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MicroRNA-489 targets XIAP to inhibit the biological progression of ovarian cancer *via* regulating PI3K/Akt signaling pathway and epithelial-to-mesenchymal transition

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Abstract. – OBJECTIVE: Ovarian cancer (OC) is a deathful malignant tumor in women worldwide, and its poor prognosis mainly results from metastasis. Recently, microRNA (miRNA/miR) has been found to exert crucial functions in the progression of multiple tumors by affecting expressions of their targets. However, the biological roles and the potential mechanism of miR-489 in OC need further elucidation.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was utilized to confirm the miR-489 expressions in OC tissue samples and cell lines. The functions of miR-489 were analyzed by performing functional assays, such as MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assays and transwell assays. The downstream target of miR-489 was confirmed by TargetScan and luciferase reporter assay. Western blot was conducted to detect the expression of indicators associated with the down-stream signaling pathway.

RESULTS: MiR-489 was prominently down-regulated in OC tissues and cells, and the decreased miR-489 expression was related to malignant clinicopathologic features and poor prognosis of OC patients. Functional assays demonstrated that miR-489 could suppress OC cell viability, invasion, and migration. X-linked inhibitor of apoptosis protein (XIAP) was identified as a target of miR-489 and partially regulated the functions of miR-489 in OC. Moreover, we found that miR-489 inhibits OC progression *via* regulating phosphatidyl-inositol 3-kinase/protein kinase B pathway (PI3K/AKT) and epithelial-to-mesenchymal transition (EMT).

CONCLUSIONS: Our results demonstrated that miR-489 inhibited OC development by directly binding to XIAP and regulating PI3K/Akt and EMT signal pathways, and miR-489 might serve as a promising biomarker for OC treatment in the future.

Key Words:
Ovarian cancer, MiR-489, XIAP, PI3K/Akt, EMT.

Introduction

Ovarian cancer (OC) is one of the most life-threatening malignancies in reproductive tracts, and its prognosis remains dismal[1]. Most OC cells are derived from epithelium, there are also germ cells and stromal tumors[2]. In general, OC is frequently silent at early stages, remaining unrecognized in most patients until the OC cells metastasize to other areas outside the ovary[3]. This reveals that extensive abdominal metastases have developed at the time of diagnosis, which is the main cause of high mortalities of OC patients[4]. Therefore, inhibiting OC cell metastasis can help improve the poor prognosis of OC patients. Recently, novel chemotherapy drugs, chemotherapy combined with radiation therapy, and traditional surgery have been applied for OC treatment[5]. However, the recurrence rates of OC patients are still high[6]. Moreover, the mechanism of OC metastases has not been fully elucidated, which has become a hot topic of recent studies.

MicroRNA (miRNA/miR) is endogenous, non-coding RNA which can regulate gene expressions frequently by targeting their 3′-untranslated regions (3′-UTRs) suppressing mRNA translation

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or stability[7]. Typically, miRs play crucial functions in different cellular events, being involved in tumorigenesis[8,9]. MiRs are promising prognostic and diagnostic biomarkers in tumors[10]. Recently, they have been found having differential expressions in multiple cancers, as well as their important effects in various tumor-associated processes, including growth and metastasis[11,12]. Several miRs are remarkably dysregulated in OC, indicating that miRs are implicated in OC genesis and development. MiR-199a-5p suppressed OC invasion and proliferation by targeting NF-kB1[13]; miR-183 regulated OC cell apoptosis and proliferation *via* the transforming growth factor-β/Mothers against decapentaplegic homolog 4 ((TGF-β)/Smad4) pathway[14]; additionally, the inhibition of miR-328-3p repressed OC cell migration and impaired cancer stem cell functions[15].

In recent years, the functional roles of miRs in tumorigenesis have received more and more attention. MiR-489 was one of the reported miRs which have close relationship with tumor biology[16]. In glioma, miR-489 was found inducing apoptosis and inhibiting cell cycle progression and cell proliferation *via* regulating spindlin 1 (SPIN1)-mediated PI3K/AKT pathway[17]. In colorectal cancer, miR-489 suppressed cancer invasion and growth by targeting histone deacetylase 7 (HDAC7)[18]. Moreover, miR-489 was confirmed to play anti-metastatic roles by binding to matrix metalloproteinase-7 in human hepatocellular carcinoma[19]. Wu et al[20] indicated that miR-489 promoted apoptosis and suppressed cell growth and cisplatin resistance in OC *via* downregulating Akt3 expressions.

X-linked inhibitor of apoptosis protein (XIAP) is currently the most potent IAP (inhibitor of apoptosis) protein in human tissues[21]. XIAP was a newly identified candidate regulator of malignancies[22]. However, the relationship between miR-489 and XIAP in OC development, such as metastasis and invasion remains unknown.

Patients and Methods

Cell Culture and Cell Transfection

Two human OC cell lines (SKOV3, OVCAR3, HO8910) and ovarian surface epithelial cell line (HOSEpiC) were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) at 37°C with 5% CO_2. MiR-489 mimics and inhibitor (GenePharma, Shanghai, China) were transfected into OC cells using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). The efficiency was analyzed 48 h after transfection.

Tissue Samples

This investigation was approved by the Ethics Committees of Zhujiang Hospital, Southern Medical University. 51 paired OC and matched normal ovarian tissue samples were collected from the OC patients who underwent surgery at Zhujiang Hospital, Southern Medical University from June 2016 to October 2018. All the patients read the consent and signed it. No patients had received any treatment prior to tissue collection. All tissues were immediately snap-frozen in liquid nitrogen and reserved at –80°C for further assays.

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

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was utilized to extract the total RNAs from OC cell lines or tissue samples based on manufacturers' specification. Taqman MicroRNA Reverse Transcription kit (Thermo Fisher Scientific, Waltham, MA, USA) was utilized to synthesize complementary deoxyribose nucleic acid (cDNA). QRT-PCR was conducted using SYBR Premix Ex Taq™ II kit (TaKaRa, Otsu, Shiga, Japan). U6 was used as a reference for miR-489 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control for XIAP. The $2^{-\Delta\Delta CT}$ method was utilized to analyze the relative expression level. The sequences of the primers were described in Table I.

Cell Proliferation Assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was conducted to determine the effect of miR-489 on OC cell viability. The OC cells were seeded in a 96-well plate. MTT solution was added into each well after the cells were incubated at 37°C 5% CO_2 for 0, 24, 48, and 72 h. Subsequently, the cells were further incubated for another 4 h. Then, the MTT solution was removed, and dimethyl sulfoxide (DMSO) was added. A microplate reader (Bio-Rad, Hercules, CA, USA) was used to measure the absorbance at 490 nm.

Transwell Assay

The transwell assays were performed to test the invasive and migratory capabilities of OC cells treated with miR-489 mimics or inhibitor using 24-well transwell chambers (8.0 μm pore

Table I. Primer sequences for qRT-PCR.

Primer	Sequence
miR-489 forward	5'-ACACTCCAGCTGGGGTGACATCACATA-3'
miR-489 reverse	5'-TGGTGTCGTGGAGTCG-3'
U6 forward	5'-CTCGCTTCGGCAGCACA-3'
U6 reverse	5'-AACGCTTCACGAATTTGCGT-3'
XIAP forward	5'-GTGCCACGCAGTCTACAAATTCTGG-3'
XIAP reverse	5'-CGTGCTTCATAATCTGCCATGGATGG-3'
GAPDH forward	5'-GGTGAAGGTCGGAGTCAACG-3'
GAPDH reverse	5'-TGGGTGGAATCATATTGGAACA-3'

U6: small nuclear RNA, snRNA; XIAP: X-linked inhibitor of apoptosis protein; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

size; BD Bioscience, San José, CA, USA) with or without Matrigel (Chemicon, Temecula, CA, USA) in the top chamber. For invasion assays, cells in serum-free medium were placed into the top chamber, whereas medium containing 10% FBS was added into the lower bottom chamber. Following incubation of 48 h, cells remained on the top filter were removed with cotton swabs. Cells which invaded to the bottom chamber surface were fixed, stained, and quantified under a microscope (Olympus, Tokyo, Japan) with three random fields. To measure the migratory capability, the same procedures mentioned above were performed except for the Matrigel-coated upper chamber.

Western Blots

The cultured cells were lysed with lysis buffer. The protein concentrations were detected with the bicinchoninic acid (BCA) assay kit (Invitrogen, Carlsbad, CA, USA). The protein was separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred on polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland), which had been blocked with 5% non-fat milk for 2 h at room temperature. Then, the membranes were incubated with the following primary antibodies against: 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), N-cadherin (1:2000), Vimentin (1:1000), and GAPDH (1:1000) overnight at 4°C, followed by incubated with secondary antibody at room temperature for 2 h. All antibodies were purchased from Abcam (Cambridge, MA, USA). The enhanced chemiluminescence (ECL) Western blot detection reagents were used to visualize the protein band. GAPDH was an internal control.

Luciferase Assay

The mutant type (MUT) or wild-type (WT) of the XIAP-3'UTR was constructed and then inserted into the pMiR-Report vectors (Thermo Fisher Scientific, Waltham, MA, USA). The OC cells were co-transfected with miR-489 mimics and the XIAP-3'UTR-WT plasmid or the XIAP-3'UTR-MUT plasmid (Promega, Madison, WI, USA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Luciferase activity was measured using Dual-Luciferase reporter assay system at 48 h after the transfection.

Statistical Analysis

All data in the current study were obtained from at least 3 independent assays. The software of Statistical analyses was carried out with Statistical Product and Service Solutions (SPSS) 18.0 version (SPSS Inc., Chicago, IL, USA) was utilized to perform the statistical analysis. Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). The survival rate was analyzed by Kaplan-Meier analysis and log-rank test. $p<0.05$ was considered to be significantly different.

Results

Decreased MiR-489 Expression Was Associated With the Poor Outcomes of OC Patients

To investigate the clinical value of miR-489 in OC patients, we first measured the expression of

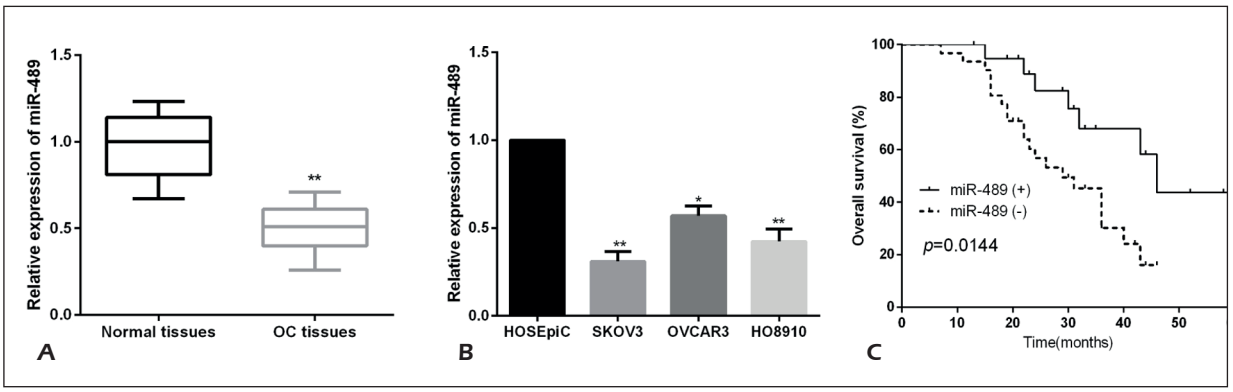

Figure 1. Downregulated miR-489 in OC was related to poor outcomes of OC patients. **A**-**B**, qRT-PCR analysis demonstrated significant decreased miR-489 expressions in OC tissues and cells. **C**, Kaplan-Meier analysis was used to analyze the survival rate of OC patients with different miR-489 expressions. *p<0.05, **p<0.01.

miR-489 in OC tissues and cell lines using qRT-PCR. MiR-489 was downregulated in OC tissue samples and cells (Figure 1A and 1B). Then, all the OC patients involved in our study were divided into high and low miR-489 groups according to the mean miR-489 expression level. Moreover, as demonstrated by Kaplan-Meier analysis, we found that the survival rate of patients in low miR-489 expression group was prominently shorter than that of the patients in the high miR-489 expression group (Figure 1C). Moreover, clinicopathologic analysis also revealed that pa-

Table II. Correlation of miR-489 expression with the clinicopathological characteristics of the ovarian cancer patients.

Clinicopathological features	Cases (n=51)	miR-489[b] expression		*p*-value
		High (n=20)	Low (n=31)	
Age (years)				0.523
> 60	27	10	17	
≤ 60	24	10	14	
Family history of cancer				0.421
Yes	26	8	18	
No	25	12	13	
Tumor size (cm)				0.335
≥ 5.0	25	9	16	
< 5.0	26	11	15	
TNM stage				0.013*
I-II	22	16	6	
III	29	4	25	
Lymph-node metastasis				0.008*
Yes	26	4	22	
No	25	16	9	
Pausimenia				
Yes	25	11	14	0.656
No	26	9	17	
FIGO stage				0.021*
I-II	21	15	6	
III-IV	30	5	25	
Distant metastasis				0.012*
Yes	27	3	24	
No	24	17	7	

TNM: tumor-node-metastasis; FIGO: International Federation of Gynecology and Obstetrics; [b]the median expression level of miR-489 was used as the cutoff; *statistically significant.

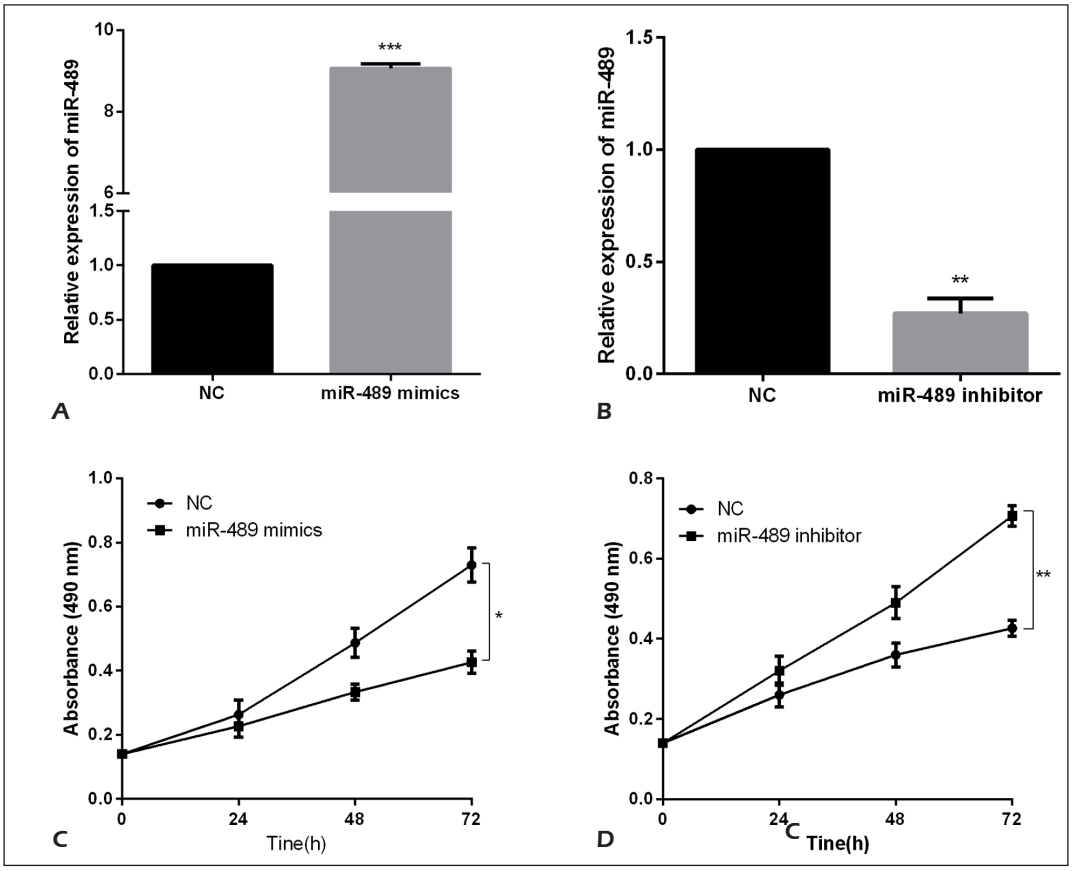

Figure 2. Overexpression of miR-489 inhibited OC cell viability. **A**-**B**, Successful overexpression or inhibition of miR-489 was confirmed by qRT-PCR analysis. **C**-**D**, MTT assays were performed to determine the roles of miR-489 in OC cell viability. *p<0.05, **p<0.01, ***p<0.001.

tients with low miR-489 levels presented malignant clinicopathologic phenotypes (TNM stage, lymph-node metastasis, FIGO stage and distant metastasis; Table II).

MiR-489 Inhibited OC Cell Viability

As we had observed that miR-489 was markedly downregulated in OC cells, we further explored its functional roles in OC progression. Firstly, we overexpressed or inhibited miR-489 expressions by transfecting miR-489 mimics or inhibitor into OC cell lines. QRT-PCR analysis was used to determine the efficiencies of the transfections. As demonstrated by qRT-PCR analysis, successful overexpression or inhibition of miR-489 in OC cells were identified (Figure 2A and 2B). Then, MTT assays were carried out to determine the influence of miR-489 on OC cell viability. Findings indicated that miR-489 upregulation prominently suppressed OC cell viability while miR-489 silence had a facilitated function in cell viability (Figure 2C and 2D).

MiR-489 Suppressed OC Cell Invasion and Migration

We next evaluated the potential functions of miR-489 in OC cell invasion and migration by performing the transwell assays. The significantly impaired invasion and migration were observed in OC cells with transfections of miR-489 mimics (Figure 3A and 3B). On the contrary, in OC cells which were treated with miR-489 inhibitor, the enhanced invasion and migration capacities were found (Figure 3C and 3D). Taken together, all above data suggested that miR-489 exerted anti-tumor functions in OC cells.

MiR-489 Regulated PI3K/Akt Signaling Pathway and EMT in OC Cells

We further explored how miR-489 influenced the biological behaviors of OC cells. In brief, Western blot was applied to detect the expression levels of PI3K/Akt-related or EMT-related indica-

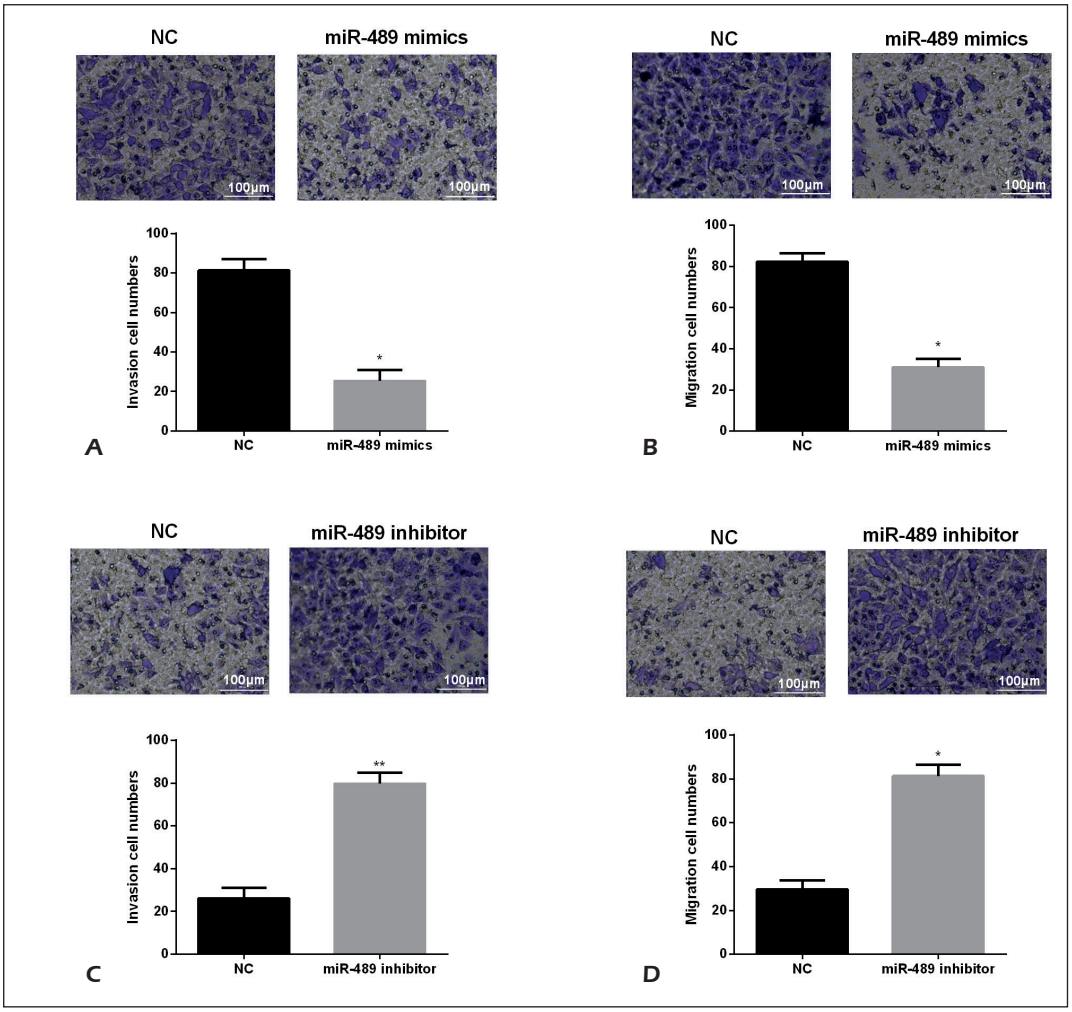

Figure 3. MiR-489 restoration suppressed OC cell invasion and migration. **A-B**, MiR-489 upregulation inhibited OC cell invasion and migration as demonstrated by transwell assays. (Magnification: 200×). **C-D**, MiR-489 silence promoted OC cell invasion and migration. (Magnification: 200 ×). *p<0.05, **p<0.01.

tors in OC cells with different transfections. As shown in Figure 4A, we found that the expressions of p-PI3K and p-Akt were significantly decreased by miR-489 mimics in OC cells. Conversely, there has been an increase in p-PI3K and p-Akt expressions of OC cells by miR-489 inhibitor (Figure 4B). Moreover, in OC cells which were transfected with miR-489 mimics, there was a prominent increase in E-cadherin expression and significant decrease in N-cadherin and Vimentin expression (Figure 4C). On the contrary, miR-489 silence was confirmed to exert suppressive function in E-cadherin expression and facilitated function in N-cadherin and Vimentin expression (Figure 4D). From the above results, we concluded that miR-489 may regulate OC progression *via* regulation of PI3K/Akt and EMT.

MiR-489 Directly Targeted XIAP in OC Cells

TargetScan was used to better explore the candidate targets of miR-489 and XIAP was identified as a potential target gene of miR-489. Then, the predicted XIAP 3′-UTR binding sites and the mutant forms were cloned into the luciferase reporter genes, respectively (Figure 5A). The above reporter genes and miR-489 mimics were co-transfected into OC cells for luciferase assay evaluation to confirm their correlation. Our results revealed that OC cells transfected with miR-489 mimics and XIAP-3′-UTR-WT presented significantly decreased reporter activities, whereas miR-489 mimics had no effects on the XIAP-3′-UTR-MUT reporter activities in OC cells (Figure 5B), indicating the direct regulation of miR-489 in XIAP. In

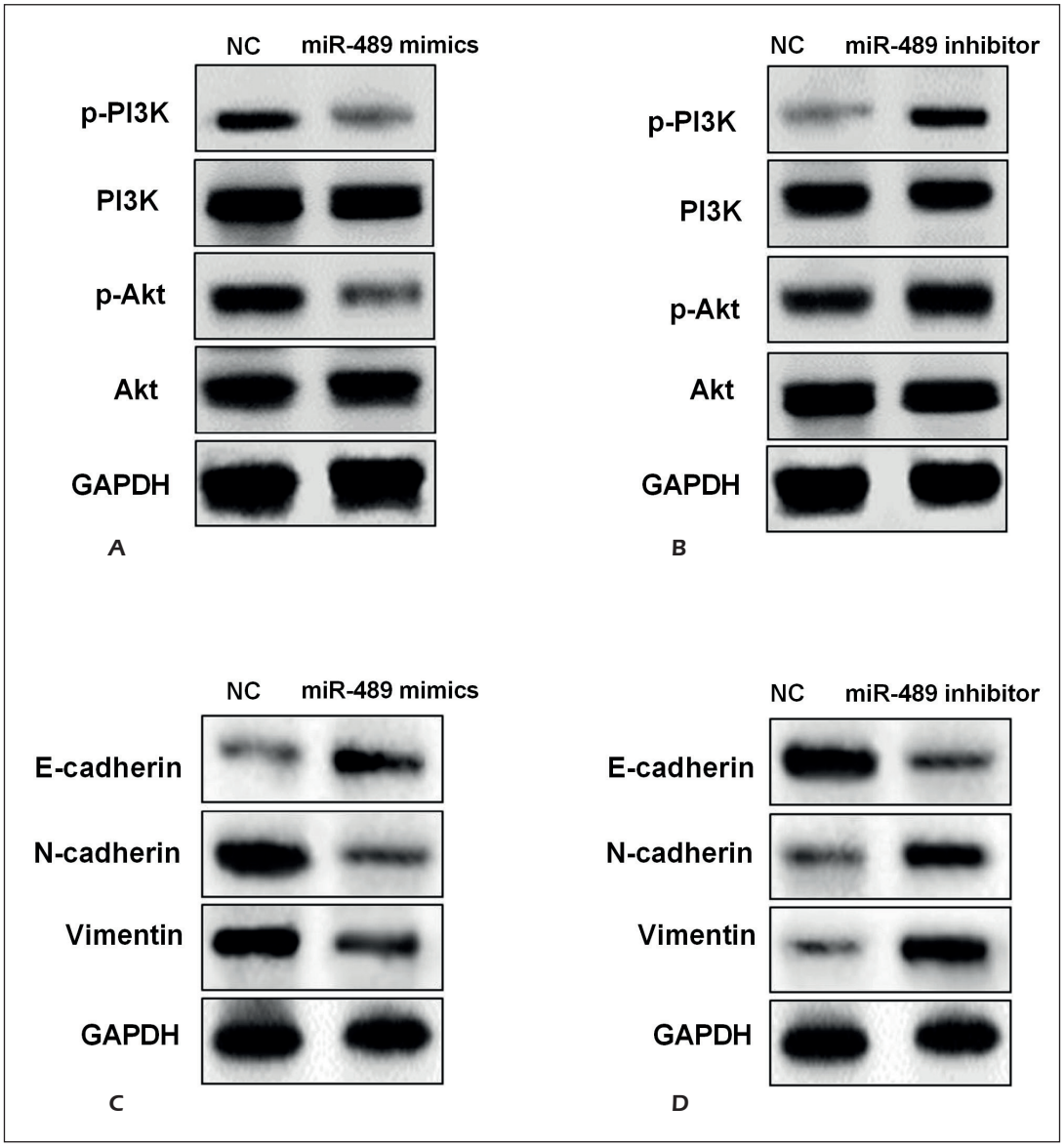

Figure 4. MiR-489 regulated OC cell PI3K/Akt and EMT. **A-B**, Western blot was performed to determine the functions of miR-489 PI3K/Akt signaling pathway of OC cells. **C-D**, Regulatory roles of miR-489 in OC cell EMT were also analyzed by Western blot.

addition, qRT-PCR was performed to indicate the regulatory roles of miR-489 in XIAP. The results showed that miR-489 overexpression in OC cells significantly reduced the XIAP expressions while miR-489 inhibition enhanced the XIAP expressions in OC cells (Figure 5C and 5D).

Upregulated XIAP in OC Indicated Poor Prognosis

As XIAP was detected to be a target of miR-489, we further analyzed the expressions and clinical values of XIAP in OC patients. Data of qRT-PCR revealed that XIAP expression was prominently increased in OC tissue samples (Figure 6A). Similarly, the increased XIAP expression in OC cells was also identified (Figure 6B). Moreover, as demonstrated by clinicopathologic analysis, we found that the overall survival of patients in high XIAP expression groups was significantly shorter than that of patients in low XIAP expression groups (Figure 6C).

Discussion

Recently, OC remains one of the world's deadliest gynecologic malignancies with markedly

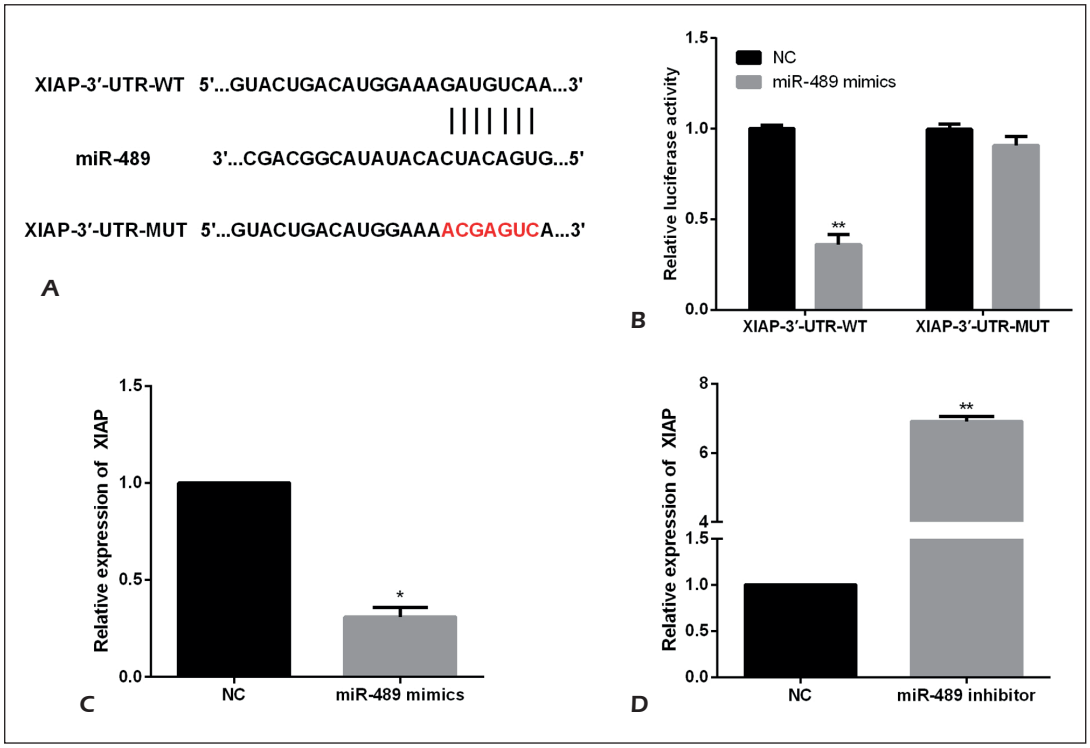

Figure 5. MiR-489 regulated XIAP expressions in OC cells. **A**, Potential binding sequences of miR-489 in XIAP 3'-UTRs. **B**, Influence of miR-489 overexpression on the luciferase activities of XIAP 3'-UTR-WT/MUT. **C-D**, Regulatory roles of miR-489 in XIAP expressions. *p<0.05, **p<0.01.

high incidence, rapid progression, and great potential for metastasis. The high fatality rate of OC largely results from early detection difficulties and lack of effective therapeutic strategies for patients in advanced or recurrent conditions[23]. Hence, novel treatment methods to inhibit OC metastasis and improve the survival rates attracted much attention in recent years. MiRs play important roles in regulating pathological and biological processes in a variety of tumors[24]. Aberrant expression of multiple miRs has been found in OC. We identified an evidently downregulated miR, miR-489, in OC tissues. MiR-489 acted as a tumor suppressor in multiple tumors[25,26], such as gastric cancer and hypopharyngeal squamous cell carcinoma. Combined with the findings in the current study, we concluded that miR-489 presented anti-tumor functions in tumorigenesis.

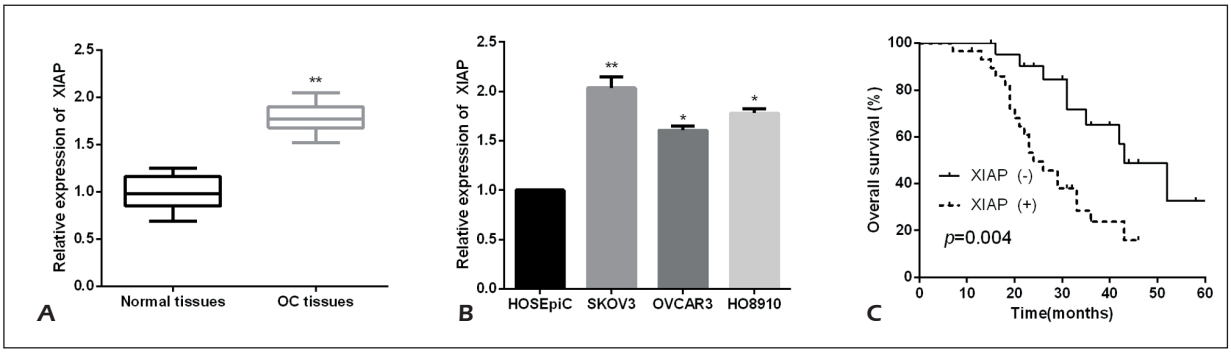

Figure 6. Upregulated XIAP in OC indicated poor prognosis. **A-B**, Increased XIAP expressions in OC tissues and cells were detected by qRT-PCR. **C**, OC patients with high XIAP expressions presented shorter overall survival as demonstrated by Kaplan-Meier analysis. *p<0.05, **p<0.01.

In the current study, miR-489 was significantly downregulated in OC. Moreover, decreased miR-489 expression was found to be associated with the aggressive clinicopathologic characteristics and poor outcomes of OC patients. As demonstrated by functional assays, we also observed that miR-489 upregulation could inhibit OC cell viability, invasion, and migration abilities. All these results proved the tumor suppressive roles of miR-489 in OC progression. Previous studies[27] revealed that the adhesion of tumor cells can reduce their migration abilities. Therefore, epithelial-mesenchymal transition (EMT), in which cell-cell adhesion is weakened and cell motility is increased, is associated with tumor metastasis[28]. In our study, miR-489 overexpression was confirmed to inhibit the EMT process *via* upregulating E-cadherin and downregulating N-cadherin/Vimentin. PI3K/Akt signaling pathway was found to play pivotal role in the occurrence and development of various tumors by affecting multiple biological processes[29,30]. We also found that miR-489 overexpression could inactivate PI3K/Akt signaling pathway.

Given the important roles of miR-489 in OC, we further studied the mechanism by which miR-489 regulated the biological behaviors of OC. TargetScan was utilized to explore the potential targets of miR-489. Since researches showed that XIAP played pivotal functions in OC tumorigenesis[31,32], it was selected for further validation as a candidate target of miR-489. It has been revealed that XIAP has vital pro-metastatic and anti-apoptotic functions[33,34]. In addition, upregulation of XIAP has been frequently identified in different tumors, including bladder cancer[35], esophageal cancer[36], and breast cancer[37]. Here, qRT-PCR analysis showed that increased XIAP expression in OC tissues indicated poor prognosis of OC patients. Moreover, the results of the Luciferase reporter assay and qRT-PCR revealed that miR-489 directly bound to XIAP 3′-UTR and regulated its expression levels.

Conclusions

In summary, this study provides strong evidence that miR-489 overexpression repressed OC cell viability, migration, and invasion *via* directly targeting XIAP and regulated PI3K/Akt and EMT. Our data support that miR-506-3p may act as a tumor suppressor in the OS which was the first time. Besides, the results in our study suggested that miR-489 exerted anti-OC functions and might serve as promising therapeutic target for OC treatment. Further studies investigating the potential applications and significance of miR-489 are ongoing.

Conflict of Interests

The authors declare that they have no conflict of interests.

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