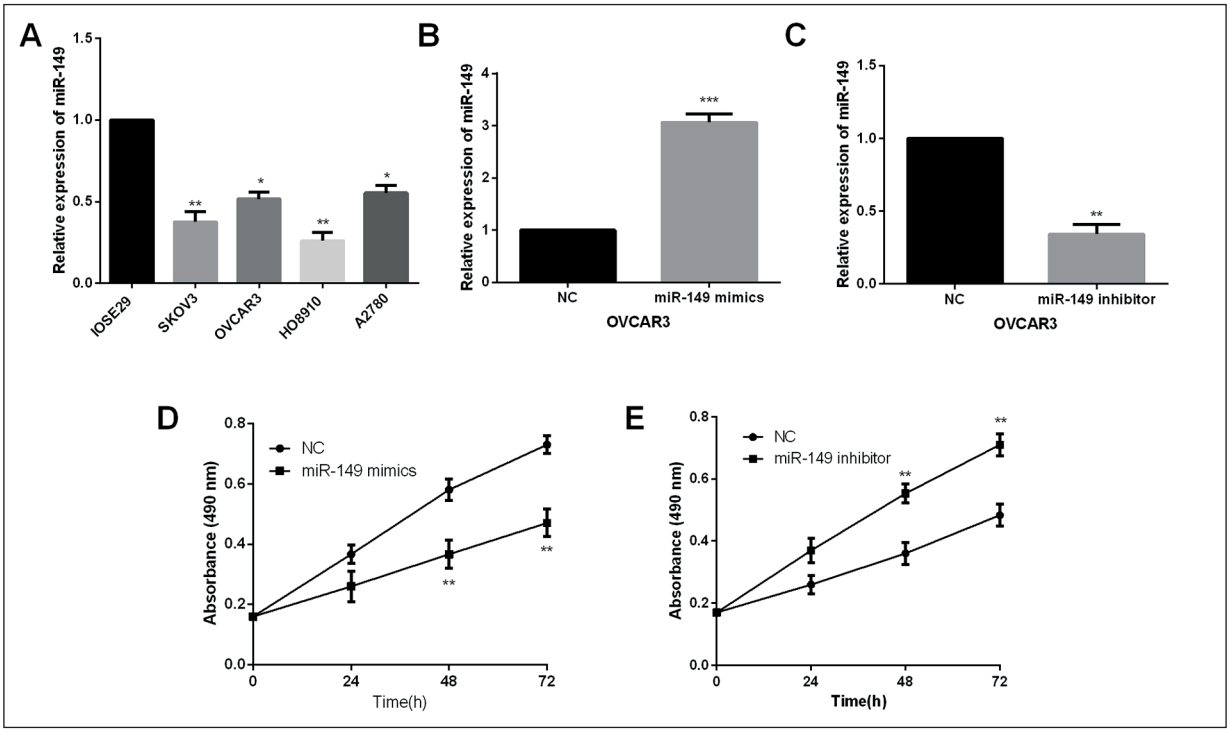

Figure 2. MiR-149 inhibited the OC cell proliferation. **A,** MiR-149 expressions in the OC cells were detected by qRT-PCR. **B, C,** MiR-149 overexpression or inhibition in the OC cells was confirmed by qRT-PCR. **D, E,** The influence of miR-149 on the OC cell viability was determined by MTT assays. ***$p<0.001$, **$p<0.01$, *$p<0.05$.

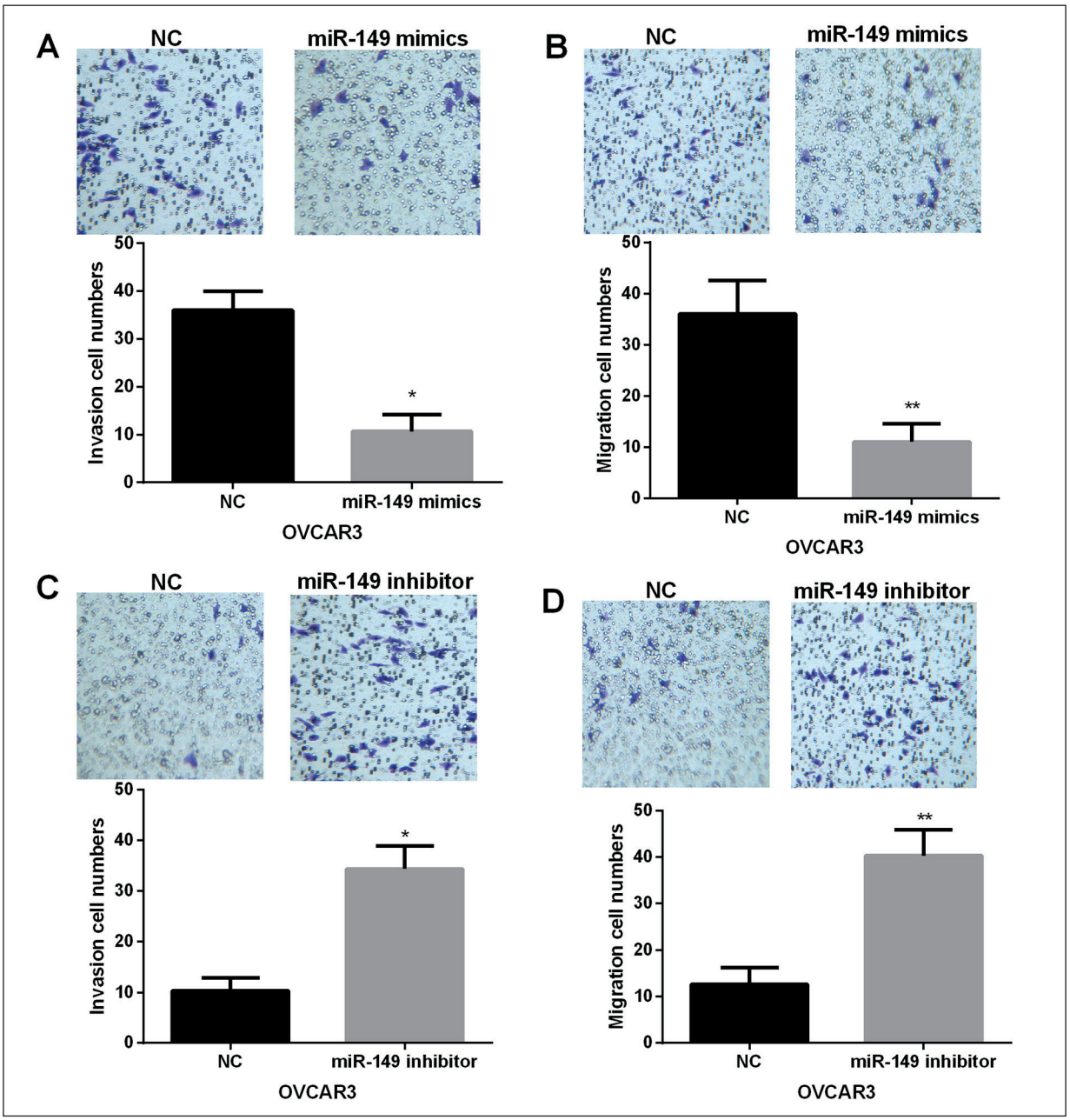

Figure 3. OC cell invasion and migration were inhibited by miR-149 overexpression. **A, B,** The transwell assays demonstrated that miR-149 overexpression impaired the invasion and migration capacities of the OC cells (magnification: 40×). **C, D,** OC cell invasion and migration abilities of the OC cells were significantly promoted by miR-149 inhibition as demonstrated by the transwell assays (magnification: 40×). $^{**}p<0.01$, $^{*}p<0.05$.

to determine the roles of miR-149 in regulating MSI2 expressions. The results revealed that the MSI2 expression in the OVCAR3 cells was significantly suppressed by miR-149 overexpression, whereas it was markedly promoted by miR-149 inhibition (Figures 4C and 4D). Taken together, these findings demonstrated that MSI2 was a target of miR-149 in OC cells.

MiR-149 Suppressed PI3K/AKT Pathway and EMT in OC Cells

As we confirmed that MSI2 was one target for miR-149 in OC cell lines, the expressions and clinical significance of MSI2 in OC were further investigated. Firstly, we measured the MSI2 expressions in the OC tissue samples, and the qRT-PCR findings demonstrated an evident increase of the MSI2 ex-

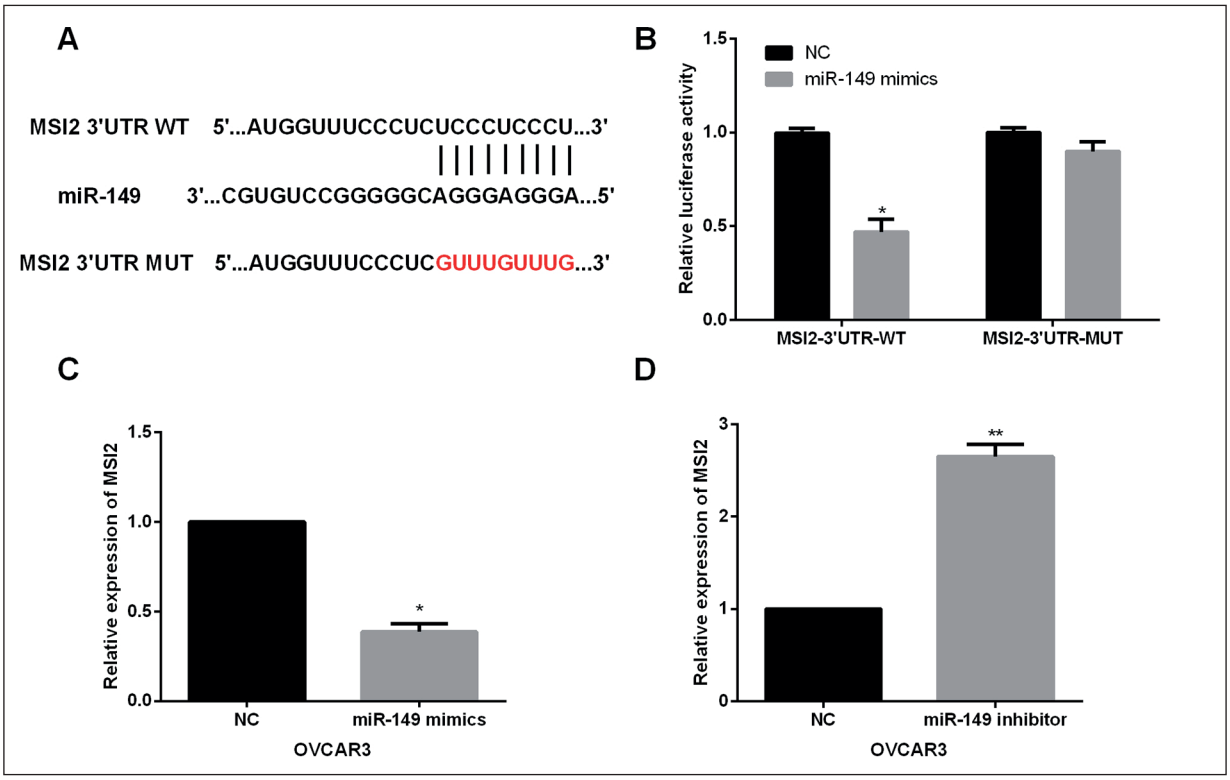

Figure 4. MSI2 was an efficient target of miR-149 in the OC cell lines. **A,** Predicted binding sequences of miR149 on MSI2-3'UTRs were presented. **B,** Luciferase activities of the OC cells with different transfections were examined. **C, D,** The regulatory roles of miR-149 in MSI2 expressions were investigated by qRT-PCR. $^{**}p<0.01$, $^{*}p<0.05$.

pressions in OC tissue samples when compared to the matched para-carcinoma tissue samples (Figure 5A). Moreover, MSI2 was also evidently upregulated in the OC cells in comparison to the normal cells (Figure 5B). Then, the clinical values of MSI2 in OC patients were analyzed by the Kaplan-Meier survival analysis. As expected, the patients who presented high MSI2 expressions had a poorer prognosis than patients who presented low MSI2 expressions (Figure 5C). We further investigated whether miR-149 affected the expressions of PI3K/AKT- and EMT-related proteins. It was shown that E-cadherin was upregulated while the expressions of vimentin and E-cadherin were decreased by miR-149 upregulation; in the meantime, p-PI3K and p-AKT expressions were downregulated by miR-149 overexpression (Figure 5D). Therefore, our results indicated that miR-149 exerted anti-tumor functions in OC *via* inhibiting PI3K/AKT and EMT.

Discussion

Ovarian cancer is becoming a serious burden, causing increasing mortality rate worldwide[18]. The deaths caused by OC are more common in developed countries[19]. Currently, chemotherapy and surgery are effective therapeutic strategies for OC patients at early stages; however, for advanced or recurrent patients, the treatments have been usually unsuccessful[20]. Moreover, the outcomes of surgery substantially differ, most of which are unexplained[21]. Therefore, exploring novel drug target and tumor biomarker to improve early diagnosis is crucial for the improvement of the survival. Emerging evidence has shown that miRs are the key regulators of various cellular processes and are aberrantly expressed in different cancers, including OC[22]. EMT is a fundamental phenomenon associated with metastasis in tumors. In this process, the epithelial cell lost celltocell adhesion, acquired invasive hallmark and increased motility to become mesenchymal cells[23]. EMT cells could degrade the surrounding microenvironment and migrate from the primary sites to the new marginal organ[24]. The PI3K/Akt pathway serves as an important driver of tumorigenesis. Akt could connect to different interrelated signaling pathways by phosphorylating many downstream targets[25]. Thus, the PI3K/Akt path-

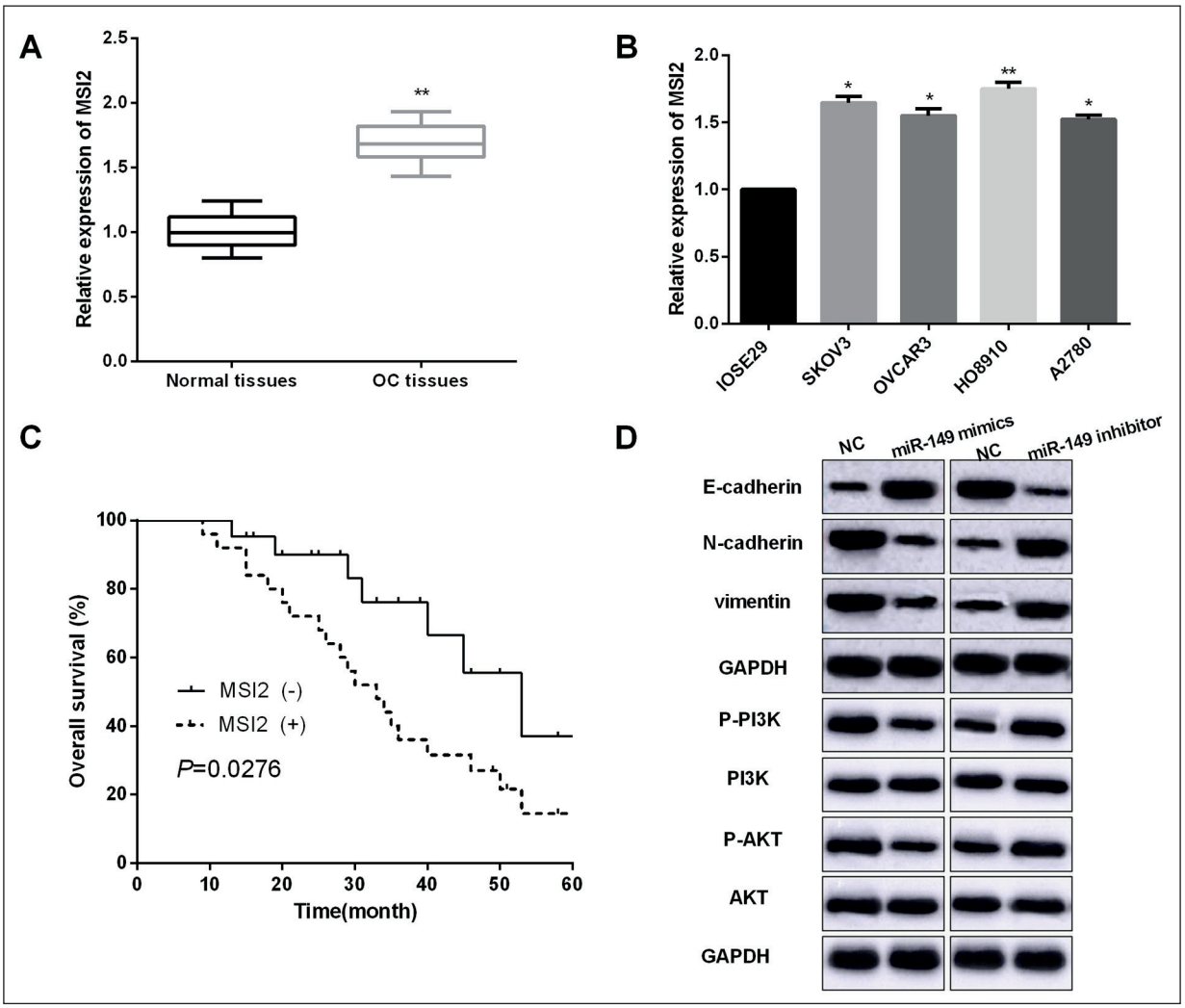

Figure 5. MiR-149 regulated PI3K/AKT pathway in the OC cells. **A, B,** MSI2 was upregulated in the OC tissues and cells as determined by qRT-PCR. **C,** The correlation between MSI2 expressions and the prognosis of OC patients was confirmed by the Kaplan-Meier survival analysis. **D,** Western blot was performed to detect the expression of PI3K/AKT-related proteins. $^{**}p<0.01$, $^{*}p<0.05$.

way is implicated in different types of processes, such as cell cycle progression, cell survival, and proliferation[26]. It is well known that the PI3K/Akt pathway is aberrantly activated in many types of human tumors, indicating poor prognosis[27]. Therefore, the present study speculated that miR-149 exhibited anti-OC abilities by inactivating the PI3K/Akt pathway.

The dysregulation of miRs is frequently correlated with tumor cell growth and metastases. MiR-149 has been demonstrated to play an important role in tumor progression. The various functions of miR-149 in different tumors depended on the target genes and the coordinated regulation between them. For example, Qian et al[28] reported that miR-149 regulated cervical carcinoma cell proliferation and apoptosis *via* regulating GIT1. Xie et al[29] proposed that miR-149 promoted human osteocarcinoma progression by regulating BMP9; additionally, Zhao et al[30] detected that miR-149 inhibited cell growth and metastases *via* downregulation of FOXM1/cyclin D1/MMP2 in human non-small cell lung cancer. In the current study, we found that miR-149 downregulation was associated with the poor prognosis and adverse clinicopathologic features of OC patients. Moreover, miR-149 upregulation could inhibit the OC cell viability and metastasis *via* the regulation of EMT and PI3K/AKT pathway.

It has been shown that MSI2 dysregulation is involved in malignant tumors of the circulatory systems and several solid tumors, promoting malignant phenotypes[16]. In the present study, MSI2 was indicated to be a functional target of miR-149 in the OC cell lines. Briefly, the MSI2 expressions in OC tissue samples and matched normal tissue samples were examined by qRT-PCR. The results demonstrated a significant up-regulation of MSI2 in the OC tissues in comparison with the adjacent noncancerous tissues. Furthermore, the Kaplan-Meier survival analysis was performed to classify the clinical significance of MSI2 in OC. It was found that MSI2 overexpression resulted in a poor prognosis in OC patients. In short, the aforementioned findings indicated that MSI2 was partially involved in the functions of miR-149 in OC, serving as a potential valuable biomarker for OC diagnosis due to its oncogenic roles.

Conclusions

We demonstrated that the downregulation of miR-149 in the OC cells was an indicator for the poor prognosis and malignant phenotypes in patients with OC. Moreover, miR-149 restoration could significantly inhibit the OC cell growth and metastasis *via* the regulating EMT and PI3K/AKT. We further explored the underlying mechanism by which miR-149 functioned in OC. The luciferase reporter assays showed that MSI2 was directly targeted by miR-149 in the OC cells, suggesting that MSI2 participated in the roles mediated by miR-149 in the OC progression. Taken together, all these results indicated that miR-149 and MSI2 might be used as novel promising targets in diagnosis and prognosis of OC patients for the effective treatment in the future.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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