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MiR-4429 suppresses the malignant development of ovarian cancer by targeting YOD1

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Abstract. – OBJECTIVE: The purpose of this study was to assess the clinical significance of microRNA-4429 (miR-4429) in the development and prognosis of ovarian cancer, and to explore the regulatory role of miR-4429 in migratory potential of ovarian cancer cells.

PATIENTS AND METHODS: MiR-4429 levels in paired ovarian cancer species and paracancerous ones were examined. Then, the relationship between miR-4429 level and clinical features of ovarian cancer patients was analyzed. Potential influences of miR-4429 on migratory potentials in CAOV3 and SKOV3 cells were explored by transwell assay. After that, the interaction between miR-4429 and its downstream gene YOD1, and their involvement in the malignant development of ovarian cancer were finally demonstrated through Luciferase assay and rescue experiments.

RESULTS: The results revealed that miR-4429 was downregulated in ovarian cancer tissues and cell lines. Its level was significantly correlated with rates of lymphatic metastasis (p=0.018) and distant metastasis (p=0.012) but not with age, tumor size, and tumor stage in ovarian cancer patients. Survival analysis uncovered that a low level of miR-4429 was unfavorable to PFS and OS in ovarian cancer patients. Besides, the overexpression of miR-4429 inhibited migratory potential in CAOV3 cells, and conversely, the knockdown of miR-4429 in SKOV3 cells obtained the opposite result. Moreover, YOD1 was proved to be the downstream gene binding to miR-4429. It was highly expressed in ovarian cancer tissues and negatively regulated by miR-4429. Rescue experiments finally identified that YOD1 was responsible for migratory potential changes in ovarian cancer cells regulated by miR-4429.

CONCLUSIONS: MiR-4429 is downregulated in ovarian cancer tissues, and its level is closely linked to metastasis and prognosis in ovarian cancer patients. By negatively regulating YOD1 level, miR-4429 suppresses the malignant development of ovarian cancer.

Key Words:
MiR-4429, YOD1, Ovarian cancer, Migration.

Introduction

Ovarian cancer cases account for only 2.4-5.6% among gynecological malignancies. However, its mortality ranks first[1-3]. It is estimated that 239,000 women worldwide were diagnosed with ovarian cancer and 152,000 died of ovarian cancer in 2018[2,3]. In China, the incidence of ovarian cancer patients remarkably increases[4,5]. Epithelial ovarian cancer is the major subtype, which accounts for about 90% of all ovarian cancer cases[2,6]. At present, tumor cytoreductive surgery combined with 6 courses of chemotherapy is the main therapeutic strategy for epithelial ovarian cancer[6,7]. Nevertheless, the prognosis of advanced ovarian cancer is extremely poor, and the 5-year survival rates in stage III and IV patients are only 29% and 13%, respectively[8]. Complications of chemotherapy and metastases of ovarian cancer remarkably limit the therapeutic efficacy. It is of significance to develop novel therapeutic strategies to improve clinical outcomes of ovarian cancer patients[9,10].

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MicroRNAs (miRNAs) are endogenous non-coding RNAs that are evolutionarily conserved[11,12]. Through complementary base pairing, miRNAs regulate target gene expressions *via* degrading or inhibiting translation of mRNAs[12-14]. The regulation of miRNAs on target genes is not one-to-one. One miRNA can directly regulate about two hundred target mRNAs, and multiple miRNAs can also regulate the same target gene[15,16]. The human genome contains thousands of miRNAs, which can regulate nearly 33% of protein-coding genes[16]. In recent years, the vital functions of miRNAs in tumor development have been discovered. They serve as oncogenes or tumor suppressors and participate in every aspect of tumor progression[17-19]. Regulatory effects of miRNAs in ovarian cancer have been previously reported as well[20-22]. MiR-4429 is predicted to be closely linked to ovarian cancer through bioinformatics analyses.

The interaction between miRNAs and their downstream genes greatly influences biological activities[22,23]. The binding sites of miRNAs that are responsible for silencing genes are generally thought to be located at the 3′ untranslated region (3′UTR) of the target mRNA, and occasionally at the coding sequence (CDS) and 5'UTR[23]. There may be false positives of target genes binding to miRNAs predicted by bioinformatics, so further experimental verifications are needed. In this paper, YOD1 was verified to be the downstream gene of miR-4429, which was responsible for the malignant development of ovarian cancer regulated by miR-4429.

Patients and Methods

Ovarian Cancer Patients and Samples

A total of 58 ovarian cancer tissues and paracancerous ones were surgically resected, pathologically confirmed, and stored at -80°C. None of the included ovarian cancer patients received pre-operative treatment. Their clinical data were recorded. In this study, tumor staging was carried out based on the guideline proposed by the Union for International Cancer Control (UICC). This investigation was approved by Ethics Committee of Zhejiang Cancer Hospital. Signed written informed consents were obtained from all participants before the study.

Cell Culture

Human ovarian cancer cell lines (SKOV3, OVCAR3, PEO1, A2780, 3AO, and CAOV3) and a normal ovarian epithelial cell line (HOSEPiCs) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in a 5% CO_2 incubator at 37°C.

Transfection

Cells were cultured to 30-40% confluence in 6-well plates and transfected with plasmids constructed by GenePharma (Shanghai, China), using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 48 hours later, the cells were collected for the following use.

Transwell Migration Assay

A total of 200 μL of suspension (5×10^5 cells/mL) were inoculated in the upper transwell chamber (Millipore, Billerica, MA, USA) inserted in a 24-well plate, with 500 μL of medium containing 10% FBS in the bottom. After 48-h incubation, the bottom cells were reacted with 15-min methanol, 20-min crystal violet, and captured using a microscope. Finally, migratory cells were counted in 5 random fields per sample.

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

RNAs extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) were purified by DNase I treatment, and reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The obtained cDNAs underwent qRT-PCR using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were the internal references. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. MiR-4429: forward: 5'-GGCCAGGCAGTCTGAGTTG-3' and reverse: 5'-GGGAGAAAAGCTGGGCTGAG-3', U6: forward: 5'-GCTTCGGCAGCACATATACTAAAAT-3' and reverse: 5'-CGCTTCACGAATTTGCGTGTCAT-3', YOD1: forward: 5'-CTTCCCTGATCCAGATACACCTCCT-3', and reverse: 5'-TCCCTTGCTTCTGCTTGTCCAGTT-3', and GAPDH: forward: 5'-AAGAAGGTGGTGAAGCAGG-3' and reverse: 5'-GTCAAAGGTGGAGGAGTGG-3'.

Western Blot

The cells were lysed for isolating cellular protein and electrophoresed. Total protein concentration was calculated by bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL, USA). The extracted proteins were separated using a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and subsequently transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). Western blot analysis was performed according to standard procedures. Subsequently, non-specific antigens were blocked in 5% skim milk for 2 hours. The membranes were reacted with primary and secondary antibodies for indicated time. Immuno-reactive bands were visualized by enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences, Foster City, CA, USA). The gray value was analyzed using Image J software (Version 1.38; National Institutes of Health, Bethesda, MA, USA).

Luciferase Assay

CAOV3 and SKOV3 cells were co-transfected with NC mimic/miR-4429 mimic and YOD1-WT/YOD1-MUT, respectively, using Lipofectamine 2000. Cells were lysed for determining relative Luciferase activity 48 h later.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM Corp., Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Differences between groups were analyzed by the *t*-test. Chi-square test was used for analyzing the relationship between miR-4299 and clinical data of ovarian cancer patients. Besides, Kaplan-Meier curves were depicted for survival analysis, followed by Log-rank test. $p<0.05$ suggested that the difference was statistically significant.

Results

MiR-4429 Was Lowly Expressed In Ovarian Cancer Species, and Correlated to Metastasis and Prognosis in Ovarian Cancer Patients

MiR-4429 levels in ovarian cancer tissues and paracancerous ones were detected by qRT-PCR. Compared with that in normal tissues, miR-4429 was markedly downregulated in ovarian cancer tissues (Figure 1A). Identically, miR-4429 was lowly expressed in ovarian cancer cell lines as well (Figure 1B). Kaplan-Meier curves were depicted for survival analyses. As the results demonstrated, lowly expressed miR-4429 was

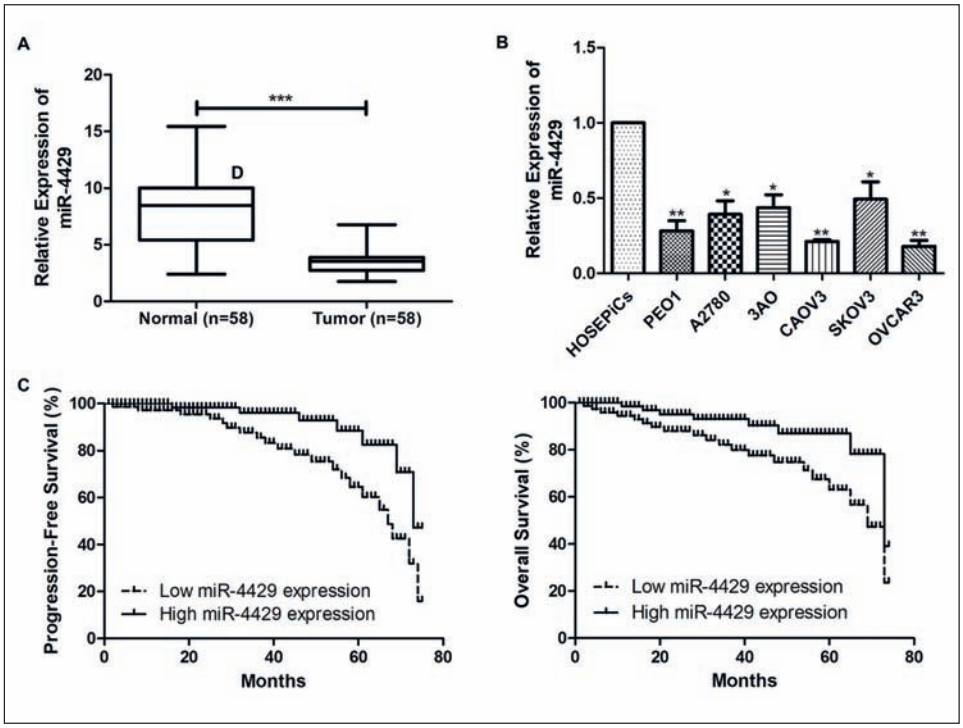

Figure 1. MiR-4429 is lowly expressed in ovarian cancer tissues. **A,** Differential expressions of miR-4429 in ovarian cancer tissues (n=58) and paracancerous ones (n=58). **B,** MiR-4429 levels in ovarian cancer cell lines. **C,** Progression-free survival in ovarian cancer patients with high or low expression of miR-4429. **D,** Overall survival in ovarian cancer patients with a high or low expression of miR-4429. Data are expressed as mean ± SD. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

unfavorable to progression-free survival (PFS) and overall survival (OS) in ovarian cancer patients (Figure 1C, 1D).

Subsequently, the influences of miR-4429 on age, tumor size, tumor staging, lymphatic metastasis, and distant metastasis in ovarian cancer patients were analyzed. It was shown that miR-4429 level was correlated with rates of lymphatic metastasis (p=0.018) and distant metastasis (p=0.012) but not with age, tumor size, and tumor stage in ovarian cancer patients (Table I). It is believed that miR-4429 may be a novel hallmark predicting the malignant development of ovarian cancer.

Overexpression of MiR-4429 Suppressed Migratory Potential in Ovarian Cancer

To explore the biological roles of miR-4429 in ovarian cancer, miR-4429 mimic and inhibitor were constructed. Their ransfection efficacy was tested in CAOV3 and SKOV3 cells, respectively (Figure 2A). Transwell assay results showed that the overexpression of miR-4429 in CAOV3 cells markedly reduced migratory cell number, and knockdown of miR-4429 yielded the opposite result in SKOV3 cells (Figure 2B).

MiR-4429 Directly Bound YOD1

Potential binding sequences in the 3'UTR of miR-4429 and YOD1 were predicted by bioinformatics method. Subsequently, Luciferase assay showed that the overexpression of miR-4429 markedly decreased Luciferase activity in wild-type YOD1 vector, rather than mutant one (Figure 3A). Hence, YOD1 was proven to be the target gene binding to miR-4429. The results of Western blotting showed that miR-4429 mimic could decrease the protein level of YOD1, but miR-4429 inhibitor could increase the protein level of YOD1, which suggest that YOD1 is negatively regulated by miR-4429 in ovarian cancer cells (Figure 3B). As expected, YOD1 was highly expressed in ovarian cancer tissues (Figure 3C) and cell lines (Figure 3D).

YOD1 Reversed Regulatory Effect of MiR-4429 on Migratory Potential in Ovarian Cancer

Rescue experiments were conducted to illustrate the involvement of YOD1 in the development of ovarian cancer. Firstly, pcDNA3.1-YOD1 and si-YOD1 were constructed, and their transfection efficacy was tested in CAOV3 and SKOV3 cells, respectively. The results of Western blotting showed that pcDNA3.1-YOD1 could increase the protein level of YOD1, but si-YOD1 could decrease the protein level of YOD1, indicating that miR-4429 level in ovarian cancer cells is negatively regulated by YOD1 (Figure 4A). In addition, qRT-PCR suggested that co-transfection with miR-4429 mimic and pcDNA3.1-YOD1 downregulated miR-4429 in ovarian cancer cells compared with those solely transfected with miR-4429 mimic. Besides, co-transfection with miR-4429 inhibitor and si-YOD1 upregulated miR-4429 in ovarian cancer cells compared with those

Table I. Association of miR-4429 expression with clinicopathologic characteristics of ovarian cancer.

Parameters	No. of cases	MiR-4429 expression		*p*-value
		High (%)	Low (%)	
Age (years)				0.419
< 60	24	13	11	
≥ 60	34	22	12	
T stage				0.419
T1-T2	34	22	12	
T3-T4	24	13	11	
Tumor size (cm)				0.539
≤ 5	35	20	15	
> 5	23	15	8	
Lymph node metastasis				0.018
No	36	26	10	
Yes	22	9	13	
Distance metastasis				0.012
No	41	29	12	
Yes	17	6	11	

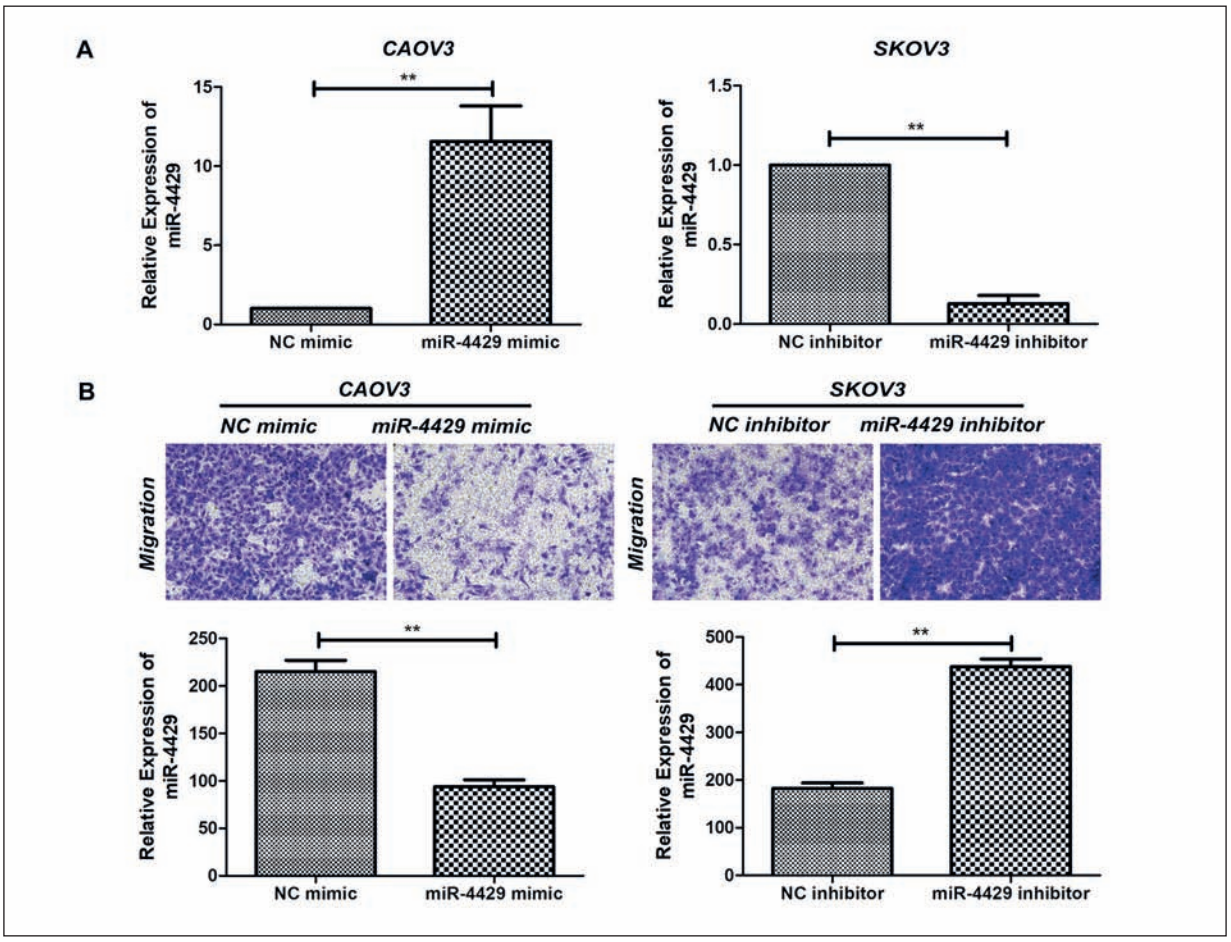

Figure 2. Overexpression of miR-4429 suppresses migratory potential in ovarian cancer. **A,** Transfection efficacy of miR-4429 mimic and inhibitor in CAOV3 and SKOV3 cells, respectively. **B,** Transwell assay shows migration in CAOV3 and SKOV3 cells with overexpression or knockdown of miR-4429, respectively (magnification: 40×). Data are expressed as mean ± SD. $^{**}p<0.01$.

solely transfected with miR-4429 inhibitor (Figure 4B). Notably, the suppressed migratory potential in CAOV3 cells overexpressing miR-4429 was abolished by overexpression of YOD1. Increased migratory cell number in SKOV3 cells with miR-4429 knockdown was reversed by silence of YOD1 (Figure 4C). It is suggested that miR-4429 regulates migratory potential in ovarian cancer by negatively regulating YOD1 level.

Discussion

Epithelial ovarian cancer is a fatal female malignancy[1-3]. Cytoreductive surgery followed by combined chemotherapy based on paclitaxel and platinum is a standard strategy applied in ovarian cancer patients[4-6]. However, chemotherapy resistance, tumor metastases and recurrence markedly restrict the therapeutic efficacy[7,8]. Tumor targeting therapy aims to introduce target genes into specific tumor tissues or cells through molecular biological technology, so as to repair defective genes or inhibit carcinogenic genes, thereafter inhibiting the growth of tumor cells without impairing normal cells[8,9]. It is a promising therapy featured by high specificity, definite efficacy and little adverse events[8-10].

MiRNAs are extensively involved in tumor development[23]. MiR-4429 is found to be downregulated in several types of tumor diseases as a tumor suppressor[24-26]. However, the mechanism of miR-4429 in ovarian cancer is not clear. Therefore, the objective of this study was firstly to elucidate the oncogenic role of miR-4429 in the progression of ovarian cancer, as well as the specific mechanism of miR-4429 regulating YOD1.

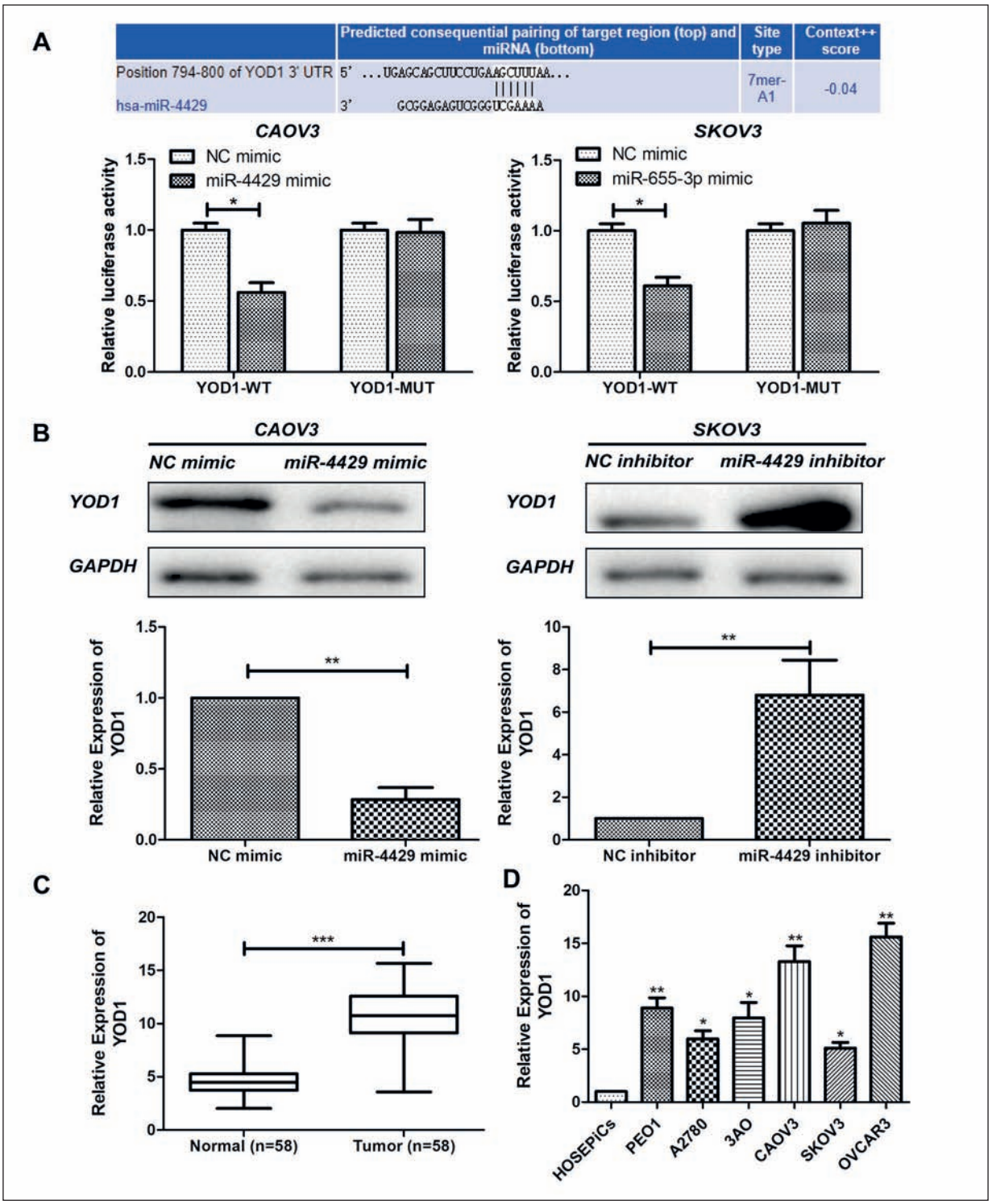

Figure 3. MiR-4429 directly binds to YOD1. **A,** Binding sequences in the 3'UTR of miR-4429 and YOD1 (upper lane). Luciferase assay in CAOV3 and SKOV3 cells co-transfected with NC mimic/miR-4429 mimic and YOD1-WT/YOD1-MUT, respectively. **B,** Protein and mRNA levels of YOD1 in CAOV3 and SKOV3 cells with overexpression or knockdown of miR-4429, respectively. **C,** Differential expressions of YOD1 in ovarian cancer tissues (n=58) and paracancerous ones (n=58). **D,** YOD1 levels in ovarian cancer cell lines. Data are expressed as mean ± SD. *p<0.05, **p<0.01, ***p<0.001.

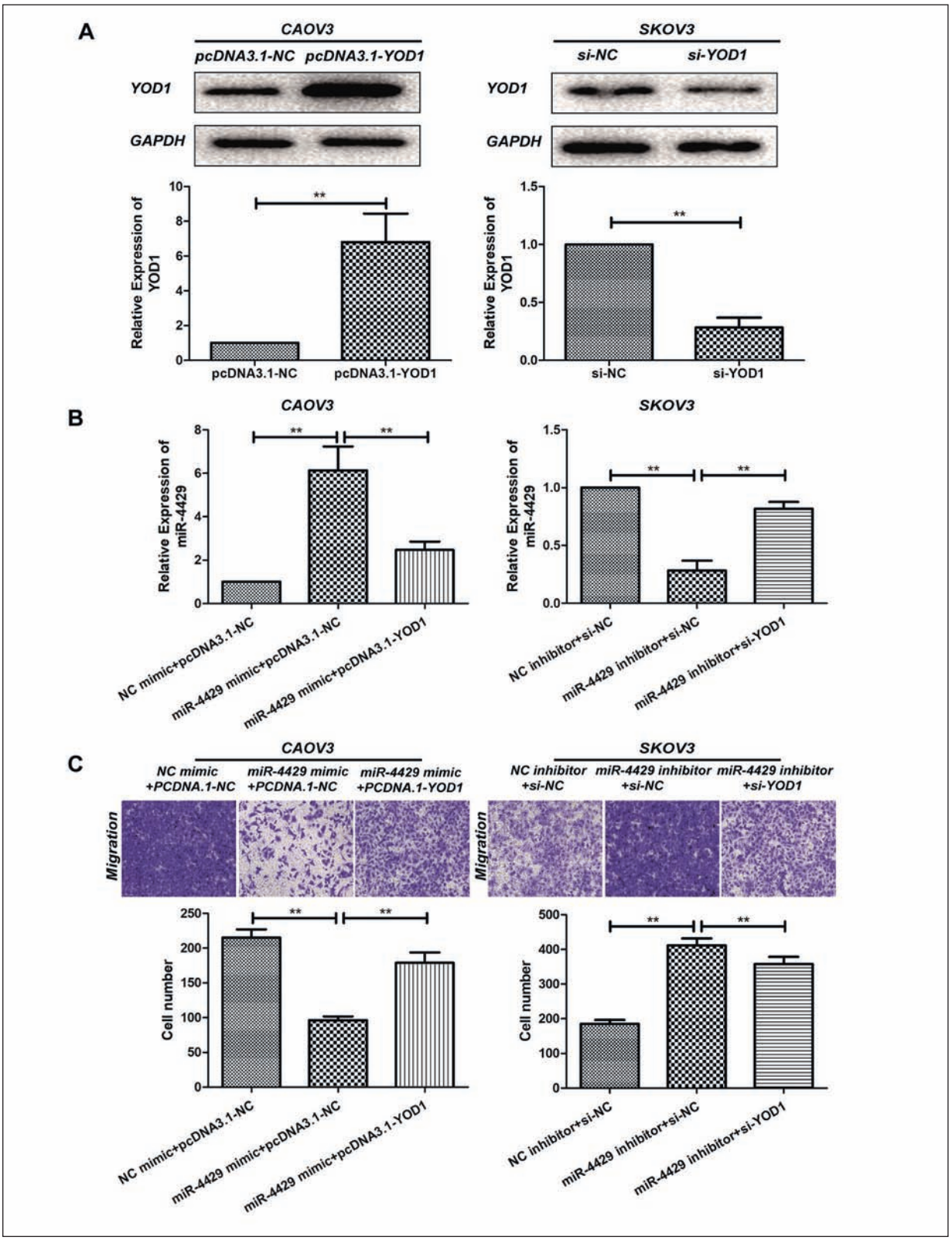

Figure 4. YOD1 reverses regulatory effect of miR-4429 on migratory potential in ovarian cancer. **A,** Transfection efficacy of pcDNA3.1-YOD1 and si-YOD1 in CAOV3 and SKOV3 cells, respectively. **B,** MiR-4429 level in co-transfected CAOV3 and SKOV3 cells. **C,** Transwell assay shows migration in co-transfected CAOV3 and SKOV3 cells (magnification: 40×). Data are expressed as mean ± SD. $^{**}p<0.01$.

The findings of this study showed that miR-4429 was lowly expressed in ovarian cancer tissues, and its level was linked to rates of lymphatic metastasis and distant metastasis, as well as survival of ovarian cancer patients. Subsequently, *in vitro* experiments demonstrated that miR-4429 suppressed migratory potential in ovarian cancer cells.

The high mortality rate of ovarian cancer patients is mainly due to chemotherapy resistance, and subsequent cancer cell invasion and metastasis[7-9]. MiRNAs display their biological functions through their downstream genes. YOD1 was proven to be the target gene binding to miR-4429. As an intracellular molecule associated with metastasis, YOD1 is involved in metastatic process[27]. In ovarian cancer tissues, YOD1 was highly expressed and negatively regulated by miR-4429. More importantly, YOD1 was capable of reversing the regulatory effect of miR-4429 on migratory potential in ovarian cancer. To sum up, a negative feedback loop miR-4429/YOD1 was verified to alleviate the malignant development of ovarian cancer.

Conclusions

Shortly, miR-4429 is downregulated in ovarian cancer tissues, and its level is closely linked to metastasis and prognosis in ovarian cancer patients. By negatively regulating YOD1 level, miR-4429 suppresses the malignant development of ovarian cancer.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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