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MiR-4282 is a tumor-suppressor gene for preventing metastasis of epithelial ovarian cancer by negatively regulating MIER1

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Abstract. – OBJECTIVE: To elucidate the biological role of miR-4282 in influencing metastasis of epithelial ovarian cancer (EOC) by regulating MIER1.

PATIENTS AND METHODS: MiR-4282 expressions in 45 cases of EOC specimens and normal controls were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The relationship between miR-4282 and clinical features in EOC patients, including pathological indicators and overall survival, was analyzed. After intervening miR-4282 level in SKOV3 and 3AO cells by plasmid transfection, changes in migratory and invasive abilities were determined by transwell assay and wound healing assay. The target gene of miR-4282 was observed by Dual-Luciferase reporter assay, followed by exploration of its involvement in EOC progression via rescue experiments.

RESULTS: MiR-4282 was downregulated in EOC specimens than normal controls. EOC patients expressing low level of miR-4282 had higher incidences of lymphatic metastasis and distant metastasis, as well as worse prognosis than those overexpressing miR-4282. Overexpression of miR-4282 in SKOV3 cells weakened metastatic ability, and conversely, knockdown of miR-4282 in 3AO cells yielded the promotive trends. MIER1 was confirmed to be the target gene binding miR-4282, which was highly expressed in EOC specimens. MIER1 was able to reverse the regulatory effect of miR-4282 on EOC cell metastasis.

CONCLUSIONS: Lowly expressed miR-4282 in EOC specimens is closely linked to the incidence of metastasis and overall survival. MiR-4282 prevents EOC metastasis by a negative regulation on MIER1.

Key Words:
MiR-4282, MIER1, Epithelial ovarian cancer, Metastasis.

Introduction

The incidence of ovarian malignant tumors ranks third in malignancies of the female reproductive system, however its mortality ranks first[1,2]. Epithelial ovarian cancer (EOC) is the major subtype, comprising about 85-90% of ovarian malignant tumors. Due to the lack of effective screening methods, most EOC patients are diagnosed as advanced stage[3,4]. EOC is featured by high malignancy, rapid disease development, easy recurrence, and difficult treatments. The prognosis of EOC is poor. Its 5-year survival is only 34-45%[4-6].

MiRNAs are single-stranded, small, non-coding RNAs that are extensively expressed in eukaryotes[7,8]. Approximately 30% of the human genome can be regulated by miRNAs that are often located in fragile sites and genomic regions, and 50% of them are frequently amplified or deleted in human cancer cells[9,10]. MiRNAs are important regulators in the biological behaviors of cell proliferation, differentiation, and apoptosis[11,12]. They have been demonstrated to be promising biomarkers for diagnosing or assessing the prognosis of tumors[13,14]. The tumor-suppressive effect of miR-4282 has been found in oral squamous cell carcinoma, breast cancer and colorectal carcinoma[15-17]. MiR-4282 participates in regulating tumor cell phenotypes to varying degrees.

So far, the role of miR-4282 in influencing EOC progression remains largely unclear. We collected clinical cases of EOC specimens for detecting differential expressions of miR-4282. In addition, the clinical significance of miR-4282 in EOC patients was analyzed. By screening out EOC cell lines with relatively high or low level of miR-4292, respectively, a series of cell functional

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experiments were conducted to uncover the role of miR-4282 in regulating EOC cell metastasis, and the underlying mechanism.

Patients and Methods

EOC Specimens

Fifty EOC specimens were collected from EOC patients during surgery, which were postoperatively confirmed by Hematoxylin-Eosin (H&E) staining and prepared into pathological sections. The normal controls were patients that were not diagnosed EOC and underwent oophorectomy with other disease, such as ovarian cyst. None of enrolled EOC patients had received preoperative chemotherapy or biological treatment. Their baseline characteristics, clinical data, pathological indicators and follow-up file were completely recorded. The tumor node metastasis (TNM) staging was confirmed according to the criteria proposed by UICC/AJCC (the 8th edition). This study was approved by the Research Ethics Committee of Yantai Yuhuangding Hospital and complied with the Helsinki Declaration. Informed consent from patients was obtained.

Cell Lines and Reagents

Human-derived ovarian cancer cell lines (SKOV3, OVCAR3, PEO1, A2780, 3AO, CAOV3) and the normal human ovarian epithelial cell line (HOSEPiCs) were provided by American Type Culture Collection (ATCC; Manassas, VA, USA). They were cultivated in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) at 37°C with 5% CO_2.

Transfection

Cells in good condition were inoculated in the 6-well plate and cultivated for 24 h. Until cells were grown to 70-80% confluence, transfection was conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Transfection plasmids were constructed by GeneCopoeia (Guangzhou, China). Transfected cells were collected at 48 h for quantitative real-time polymerase chain reaction (qRT-PCR) and cell function experiments.

Transwell Migration and Invasion Assay

200 μL of serum-free suspension (2×10^4 cells) was applied on the top of transwell chamber. In the bottom, 500 μL of complete medium was applied. After 24 h of incubation, cells in the bottom were fixed in methanol for 15 min, dyed with crystal violet for 30 min and counted using a microscope. Invasion assay was conducted using transwell chambers precoated with Matrigel (diluted in serum-free medium at 9:1).

Wound Healing Assay

Cells were prepared into suspension with 5×10^5 cells/mL, and implanted in 6-well plates. Until 90% of cell attachment, an artificial wound was made using a sterilized pipette tip. Cells were washed in phosphate-buffered saline (PBS) for 2-3 times and cultured in the medium containing 1% FBS. 24 hours later, wound closure was captured for calculating the percentage of wound healing.

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

Cellular or tissue RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan). SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) was used for qRT-PCR. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. miR-4282: Forward: 5'-AGGATGATGTTCCTGGATGC-3', Reverse: 5'-GGTGAAGTTCCAGGGGAAGAT-3'; U6: Forward: 5'-GCTTCGGCAGCACATATACTAAAAT-3', Reverse: 5'-CGCTTCACGAATTTGCGTGCAT-3'; MIER1: Forward: 5'-ATGGTGTGGTCGCTCGATTC-3', Reverse: 5'-TGTTGCTGAACCTCCTGGAC-3'; GAPDH: Forward: 5'-GCTGCCCAGAACATCATCC-3', Reverse: 5'-GTCAGATCCACGACGGACAC-3'.

Western Blot

Cells were lysed in radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) on ice for 30 min for isolating total protein. The concentration of cellular protein was determined by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). Protein samples were denaturized by water bath at 100°C for 5 min, and adjusted to the same concentration by adding loading buffer. Protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; 100 V for 1-2 h) and loaded on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) using sandwich method (100 V for 1 h). The membrane was cut into small pieces according to the molecular size

and blocked in 5% skim milk for 2 h. They were incubated with primary and secondary antibodies, followed by band exposure and grey value analyses using Image J (Rawak Software, Inc., Hamburg, Germany).

Dual-Luciferase Reporter Assay

HEK293T cells were inoculated in a 24-well plate. Consequential pairing of MIER1 and miR-4282 was predicted using online tool, followed by construction of pmirGLO-MIER1-WT and pmirGLO-MIER1-MUT. They were co-transfected in HEK293T cells with NC mimic or miR-4282 mimic. Luciferase activity was measured after 48 h of co-transfection.

Statistical Analysis

Data were expressed as mean ± SD (Standard Deviation), and processed by Statistical Product and Service Solutions (SPSS) 22.0 (SPSS Chicago, IL, USA). Chi-square test was applied for evaluating the influence of miR-4282 on clinical features in EOC patients. Differences between groups were compared by the *t*-test. $p < 0.05$ was considered as statistically significant.

Results

MiR-4282 was Lowly Expressed in EOC

Compared with normal controls, miR-4282 was downregulated in EOC tissues (Figure 1A). In addition, it was lowly expressed in EOC cell lines (Figure 1B). According to miR-4282 level in EOC tissues, enrolled EOC patients were classified into high miR-4282 expression group and low miR-4282 expression group, respectively. Chi-square test uncovered significant differences in the incidences of lymphatic metastasis and distant metastasis between two groups (Table I).

MiR-4282 Suppressed Metastasis in EOC

MiR-4282 overexpression and knockdown models were respectively generated in SKOV3 and 3AO cells by transfection of miR-4282 mimic and inhibitor. Transfection efficacy was examined by qRT-PCR (Figure 2A). Transwell assay uncovered that both numbers of migratory and invasive cells were elevated by overexpression of miR-4282 in SKOV3 cells. On the contrary, migratory and invasive cell numbers were declined after knockdown of miR-4282 in 3AO cells (Figure 2B). Similarly, wound healing assay showed that the overexpression of miR-4282 decreased wound closure percentage at 24 h, and the knockdown of miR-4282 yielded the opposite result (Figure 2C).

MiR-4282 Negatively Regulated MIER1

Through bioinformatic analysis, MIER1 was the potential target of miR-4282 (Figure 3A). Subsequently, we found that the Luciferase activity was declined in pmirGLO-MIER1-WT by overexpression of miR-4282. In pmirGLO-MIER1-MUT, Luciferase activity was not influenced by overexpressed miR-4282 (Figure 3B). It is confirmed that miR-4282 could bind MIER1.

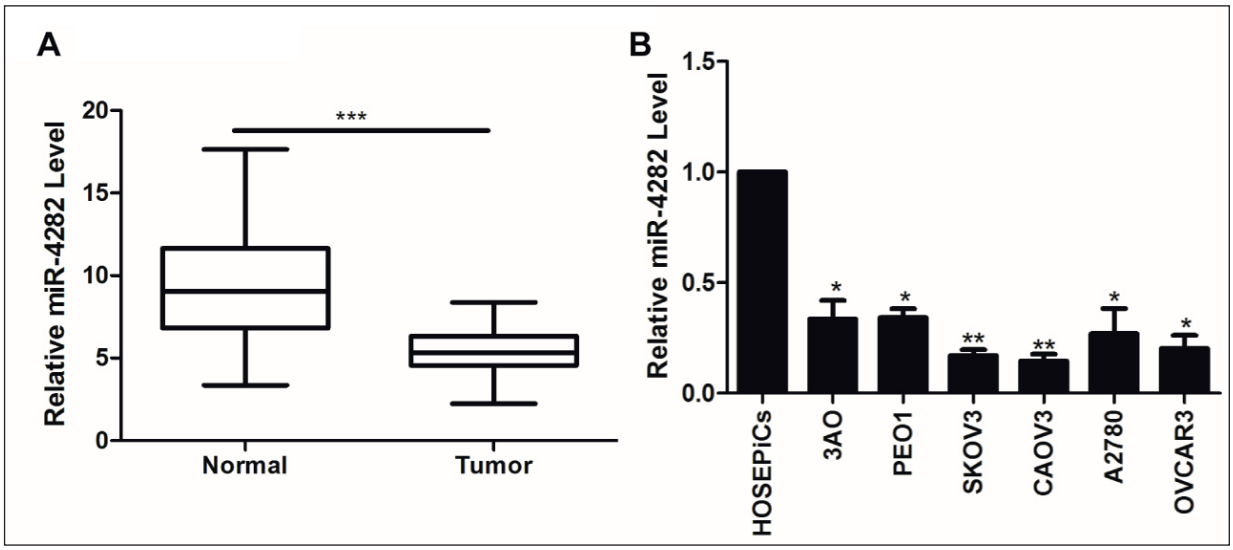

Figure 1. MiR-4282 was lowly expressed in EOC. **A,** Differential expressions of miR-4282 in EOC and normal specimens; **B,** MiR-4282 levels in EOC cell lines; Data were expressed as mean±SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Table I. Clinicopathologic characteristics of the patients with ovarian cancer in Low- and high-miR-4282 expression groups.

Parameters	Number of cases	miR-4282 expression High (n=25)	Low (n=20)	*p* -value
Age (years)				0.736
<60	19	10	9	
≥60	26	15	11	
Tumor size (cm)				0.423
≤3	21	13	8	
>3	24	12	12	
TNM stage				0.841
I+II	24	13	11	
III+IV	21	12	9	
Lymph node metastasis				0.031
No	26	18	8	
Yes	19	7	12	
Distance metastasis				0.015
No	29	20	9	
Yes	16	5	11	

QRT-PCR data detected higher level of MIER1 in EOC specimens than normal controls, and it was negatively correlated to miR-4282 level (Figure 3C). MIER1 was identically upregulated in EOC cell lines (Figure 3D). Protein level of MIER1 was downregulated in SKOV3 cells overexpressing miR-4282, and it was upregulated in 3AO cells transfected with miR-4282 inhibitor (Figure 3E).

MiR-4282 Alleviated Malignant Progression of EOC by Competitively Binding MIER1

The above results have demonstrated the negative interaction between miR-4282 and MIER1. We thereafter speculated that MIER1 could be involved in EOC metastasis mediated by miR-4282. Transfection efficacy of pcDNA3.1-MIER1 and si-MIER1 was examined in SKOV3 cells overexpressing miR-4282 and 3AO cells with miR-4282 knockdown, respectively (Figure 4A). Compared with SKOV3 cells overexpressing miR-4282, the numbers of migratory and invasive cells, and wound closure percentage were higher in those co-overexpressing miR-4282 and MIER1 (Figure 4B and 4C, left). Meanwhile, lower migratory and invasive abilities were determined in 3AO cells with co-silence of miR-4282 and MIER1 than those with miR-4282 knockdown (Figure 4B and 4C, right). It is suggested that MIER1 was responsible for EOC cell metastasis regulated by miR-4282.

Discussion

EOC is a common malignant tumor in the female reproductive system[1-3]. Surgery combined postoperative chemotherapy is preferred to EOC. However, the mortality of EOC remains high due to the advanced stage and drug resistance[4]. It is of significance to screen drug-resistance indicators and effective therapeutic targets for EOC[4-6]. In recent years, miRNAs have been identified to be related to EOC progression[15-17]. By detecting differential expressions of miR-4282 in EOC and normal specimens, miR-4282 was found to be downregulated in EOC tissues. Meanwhile, it was correlated to lymphatic metastasis, distant metastasis and overall survival in EOC patients. We thereafter speculated that miR-4282 may be a tumor-suppressor gene involved in EOC progression.

MiRNAs have a relation to tumor progression and metastasis[13,14]. Cancer cells are seeds that can spread to somewhere else, and metastases are the soil where seeds are sown[18]. Distant metastases of tumors have organ tendency. For example, lung cancer tends to metastasize to the adrenal gland and brain. Thyroid cancer, renal cancer and prostate cancer are easy to metastasize to bones. Breast cancer often metastasizes to lung, liver, bone, ovary, adrenal gland, etc.[19]. Organ tendency of tumor is unclear, which may be attributed to ligands on the endothelium that can specifically bind to adhesion molecules on the surface of cancer cells entering the blood circulation. Chemical attractants released by target organs that attract cancer cells are also responsible for the organ tendency[20,21]. Besides, some specific organs create a favorable microenvironment for cancer cell growth[22]. To elucidate the influence of miR-4282 on EOC cell functions, we generated miR-4282 overexpression and knockdown model in SKOV3 and 3AO

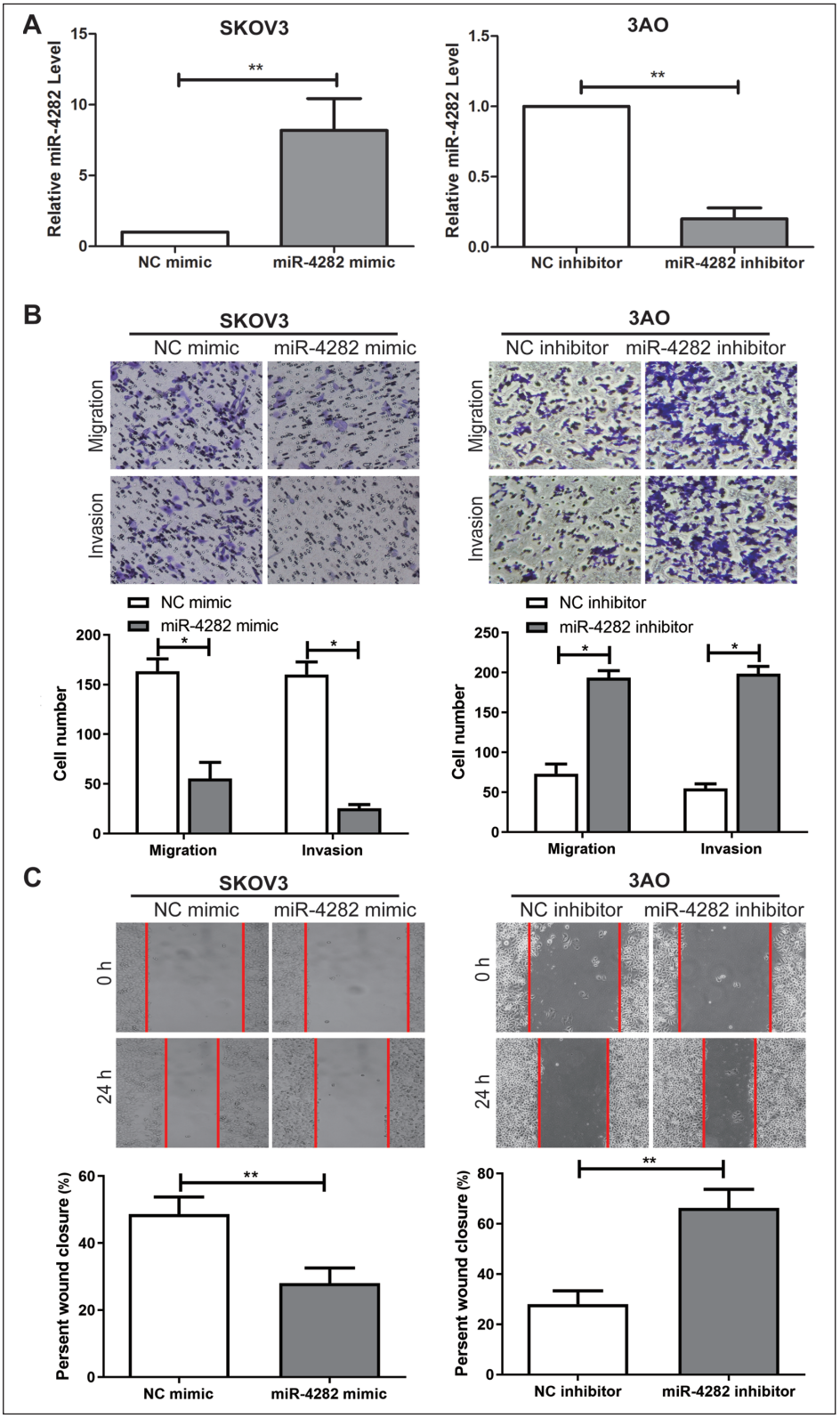

Figure 2. MiR-4282 suppressed metastasis in EOC. **A,** Transfection efficacy of miR-4282 mimic and miR-4282 inhibitor in SKOV3 and 3AO cells, respectively; **B,** Migration and invasion in SKOV3 and 3AO cells with overexpression and knockdown of miR-4282, respectively (20×); **C,** Wound closure percentage in SKOV3 and 3AO cells with overexpression and knockdown of miR-4282, respectively (20×). Data were expressed as mean±SD. *$p < 0.05$, **$p < 0.01$.

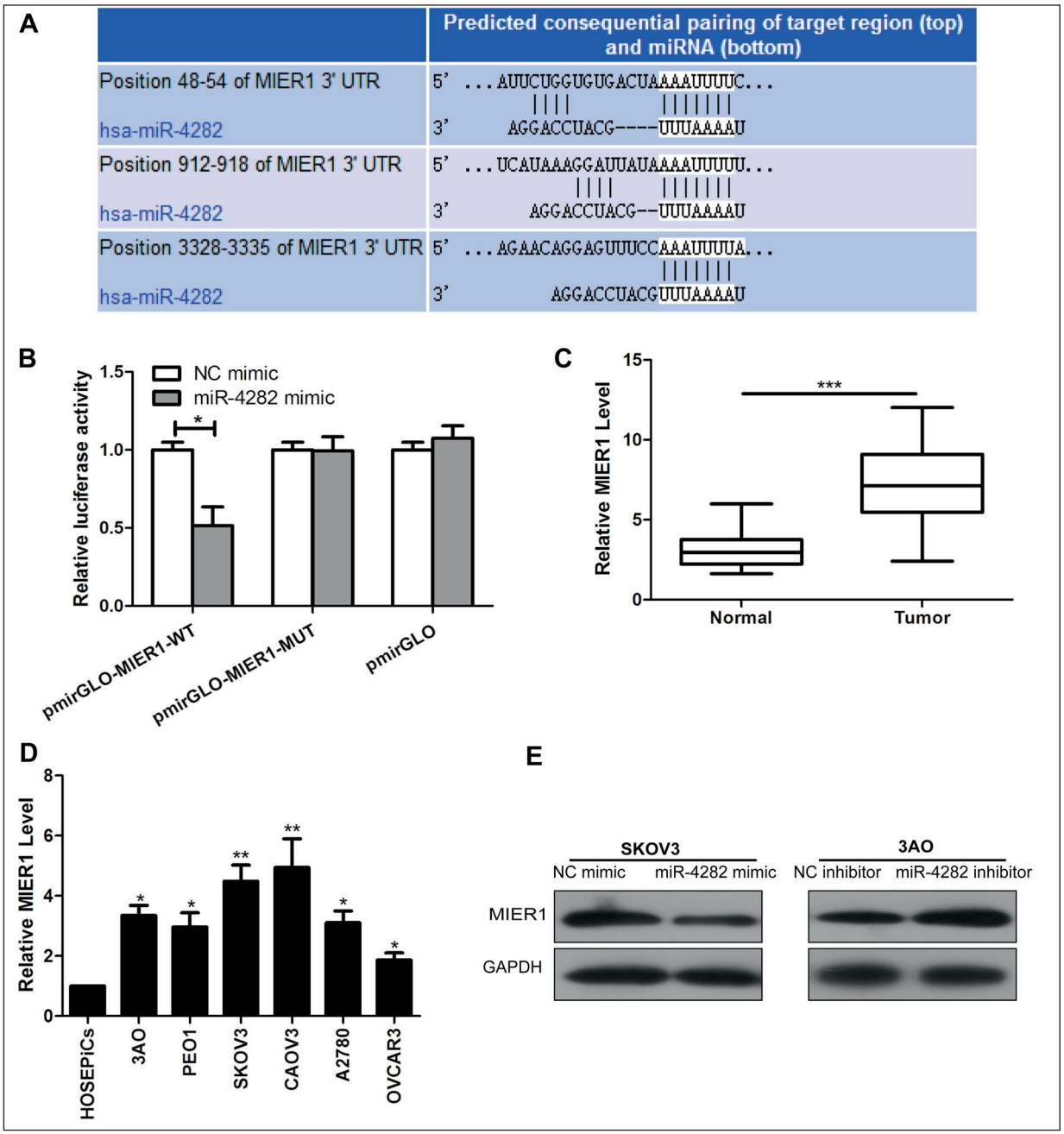

Figure 3. MiR-4282 negatively regulated MIER1. **A,** Predicted consequential pairing of MIER1 and miR-4282; **B,** Luciferase activity by co-transfection of pmirGLO-MIER1 vector and miR-4282 mimic; **C,** Differential expressions of MIER1 in EOC and normal specimens; **D,** MIER1 levels in EOC cell lines; **E,** Protein level of MIER1 in SKOV3 and 3AO cells with overexpression and knockdown of miR-4282, respectively. Data were expressed as mean±SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

cells, respectively. Both transwell and wound healing assay indicated the inhibitory effect of miR-4282 on migratory and invasive abilities in EOC.

About one third of human genomes are associated with miRNA regulation. There are still many undiscovered functions of miRNA[7,8]. MiRNAs are responsible for regulating cell phenotypes by mediating expression levels and biological functions of target genes[9-12]. The MIER family consists of three genes encoding proteins with common conserved primary sequence, especially in the ELM2 and SANT domains. Mier1 was identified as a fibroblast

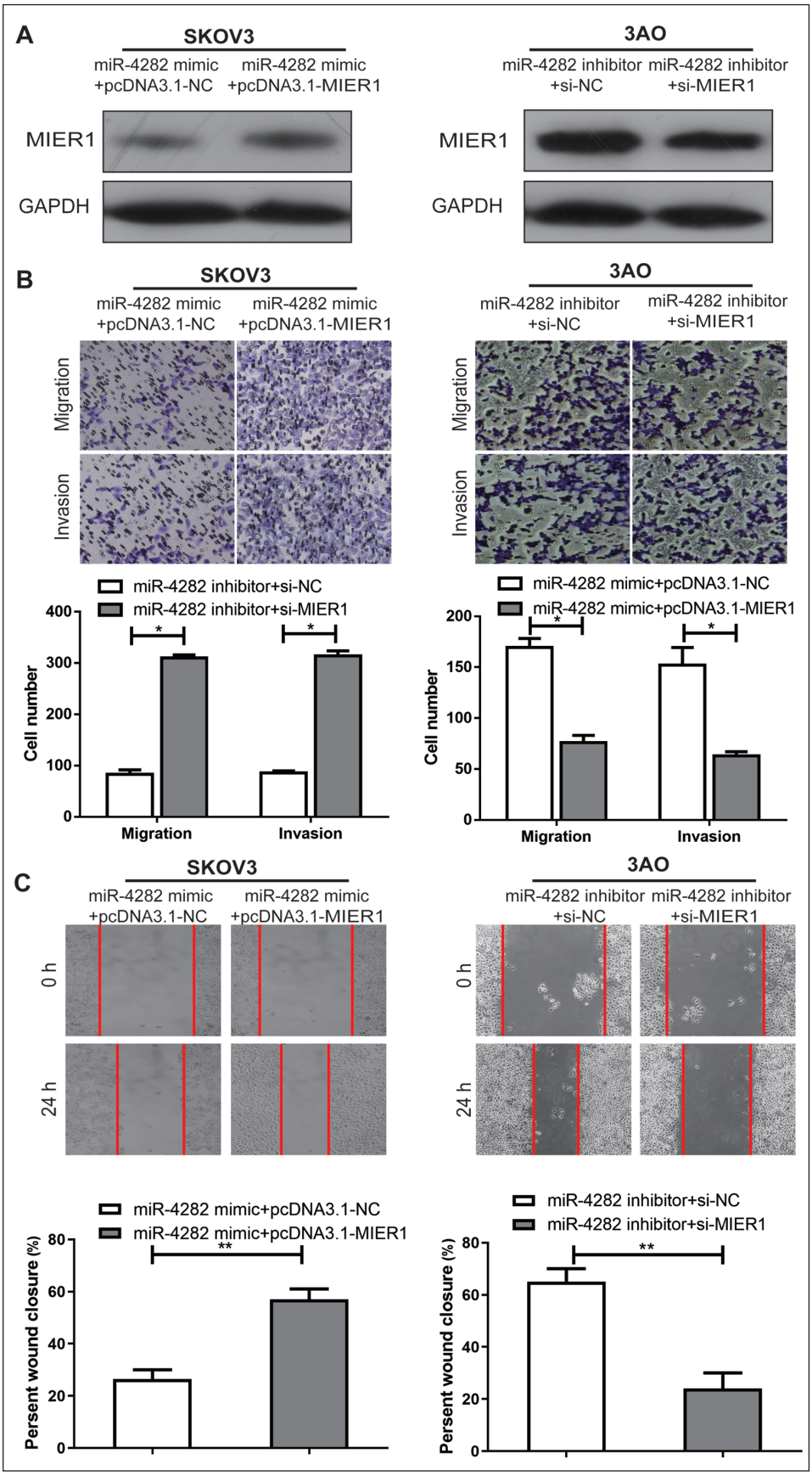

Figure 4. MiR-4282 alleviated malignant progression of EOC by competitively binding MIER1. **A,** Transfection efficacy of pcDNA3.1-MIER1 and si-MIER1 was examined in SKOV3 cells overexpressing miR-4282 and 3AO cells with miR-4282 knockdown, respectively; **B,** Migration and invasion in SKOV3 and 3AO cells co-regulated by miR-4282 and MIER1 (20×); **C,** Wound closure percentage in SKOV3 and 3AO cells co-regulated by miR-4282 and MIER1 (20×). Data were expressed as mean±SD. *$p < 0.05$, **$p < 0.01$.

growth factor early response gene [23]. Several MIER1 isoforms have been characterized [24]; ELM2 and SANT domains with divergent N- & C- termini were include in each isoform.

Studies shown α that under the control of the Tre promoter in T47D breast carcinoma cells, the α isoform interacts with estrogen receptor α (ERα) and that stable expression of MIER1α which inhibited estrogen-stimulated colony growth. IHC assay of breast tumour samples revealed a shift in the subcellular localization of MIER1α from the nucleus to the cytoplasm during breast cancer progression [25]. Here, we have demonstrated that MIER1 was the target gene binding miR-4282. MIER1 was upregulated in EOC specimens, presenting a negative correlation to miR-4282 level. Protein level of MIER1 was negatively regulated by miR-4282 in SKOV3 and 3AO cells as well. Rescue experiments showed that MIER1 was capable of reversing the regulatory effect of MIER1 on migratory and invasive abilities in EOC cells. MiR-4282 may be a promising biomarker for EOC.

Conclusions

In brief, lowly expressed miR-4282 in EOC specimens is closely linked to the incidence of metastasis and overall survival. MiR-4282 prevents EOC metastasis by a negative regulation on MIER1.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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